Using a native plant-pathogen system as a model to investigate success of the invasive mountain pine beetle in jack pine

By

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Abstract

Tree-infesting organisms have recently expanded their ranges into many novel habitats where they will not only interact with new host tree species, but also with a myriad of other organisms that also share these hosts. Understanding the major factors and mechanisms that mediate plant-herbivore-pathogen interactions, such as plant defenses, will be important for determining the impact of invading organisms. My research investigates the range expansion of mountain pine beetle (*Dendroctonus ponderosae* (Hopkins), Coleoptera: Curculionidae, Scolytinae) into the novel host jack pine (*Pinus banksiana* Lamb.), which is an ecologically and economically important component of the Canadian boreal forest. First, I assessed the effects of drought on induced plant defense responses in jack pine to phytohormones (as a proxy for different classes of biotic disturbances) and a pathogenic fungal associate of mountain pine beetle, *Grosmannia clavigera* (Robinson-Jeffrey & Davidson). Prior induction from phytohormones resulted in systemic cross-induction of resistance to *G. clavigera* under normal watering treatment, but susceptibility under low watering treatment. Next, I identified the impact of multiple classes of induced host defense compounds due to the infection by a widespread native parasitic plant (dwarf mistletoe, *Arceuthobium americanum* Nutt. ex Engelm.) on the success of mountain pine beetle and *G. clavigera*. Systemically, there was a non-linear effect of dwarf mistletoe infection on monoterpenes, with increasing concentrations of monoterpenes at moderate severities and decreasing concentrations at high severities. Dwarf mistletoe-induced changes in monoterpenes seem to result in the systemic induced resistance as trees with moderate mistletoe severity were most resistant to *G. clavigera.* In contrast, phenolic compounds increased in amount with greater dwarf mistletoe infection severity but decreased after inoculation with *G. clavigera*. This inverse response to infection between monoterpenes and phenolics suggests that phenolics are detoxified

by the fungus or there are tradeoffs between these two major defense classes. Furthermore, dwarf mistletoe-induced changes in defensive and physical characteristics reduced the competitive advantage of the subcortical insect community on mountain pine beetle performance. Tree-mediated interactions between biotic disturbances, such as dwarf mistletoe, and *G. clavigera* may impact mountain pine beetle establishment or maintenance in novel jack pine forests through systemic effects and coordination of defense chemicals.

Preface

This thesis presents four studies (Chapters 2, 3, 4, and Appendix D) that are either published, submitted, or intended for publication. These studies represent collaborative work with Dr. Nadir Erbilgin of the University of Alberta. For all studies, I was responsible for concept formation, research design, data collection and analysis, and writing of thesis and manuscripts. Dr. Erbilgin was involved with concept formation for the original research, advice on research design, and help with manuscript composition. All permits and protocols from the Government of Alberta were followed for field work. This included covering inoculation points with screening, cutting inoculated trees, and removing and destroying infected material.

Chapter 2 of this thesis was submitted for publication as J. G. Klutsch, Simon F. Shamoun, N. Erbilgin, "Drought stress leads to systemic induced susceptibility to a nectrotrophic fungus associated with mountain pine beetle in *Pinus banksiana* seedlings" in PLOS ONE. I was responsible for research design, data collection, most data analysis, running chemical analyses, and writing the manuscript. Dr. Erbilgin assisted in research design and manuscript composition. Dr. Shamoun of Natural Resources Canada, Pacific Forestry Centre, provided advice on research design.

Chapter 3 of this thesis is accepted for publication as J. G. Klutsch, Ahmed Najar, Patrick Sherwood, Pierliugi Bonello, and N. Erbilgin, "A native parasitic plant systematically induces resistance in jack pine to a fungal symbiont of invasive mountain pine beetle," in Journal of Chemical Ecology. I was responsible for research design, data collection, most data analysis, running monoterpene and carbohydrate analyses, and writing the manuscript. Dr. Erbilgin was involved in concept formation, assisted in research design and manuscript composition. Ahmed Najar helped with monoterpene chemical analysis. Dr. Sherwood under the supervision of Dr.

Bonello (The Ohio State University) performed all phenolic analyses and provided edits to the manuscript.

Chapter 4 of this thesis has been published as J. G. Klutsch, A. Najar, Jonathan A. Cale, and N. Erbilgin, "Direction of interaction between mountain pine beetle (*Dendroctonus ponderosae*) and resource‐sharing wood‐boring beetles depends on plant parasite infection," Oecologia, 2016, 182:1-12. I was responsible for research design, data collection, most data analysis, running the chemical analysis, and writing the manuscript. Dr. Erbilgin assisted in research design and manuscript composition. Dr. Cale of the University of Alberta assisted with a portion of the data analysis. Assistance with chemical analysis was provided by Ahmed Najar.

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Table of Abbreviations

DMR	dwarf mistletoe rating
GC/MS	gas chromatograph/mass spectrometer
HPLC	high-performance liquid chromatography
MJ	methyl jasmonate
MPB	mountain pine beetle
MS	methyl salicylate
NMDS	non-metric multidimensional scaling
PDA	photo diode array
RII	relative interaction index
SIR	systemic induced resistance
SIS	systemic induced susceptibility
UPLC	using ultra-high pressure liquid chromatography

Chapter 1

Introduction

In recent decades, there have been shifts in the ranges of species due to climate change (Parmesan and Yohe 2003). The range expansion of species has the potential to significantly affect ecosystem dynamics and natural resilience. Understanding the interactions between native and invasive species is important to determine how range expansions of species affect naïve ecosystems.

The recent outbreak of mountain pine beetle (*Dendroctonus ponderosae* Hopkins, MPB) in western Canada has killed 16.3 million ha of lodgepole pine (*Pinus contorta* Dougl. ex Loud.) forests in British Columbia over a ten-year period (Safranyik et al. 2010). Much of this outbreak is in areas that are historically north of the endemic MPB (Safranyik et al. 2010); this rapid range expansion is thought to have been driven by climate change. As the MPB expands eastwardly into Alberta, it has spread across the lodgepole × jack pine hybrid zone, threatening to expand further into pure jack pine (*Pinus banksiana* Lamb.) forests (Cullingham et al. 2011), which extend from Alberta to eastern Canada. Several laboratory and field studies confirmed that jack pine is a host for the MPB or its associated fungi (Rice et al. 2007; Colgan and Erbilgin 2010; Lusebrink et al. 2011, 2016; Erbilgin et al. 2014a, 2017). While attacking a tree, MPB introduces its associated fungal symbionts, such as *Grosmannia clavigera* (Robinson-Jeffrey & Davidson), which deplete tree defenses and restricts water and nutrient flow between foliage and roots (Raffa and Berryman 1983a, b). The combined impacts of beetle damage to the phloem and fungal inoculation are integral for beetle success and reproduction (Raffa and Berryman 1983b;

Six and Wingfield 2011). Furthermore, these pathogenic fungal associates provide nutrition to developing beetles (Klepzig and Six 2004; Goodsman et al. 2012). Mountain pine beetle was recently intercepted just west of the Saskatchewan-Alberta border and just north of the Northwest Territories-Alberta border.

The historical host pines for MPB have physical and chemical defenses that protect them from a MPB, MPB-associated fungi, and the multitude of other attacking organisms they experience over their long-life (Langenheim 1994; Franceschi et al. 2005; Keeling and Bohlmann 2006; Eyles et al. 2010). As an important class of defensive chemicals, monoterpenes are toxic to both MPB and its associated fungi (Raffa and Berryman 1983b; Langenheim 1994; Raffa et al. 2005, 2008, 2013; Goodsman et al. 2013). For example, some individual compounds including 3-carene, α-pinene, limonene, and the phenylpropanoid 4-allyanisole have been shown to be particularly toxic at high doses or associated with tree resistance to either MPB or its associated fungi (Raffa and Berryman 1983; Raffa et al. 2005, 2013; Emerick et al. 2008). Furthermore, diterpenes are also an important component of resin and act as physical and chemical defenses against pathogens and insects (Keeling and Bohlmann 2006). Monoterpenes, can also be detoxified and even used as a carbon source by *G. clavigera* (DiGuistini et al. 2011; Wang et al. 2013, 2014). Furthermore, as covered below, some monoterpenes are synergists to MPB pheromones and α-pinene is required for some pheromone production by MPB (Borden et al. 2008; Blomquist et al. 2010).

These chemical defenses can be constitutive and induced along with targeted to a specific attacking organism or broad-based. For example, conifers being infested with bark beetles and their associated fungi have constitutive resin ducts filled with terpenoids toxic to the attacking bark beetles (Franceschi et al. 2005). Conifers also have induced chemical responses at the site of attack that result in a necrotic and resinous lesion filled with terpenoid compounds (Fig 1.1)

(Franceschi et al. 2005; Goodsman et al. 2013). In instances of a successful tree defense response to attack, these lesions compartmentalize both insect and fungal spread (Lieutier 2002; Franceschi et al. 2005). Lesion length is generally interpreted to be a proxy for a resistant or susceptible response in pine-bark beetle-associated fungi interactions. Most researchers have considered shorter lesion lengths be an expression of more efficient defenses and a less susceptible response to attack (Bonello et al. 2006; Krokene et al. 2008; Lusebrink et al. 2011; Goodsman et al. 2013; Arango-Velez et al. 2016). However, *G. clavigera* and jack pine do not have a co-evolved relationship. Therefore, a targeted resistant response by the tree to a non-native pathogen may require recognition of the pathogen by the plant (e.g., Gabriel and Rolf 1990; Keen 1990). Furthermore, others have associated larger lesions with more resistant responses in *Eucalyptus* spp. infected with a canker fungus (*Chrysophorte* spp.) (da Silva Guimarães et al. 2010). However, Erbilgin and Colgan (2012) found that longer lesion lengths from *G. clavigera* inoculation had lower monoterpene concentration in jack pine, which potentially supports the interpretation of shorter lesion lengths indicates a more efficient defense response in jack pine.

Along with this local response at the site of inoculation, there are systemic increases in defense chemicals around the lesion (Goodsman et al. 2013). The lesion expands mostly above and below the site of fungal inoculation and is therefore vertically oriented on the tree (Fig. 1.1). The phloem tissue just above and below this expanding lesion would be the next tissue invaded by the infecting fungus. Therefore, throughout this thesis, I call this phloem the 'defensive zone' and consider it the area where the tree could selectively allocate resources towards the production of defenses in an effort to limit lesion expansion (Figure Appendix B.1). Along with acting in a defensive role, tree chemicals can play a number of ecological roles in the bark beetle-fungus-tree relationship. Furthermore, systemic responses to initial attack by an insect or

pathogen can alter the tree response to subsequent attack by the same or different organism in different parts of the tree (Bonello et al. 2006; Eyles et al. 2010; Colgan and Erbilgin 2011; Klutsch et al. 2016). This phenomenon is termed systemic induced resistance (SIR) and has been reported for a number of conifer-pathogen systems (Wallis et al. 2008; Villari et al. 2014). The original hypothesis from Bonello et al. (2006) states that after an initial induction event, trees can respond to subsequent attack with an induced resistant response as compared to before the initial induction event. However, as symptoms start manifesting, the stress of infection becomes too great, or environmental conditions limit defense production, a tree may then have a reduction in its relative resistance to the point of becoming more susceptible to attack than before the initial induction event (Bonello et al. 2006). This increase in relative susceptibility is termed systemic induced susceptibility (SIS) (Bonello et al. 2006). This relative resistance is not to say that the tree is resistant to attack by MPB and its associated fungi. Instead, a resistant response, as used in this thesis, only refers to relative resistance compared to trees without the initial induction event. Therefore, a tree that expresses SIR to *G. clavigera* would be less optimal for fungal infection than a tree without the initial inducing event but the tree could still be overcome by MPB or its fungi. The mechanisms that can underlie induced resistant or susceptible responses are defense chemicals (Bonello et al. 2006). However, how the production and allocation of resources towards defense chemicals is regulated or coordinated is not understood well in conifers.

Populations of MPB range from endemic to epidemic levels in its historical host range (Raffa 1988; Safranyik and Carroll 2006). At high population levels, MPB is able to attack trees at high densities, which allows them to overcome defenses of large and healthy host trees (Raffa 1988). During these outbreak population phases, MPB is able to cause extensive host mortality over large areas of forest. However, MPB normally exists in endemic populations and is restricted to attack weakened, suppressed or damaged trees (Safranyik and Carroll 2006; Smith et

al. 2011). Mortality of host trees from endemic populations of MPB is usually scattered throughout forests (Carroll and Safranyik 2006). The switch between the endemic into epidemic phase, which is termed as the incipient epidemic phase, involves a change in MPB's colonization behavior (Carroll and Safranyik 2006). During the incipient epidemic phase, the increase in beetle population can be due to drought or other disturbances that stress trees and therefore increase the success of beetles in trees and increase the number of susceptible trees (Raffa et al. 2008). Also, the expansion of host selection behavior by MPB can be due to the increase the concentration of the aggregation pheromones within an area as beetle populations increase (Carroll and Safranyik 2006). This is important because beetles use semiochemicals to attract conspecifics that increases attack densities and help overcome tree defenses (i.e., aggregation pheromones) (Safranyik and Carroll 2006). Mountain pine beetles also coordinate and direct attacks to neighboring trees to minimize intraspecific competition within an attacked host tree (i.e., anti-aggregation pheromones) (Safranyik and Carroll 2006). The first attacking female MPB produce the aggregation pheromone *trans*-verbenol that preferentially attracts male MPB (Safranyik and Carroll 2006). Another aggregation pheromone (*exo*-brevicomin) is produced by the attracted males at low concentrations, which together with trans-verbenol attracts more MPB (Safranyik and Carroll 2006). As densities of attacking beetles reach an optimum density, the concentrations of *exo*-brevicomin and frontalin by males increase, which act as deterrents, and beetles produce the anti-aggregation pheromone verbenone (Raffa and Berryman 1983a; Safranyik and Carroll 2006). This regulation of attack density minimizes competition among developing brood MBP and directs further attacks to neighboring trees. Tree chemicals are also integral in the aggregation process, as a number of tree monoterpenes (e.g., α-pinene, myrcene and terpinolene) act as synergists to MPB aggregation pheromones (Borden et al. 2008).

Furthermore, trans-verbenol and verbenone are oxidative products of the host tree monoterpene α-pinene (Blomquist et al. 2010).

Although colonization of jack pine forests has initially occurred as mass dispersal of MPB from British Columbia and western Alberta, subsequent MPB generations will most likely establish and persist at low (≈endemic) levels, and population growth will likely be constrained for many years by low winter temperatures (Régnière and Bentz 2007). Therefore, population persistence will depend on weakened or stressed trees, and MPB spread and survival will be affected by interactions with other organisms that are the primary stressors of jack pine in the western boreal forest. Boone et al. (2011) demonstrated that tree induced defenses are a crucial determinant of MPB success at low population densities, and hence of whether beetle populations can transition into outbreaks.

Earlier studies in MPB-jack pine interactions have found that (1) attacks of jack pine budworm (*Choristoneura pinus pinus* Freeman, Lepidoptera: Tortricidae), a prominent insect pest of jack pines, increased host resistance to one of the fungal associates of the beetle, *Grosmannia clavigera* Robinson-Jeffrey & Davidson, and (2) the sequence of attack can be important in determining jack pine susceptibility to budworm or *G. clavigera* (Colgan and Erbilgin 2011). Thus, the type of interaction (synergistic or antagonistic) between native insects and MPB could influence the MPB invasion process in the boreal ecosystem. Along with tree responses to biotic attack, drought can lead to increased susceptibility of historical host species to attack by MPB (Raffa et al. 2008; Bentz et al. 2010; Creeden et al. 2014; Kolb et al. 2016). When drought conditions are experimentally applied to jack pine, trees have lowered chemical defense responses than lodgepole pine trees (Lusebrink et al. 2011; Arango-Velez et al. 2016; Erbilgin et al. 2017), which indicates the potential for increased susceptibility of jack pine to MPB or its associated fungi. Such investigations can be done proactively, which is of great

advantage for identifying jack pine stands susceptible to MPB and for developing pre-emptive management strategies prior to MPB arrival.

Lodgepole dwarf mistletoe (*Arceuthobium americanum* Nutt. ex Engelm.), a parasitic plant, is the most widespread tree pathogen in the jack pine forest. Dwarf mistletoe infections slowly progress and intensify with time, resulting in reduced tree vigor and increased probability of mortality (Hawksworth and Wiens 1996). Dwarf mistletoe interacts with several bark beetles in lodgepole pines, including MPB (Johnson et al. 1976; Kenaley et al. 2006; Smith et al. 2011), and it may precondition trees for MPB attack and thereby promote invasion into the boreal forest. Dwarf mistletoe currently occurs at high levels in Alberta, Saskatchewan and Manitoba (Brandt et al. 1998). Therefore, it is particularly critical to understand their interactions because the eastward expanding wave of MPB will likely first encounter highly abundant dwarf mistletoe-infected jack pine trees and colonize them upon arrival.

Furthermore, MPB has important interactions with competing insects in its historical hosts (Safranyik et al. 1999). These insects share the same subcortical environment and are limited by the same resources (Safranyik et al. 1999, 2010; Allison et al. 2001, 2004). However, while bark beetles, such as MPB and *Ips pini* (Say) primarily feed on the phloem of their hosts during the larval stage, woodboring beetle larvae (Coleoptera: Ceramybicidae) begin their development by feeding on phloem, and migrate into xylem as they develop. While *I. pini* has been studied as a common competitor to MPB in its historical range (e.g., Safranyik et al.1999) and is present in jack pine forests (Schenk and Benjamin 1969), interactions between MPB and other subcortical insect herbivores, including woodboring beetles (Coleoptera: Curculioniade and Cerambyicidae) can also be important as they both rely on stressed host trees to infest. There is a close association between bark and woodboring beetles in terms of host colonization as some woodboring beetles, particularly in the family cerambycidae, use bark beetle pheromones to

detect suitable host trees, which usually lead to asymmetric competition between these two groups in favor of woodboring beetles (Allison et al. 2001, 2004). Competition for phloem resources and even in some cases predation by woodboring beetle larvae on early developmental stages of MPB are therefore expected to occur between these beetles (Safranyik et al. 1999; Dodds et al. 2001; Schoeller et al. 2012). In some cases, however, woodboring beetles have been shown to facilitate bark beetle development (Smith et al. 2011). Also bark and woodboring beetles may be affected by secondary compounds of host plants, deployed in combinations of constitutive (pre-existing) and induced (post-attack) structural and biochemical mechanisms (Franceschi et al. 2005; Colgan and Erbilgin 2011; Erbilgin and Colgan 2012). Therefore, host condition may impact interactions among subcortical insects that share the same resources and thus impact the success of MPB in jack pine.

1.1 Thesis aims

The aim of my thesis research is to better understand factors that influence jack pine susceptibility to MPB as it expands into the boreal forest. This project uses the MPB range expansion as a model system to proactively investigate how interactions with native organisms (e.g., dwarf mistletoe) may influence the establishment of a non-native bark beetle. I hypothesize that tree response to dwarf mistletoe infection will be dependent on the intensity of infection, with low intensity infections of dwarf mistletoe inducing resistance to MPB attack, and high intensity infections leading to greater susceptibility to attack by MPB. The following objected will be tested: (1) explore coordination and trade-offs of defense chemical production; (2) quantify pathogen-induced changes in jack pine chemistry; (3) identify how these changes influence MPB success; and (4) examine the effect of a native pathogen on intra-guild interactions between MPB and native insect competitors. The resulting information from such

tripartite interactions among trees, pathogens and insects will enhance our understanding of complex interactions that likely occur in all forest ecosystems.

In Chapter 2, I examine the cross-induction of resistance from abiotic and biotic disturbances on jack pine defenses to a MPB-associated fungus in a greenhouse study. I show that prior induction from phytohormones (methyl salicylate and methyl jasmonate) results in systemic cross-induction of resistance to the MPB-associated fungus (*G. clavigera*) under normal watering treatment, but susceptibility under low watering treatment. These results demonstrate that drought can affect interactions among tree-infesting organisms through systemic induced susceptibility. This chapter is titled "Drought stress leads to systemic induced susceptibility to a nectrotrophic fungus associated with mountain pine beetle in jack pine seedlings" and was submitted for publication in a PLOS ONE.

In Chapter 3, I identify the impact of dwarf mistletoe-induced defenses on the MPB-associated fungus *G. clavigera*. Dwarf mistletoe infection had a non-linear systemic effect on monoterpene production, with increasing concentrations of monoterpenes at moderate severities and decreasing at high severities. Inoculation with *G. clavigera* resulted in 33 times greater concentration of monoterpene production and half the level of phenolics in the necrotic lesions compared with control trees not inoculated with *G. clavigera*. Monoterpene production following dwarf mistletoe infection seemed to result in systemic induced resistance, as trees with moderate disease severity were most resistant to *G. clavigera*, as evident from shorter lesion lengths. These results demonstrate that interactions between biotrophic and necrotrophic fungi may impact MPB establishment in novel jack pine forests through systemic effects mediated by the coordination of jack pine defense chemicals. This chapter is titled "A native parasitic plant systemically induces resistance in jack pine to a fungal symbiont of invasive mountain pine

beetle" and is currently accepted for publication in the Journal of Chemical Ecology (See Preface).

In Chapter 4, I examine the interspecific herbivore interactions to determine the competitive effect of intra-guild insects on MPB performance as a function of dwarf mistletoe infection. Mountain pine beetle performance was negatively associated with woodboring beetle feeding and dwarf mistletoe severity when reared separately. However, when both woodboring beetles and a high severity of mistletoe infection occurred together, MPB escaped from competition and improved its performance (increased brood production and feeding). Species-specific responses to changes in tree defense compounds (monoterpenes) and quality of resources (available phloem) were likely mechanisms driving this change of interactions between the two beetle groups. This chapter is titled "Direction of interaction between mountain pine beetle (*Dendroctonus ponderosae*) and resource-sharing woodboring beetles depends on plant parasite infection" and is published in Oecologia (2016, 182: 1-12) (See Preface).

Figure 1.1. Lesion formation six weeks after inoculation by *Grosmannia clavigera* in *Pinus banksiana* phloem tissue. Bar is 1 cm.

Chapter 2

Drought stress leads to systemic induced susceptibility to a nectrotrophic fungus associated with mountain pine beetle in *Pinus banksiana* seedlings

2.1 Introduction

Conifers have physical and chemical defenses that act to protect them from attacks by both insects and pathogens. However, due to the high energetic cost of defense responses, host tree susceptibility can be dependent on predisposing factors such as drought (e.g., Gaylord et al. 2013). Recent changes in drought patterns in western and boreal forests in North America have altered conifer susceptibility to a number of pest insects and pathogens (Brashears et al. 2009; Allen et al. 2010; Sturrock et al. 2011; Preisler et al. 2012; Weed et al. 2013; Kolb et al. 2016). For example, intense droughts have led to increased susceptibility of trees to attack by bark beetles, including mountain pine beetle (*Dendroctonus ponderosae* Hopkins, MPB) (Coleoptera: Curculionidae, Scolytinae) (Raffa et al. 2008; Bentz et al. 2010; Creeden et al. 2014; Kolb et al. 2016). During the last outbreak, MPB killed millions of trees, mainly lodgepole pine (*Pinus contorta* Dougl. ex Loud.), in western Canada and has recently spread into the novel host jack pine (*Pinus banksiana* Lamb.) in the boreal forest and threatens to expand to the eastern coast of North America (Cullingham et al. 2011; Erbilgin et al. 2014a). Along with drought being a predisposing factor affecting tree susceptibility to MPB, there are also multiple attacking insects and pathogens that can elicit defense responses in host trees and subsequently can impact host susceptibility MPB (Colgan and Erbilgin 2011; Klutsch et al. 2016). Thus, understanding the role of drought on host-pathogen-insect interactions can give insight to the mechanisms that influence

multitrophic interactions under a changing climate with factors that predict the susceptibility of jack pine to the invasive MPB.

Conifers rely on chemical defense against bark beetles and their associated pathogenic fungi. Conifer defense chemicals can be constitutive and act as anti-feedants and toxins such as terpenoid and phenolic compounds (Franceschi et al. 2005; Raffa et al. 2005; Keeling and Bohlmann 2006) that repel infestation by attacking organisms (Larsson 2002; Franceschi et al. 2005). After the initial attack by an insect or pathogen, host defense chemicals can be induced both locally (at the site of attack) and systemically (in distant parts of the tree). These defense responses negatively impact the performance of the attacking organism and deter further attacks in other parts of the tree (Franceschi et al. 2005; Eyles et al. 2010). The initial attack by an organism can therefore have cascading effects on the success of further colonization by the same or different organisms sharing the same host plant though systemically induced tree responses (Eyles et al. 2007; Colgan and Erbilgin 2011; Tack et al. 2012; Klutsch et al. 2016).

The outcome of systemic responses can make plants either resistant or susceptible to biotic attack agents. Systemic induced resistance (SIR) is when initial attack can make a tree resistant to subsequent attacks (Bonello et al. 2006). This is usually expressed at the early phases of pathogen infection or insect infestation, when the host defensive capabilities are not substantially impaired (Bonello et al. 2006; Klutsch et al. 2016; Sherwood and Bonello 2016). As the initial attack progresses, however, the defense machinery of the tree may break down and the tree expresses systemic induced susceptibility (SIS) to subsequent attack (Bonello et al. 2006). The switch from SIR to SIS is dependent on the availability of resources, such as carbohydrates, where plants with limited ability to acquire additional resources may become susceptible to further attack by organisms (Arnold et al. 2004; Sherwood et al. 2015). Drought conditions can reduce stomatal conductance and photosynthetic rate in trees, which negatively

impacts the acquisition of photosynthates needed for defense metabolite production (Llusià and Peñuelas 1998; Lusebrink et al. 2011; Arango-Velez et al. 2016). Furthermore, carbohydrates that are stored or translocated from other tissues are required for biosynthesis of induced defense metabolites against insect and pathogen attack or tolerance mechanisms, such as compensatory growth, which also creates a demand for additional resources (Goodsman et al. 2013; Erbilgin et al. 2014b). Since drought conditions can be concurrent with the attack by multiple organisms, drought can potentially alter insect or pathogen-induced host responses to subsequent attacks.

After a biotic or abiotic stress event, several phytohormones, such as salicylic acid, jasmonic acid and their methylated forms, signal the induction of host defense chemicals that can be dependent on the feeding habit of an attacking organism (Metraux et al. 1990; Truman et al. 2007; Bari and Jones 2009). Infection by biotrophic and hemi-biotrophic pathogens (organisms that acquire nutrients from live plant cells, and in the case of hemi-biotrophs can at times also use dead plant cells) and feeding from phloem-sucking insects triggers the plant defense signaling pathway characterized by accumulation of methyl salicylate (MS) (Walling 2000; Bari and Jones 2009; Arango-Velez et al. 2016). Induction of the salicylic acid pathway through attack by organisms or exogenous application of MS has been shown in several annual and perennial plant systems to increase resistance to subsequent attack by pathogens and insects (Kozlowski et al. 1999; Davis et al. 2002; Bari and Jones 2009; Thaler et al. 2010). Evidence for MS-dependent defense chemical responses or the effects on subsequent resistance to attack, however, is scarce in conifers (Hudgins and Franceschi 2004; Corcuera et al. 2012; Erbilgin and Colgan 2012; Arango-Velez et al. 2016). In contrast, the jasmonic acid signaling pathway is associated with host defense against necrotrophic pathogens (microorganisms that kill plant tissue and acquire nutrients from dead cells) and herbivorous insects (Bari and Jones 2009; Arango-Velez et al. 2016). Application of methyl jasmonate (MJ) on conifers results in

anatomical changes, such as production of traumatic resin ducts, and induction of terpenoid and phenolic compound accumulation (Herrmann and Weaver 1999; Franceschi et al. 2005; Erbilgin et al. 2006; Krokene et al. 2008; Erbilgin and Colgan 2012).

There are several symbiotic fungi associated with MPB that are generally thought to be important contributors to tree mortality, which is necessary for successful beetle reproduction (Raffa and Berryman 1983b). These pathogenic fungi block transport of water and nutrients in the tree, help the beetle overwhelm tree defenses, detoxify some defensive compounds, and even provide nutrition to developing beetles (Raffa and Berryman 1983b; Paine et al. 1997; Klepzig and Six 2004; Lieutier et al. 2009). Furthermore, MPB-associated fungi have been shown to induce chemical changes in multiple pine species, such as decreased concentrations of non-structural carbohydrates and increased concentration of monoterpenes (Lusebrink et al. 2011; Goodsman et al. 2013; Arango-Velez et al. 2016; Keefover-Ring et al. 2016). Infection also induces the formation of a resin-filled lesion with high concentrations of monoterpenes that can be toxic to both MPB and fungi, with shorter lesions indicating greater resistance to fungal spread (Krokene et al. 2008).

We used jack pine seedlings and the MPB-associated fungus *Grosmannia clavigera* (as a proxy for MPB) to investigate the impact of drought on plant induced responses elicited from multiple signaling pathways and the SIR or SIS of trees to subsequent attack. Our objectives were to: (1) determine whether the local and systemic effect of different initial induction elicitors (*G. clavigera*, MS, and MJ) on monoterpene defenses of jack pine depends on drought stress, and (2) examine whether the combination of prior induction of defenses from initial induction elicitors and drought stress affect jack pine responses to subsequent challenge from *G. clavigera*.

2.2 Methods

One-year-old jack pine seedlings (n = 200) were obtained from Pineland Forest Nursery, Manitoba, Canada, in spring of 2012 (Seed lot #0-10-04.1-I-1635). Seedlings were planted in 4 L pots filled with Sunshine Mix #4 (Sungro, Vancouver, BC, Canada) and maintained in a greenhouse at the University of Alberta under ambient light supplemented with full spectrum lighting (light:dark of 12:12) from March 2012 to July 2013. To meet dormancy requirements, seedlings had an eight week period of cold hardening in a cold room (4°C with light:dark of 10:14 starting 13 November 2012) before the second growth cycle. Throughout the growth and dormancy periods, seedlings were watered once a day with acidified water (pH of 5.5). During the growth period and up to the time of the initiation of the watering treatments (13 May 2013), fertilizer (17 N - 5 P - 19 K at 175 ppm N plus periodic micronutrient fertilization) was applied weekly. During the last four-weeks, a conditioning phase was applied before cold hardening and seedlings received a different fertilization regime (8 N - 20 P - 30 K at 50 ppm N).

After cold hardening (mid-January 2013), the seedlings were returned to the greenhouse and growth period conditions were resumed. The seedlings were randomly divided into eight blocks with 24 seedlings per block (192 seedlings total). A three-factorial design in a randomized-block arrangement was used for the application of the following treatments: 3 levels of watering treatment (normal, moderate, and low) x 4 types of initial induction treatment (control, *G. clavigera* inoculation, MJ, and MS application) x 2 types of challenge inoculation with *G. clavigera* (non-challenged vs. challenged). The *G. clavigera* used in this study was grown on malt extract agar and was originally isolated from MPB from Fox Creek, AB (54°24'N, 116°48'W) by AV Rice at Northern Forestry Centre, Canadian Forest Service, Edmonton, AB and is housed and maintained by N Erbilgin at University of Alberta, Edmonton,

AB. This design resulted in a total of eight seedlings per watering x induction x challenge treatment with each treatment combination being represented once in each block. To standardize watering treatments and prevent water from draining from the pots, each pot was placed on individual pot saucers. The low watering treatment was initiated on 13 May 2013 and continued to the end of the experiment (56 days) on eight seedlings per block and consisted of the daily application of water at 10-20 % volume of the normal watering treatment. The moderate watering treatment, which consisted of daily application of 30-40 % volume of water of the normal watering treatment, was initiated on eight seedlings per block on 24 May 2013 to the end of the experiment (45 days). The different extent and intensity of water limitation between low and moderate watering treatments reflects the predictions from climate models for the next century that there will be an increase in drought frequency, extent, and intensity in many forest ecosystems (Sheffield et al. 2012; Fischer et al. 2013). The remaining seedlings were assigned the normal watering treatment, which consisted of about ~200 ml of water daily. Watering treatments were maintained until harvesting.

The initial induction treatments (i.e., *G. clavigera*, MJ, and MS) were applied on the lower third of the stem to all seedlings on 10 June 2013 (i.e., 4 and 2 weeks after initiation of low and moderate watering treatments, respectively). We did not apply a wounding treatment without inoculation because experiments that used the same jack pine seedling system (e.g., Lusebrink et al. 2011; Erbilgin and Colgan 2012; Arango-Velez et al. 2016) found smaller lesions from wounding than inoculation with *G. clavigera* and similar physiological and hormone, and defensive responses, as non-wounded controls. Within blocks, seedlings were arranged into nested groups by their induction treatment to minimize any possible interaction between treatments. Seedlings in the control treatment did not receive any induction treatment. The fungal inoculation involved the removal of three disks of bark (4 mm dia.) that were spaced about 2 cm

apart vertically and equally distributed around the stem (Erbilgin and Colgan 2012). The wounds were immediately inoculated with an agar plug of *G. clavigera* and covered with Parafilm (Pechiney Plastic Packaging Company, USA). For the MJ and MS applications, 100 mM solutions with 0.1% (v/v) Tween 20 were applied to the bottom third of trees using foam brushes. Application of the signaling hormones was conducted in separate rooms and the solutions were allowed to dry and absorb on trees for 24 hrs before re-assembling seedlings into blocks. While there is a potential for volatiles from MJ or MS applications to interact with other trees, others have successfully used similar methods in greenhouse experiments (e.g., Erbilgin and Colgan 2012) and we nested induction treatments within blocks as an attempt to minimize these interactions.

On 24 June 2013, half of the seedlings in each of the watering x induction x challenge treatments were challenged with a *G. clavigera* inoculation on the middle third of the stem. The same inoculation procedure was used as the initial induction from *G. clavigera*. These fungal challenge inoculations were on average 17.6 cm (SE=0.3) above the initial induction inoculations. Two weeks after fungal challenge (8 July 2013), all seedlings were harvested. Bark (including phloem tissue) was separately removed from the lowest third of stems, middle third of stems, and fungal-induced necrotic and resin-filled lesions (Table 2.1). The length of each lesion was measured and averaged by either induction treatment or fungal challenge treatment. All tissues were immediately placed in liquid nitrogen and stored at -40°C until processing. Tissues were separately ground using mortar and pestle with liquid nitrogen. Seedling height and stem base diameter were also measured.

2.2.1 Soil water content and stomatal conductance

To monitor the effect of watering treatments, we measured soil water content and stomatal conductance every week starting the day of initial induction treatment application on two randomly selected blocks. Soil water content was measured using time-domain reflectometry with a Tektronix 1502B (Beaverton, OR, USA). The empirical equation for organic soils (Robinson et al. 2003) was used to convert the apparent dielectric constant of the soil to water content. An AP4 Leaf Porometer (Delta-T Devices Ltd., Cambridge, UK) was used to measure stomatal conductance on two current year needles of each tree within the selected block, which was corrected for needle area and averaged per tree.

2.2.2 Monoterpene analysis

Dichloromethane extracted compounds, which mainly consist of monoterpenes, were measured using established methods (Klutsch et al. 2016). Briefly, ground tissue samples (100 mg) were extracted twice with 0.5 ml of dichloromethane and an internal standard of 0.004 % tridecane. Samples with solvent were vortexed for 30 s, sonicated for 10 min, and centrifuged at 16,100 rcf at 0°C for 15 min. Sample extract (1 µl) was injected into a Gas Chromatograph/Mass Spectrometer (Agilent 7890A/5062C, Agilent Tech., Santa Clara, CA, USA) equipped with a HP-INNOWax column (I.D. 0.25 mm, length 30 m) (Agilent Tech.) with He carrier gas flow at 1 ml min^{-1}, and a temperature of 50°C for 0.5 min, increased to 60°C by 2°C min^{-1} and held for 1 min, increased to 120°C by 10°C min^{-1} and held for 1 min, and then increased to 250°C by 30°C min^{-1}. To quantify individual and total compounds (ng mg^{-1} of fresh tissue, hereafter concentration), the following 14 standards were used: α-terpinene, γ-terpinene (Sigma-Aldrich, St. Louis, MO, USA), 3-carene, terpinolene, α-pinene, β-pinene, limonene, myrcene, camphene, *p*-cymene, 4-allylanisole (Fluka, Sigma-Aldrich, Buchs, Switzerland), bornyl acetate (SAFC

Supply Solutions, St. Louis, MO, USA), and β-phellandrene (Glidco Inc., Jacksonville, FL, USA).

2.2.3 Data analysis

ANOVAs with random effects for blocks and nesting of induction treatments within blocks were separately run to compare the effect of induction and watering treatments, and their interaction on monoterpene concentration and proportion within the following tissues: 1) the lowest third of the stem without fungal challenge (i.e., area of induction treatment application), 2) middle third of the stem in trees without fungal challenge (i.e., systemic to the area of induction treatment), 3) middle third of stem in trees with fungal challenge (i.e., area surrounding fungal lesion), and 4) lesions of the fungal challenge. Lesion length from fungal challenge was also compared between induction and watering treatments (PROC MIXED in SAS, ver. 9.3). The random effect of induction treatment nesting within blocks was removed from all models because the Akaike's information criterion was lower when accounting for blocks alone. Where the interaction term was not significant, it was removed from the model. Tree height and stem base diameter were not significant covariates in any models and thus were not included in the final analyses. Where interaction terms were significant, partial ANOVA models with blocking were used to determine the effect of induction treatment on a response variable at each watering treatment level. Tukey's HSD tests were performed for multiple comparisons among induction treatments. A contrast statement was used to compare monoterpene concentration in control seedlings to those treated with MJ or MS. At each measurement date for soil water content and stomatal conductance, an ANOVA with blocking was used to test whether there was an effect of watering treatment. To meet assumptions of normality and homogeneity of variance, monoterpene concentration and lesion length were natural log transformed. Back-transformed least square means with 95%

confidence intervals are presented. Non-metric multidimensional scaling (NMDS) with Bray-Curtis distance and perManova with blocking were used to see whether the profile of monoterpenes varied with induction and watering treatments (R software, ver. 3.2.1). Significance level for ANOVAs, perManovas, and Tukey's HSD tests are at $\alpha=0.05$ unless otherwise reported.

2.3 Results

2.3.1 Soil water content and stomatal conductance

At the time of induction treatment application, the soil water content was almost 3 and 10 times lower in the moderate and low watering treatments than in the normal watering treatment, respectively (Fig. 2.1A). The differences between normal and low watering treatments were maintained 28 days after the application of the induction treatment until the time of tissue sampling. Furthermore, trees in the low watering treatment had almost 3 times lower stomatal conductance than trees with normal watering treatment at the time of induction treatment application (Fig. 2.1B). Similarly, stomatal conductance was consistently lower in trees with low and moderate watering treatments compared with normal to the time of tissue sampling.

2.3.2 Effect of water availability on local response to induction treatment

Seedlings treated with MJ experienced high mortality, with the final number of live trees in the normal, moderate and low watering treatments being 6, 5, and 4, respectively. There was no mortality of seedlings in the other induction treatments. Across all watering treatments, total monoterpene concentration in the bark of the lower third of seedlings (at the site of induction

treatment application) varied with initial induction treatment (Fig. 2.2A). In contrast, across all induction treatments, monoterpenes did not vary with watering treatments in seedlings (Fig 2.2B). There was no interaction effect between induction and watering treatments on monoterpene responses. The lesion tissue had 5.6 times greater concentration of total monoterpenes than in control trees and was also greater than bark treated with signaling hormones (Fig. 2.2A). Four individual monoterpenes (α-pinene, β-pinene, β-phellandrene, camphene) were also greater in the lesion than in trees with other induction treatments. Furthermore, fungal lesions had greater concentrations of myrcene, limonene, γ-terpinene and *p*-cymene than control and MS treated trees, but not MJ treated trees. The bark treated with MJ had two times greater concentration of total monoterpenes, along with greater concentration of five individual monoterpenes (α-pinene, β-pinene, β-phellandrene, camphene, myrcene), compared with control trees and MS treated bark. The concentration of total monoterpenes in bark treated with MS was the same found in control trees.

Across all watering treatments on the lowest third of trees, the relative proportion of individual monoterpenes varied with induction treatment, not with water treatment. There was proportionally more β-pinene in fungal lesions (49.3%, $CI_{95\%}$=47.2-51.4%) compared to control trees (38.1%, $CI_{95\%}$=36.1-40.2%), MJ (44.4%, $CI_{95\%}$=41.8-47.0%), and MS (39.1%, $CI_{95\%}$=37.0-41.2%) treated trees ($F_{3,72}$=24.0, P<0.001). Furthermore, the proportion of camphene in lesions (1.5%, $CI_{95\%}$=1.3-1.6%) was greater than in control (1.2%, $CI_{95\%}$=1.0-1.3%) and MS (1.1%, $CI_{95\%}$=1.0-1.3%) treated trees ($F_{3,72}$=3.93, P=0.012). In contrast, fungal lesions had the lowest percent proportion of both myrcene (1.2%, $CI_{95\%}$=0.0-2.4% [$F_{3,72}$=2.83, P=0.044]) and β-phellandrene (0.9%, $CI_{95\%}$=0.8-1.0% [$F_{3,72}$=40.78, P<0.001]) compared to control trees (3.5%, $CI_{95\%}$=2.3-4.7%, and 1.3%, $CI_{95\%}$=1.2-1.4%, respectively). Fungal lesions also had the lowest proportion of β-phellandrene compared to trees treated with signaling hormones.

Only the phenylpropanoid 4-allylanisole varied in the bark of the lower third of trees with watering treatment ($F_{2,72}$=5.61, P=0.005) along with induction treatment ($F_{3,72}$=3.80, P=0.014). The bark treated with MJ had 12 times greater concentration of 4-allylanisole (2.4 ng mg^{-1} [$CI_{95\%}$=1.7-5.6 ng mg^{-1}]) compared to fungal lesion tissue (0.2 ng mg^{-1} [$CI_{95\%}$=0.1-0.3 ng mg^{-1}]), but was not different than control or MS treated bark. Also, across all induction treatments, trees in the moderate watering treatment had more than seven times greater 4-allylanisole concentration compared to trees in the low watering treatment (t=3.24, P=0.005), and four times more than trees in the normal watering treatment (t=-2.33, P=0.058).

The monoterpene profile at the lowest third of trees varied with induction treatment (perManova $F_{3,73}$=40.93, P=0.001) and not with watering treatment ($F_{2,73}$=1.77, P=0.156). This pattern is illustrated in the NMDS analysis where the concentrations of many monoterpene compounds (represented by arrow vectors) were positively associated with trees treated with *G. clavigera* or MJ (Fig. 2.3A).

2.3.3 Effect of water availability and induction treatment on local response to fungal challenge

For seedlings challenged with *G. clavigera* after initial induction, mortality was high in the ones treated with MJ, with the final number of live trees in the normal, moderate and low watering treatments being 7, 6, and 4, respectively. There was no mortality of seedlings in the other induction treatments. The accumulation of total monoterpenes in lesions formed by the fungal challenge inoculation did not vary with initial induction treatment (Fig. 2.2A) but instead only varied with watering treatment (Fig. 2.2B). There was no interaction effect between induction and watering treatments on monoterpene responses. From Tukey's HSD tests (α=0.1), the concentrations of total monoterpenes (Fig. 2.2B) and camphene ($F_{2,76}$=2.54, P=0.086) were

weakly greater in seedlings in the normal watering treatment compared to moderate and low watering treatments. In addition, from a Tukey's HSD test with α=0.05, the concentration of β-pinene was 55% greater in normal watering treatment compared to trees in low watering treatment ($F_{2,76}$=4.67, P=0.012). Similarly, β-pinene made up 50.1% ($CI_{95\%}$=48.3 – 51.9%) of total monoterpenes in seedlings in normal watering treatment, which was significantly greater than in the low watering treatment (45.0%, $CI_{95\%}$=43.1 – 46.9%; $F_{2,76}$=7.48, P=0.001). In contrast, while the concentration of 3-carene also significantly varied with watering treatment ($F_{2,76}$=3.59, P=0.033) across all induction treatments, a Tukey's HSD test (α=0.1) showed that trees treated with moderate and low watering treatments had greater concentrations than normal watering treatment. There was also an effect of induction treatment on one dichloromethane extracted compound, bornyl acetate. Methyl jasmonate treated seedlings had nearly four times greater concentration of bornyl acetate in the fungal challenge lesion than control seedlings and bornyl acetate concentrations were the same in MJ and fungal induction treated seedlings ($F_{3,76}$=4.63, P=0.005).

In the multivariate analysis of the profile of monoterpenes in the fungal challenge lesions, the concentrations varied by treatment (perManova $F_{3,88}$=2.23, P=0.039) and weakly by water treatment (perManova $F_{2,88}$=2.04, P=0.067). Most monoterpene concentrations in the fungal challenge lesion were lowest in the fungal and MJ induction treatments compared to control and MS treated seedlings. Furthermore, the concentrations of most monoterpenes were higher in the normal watering treatment seedlings than in the moderate and low.

2.3.4 Effect of water availability on systemic response to induction treatment

Above the induction treatment application area (in the middle third of trees) MJ treated trees had 1.5 times greater concentration of total monoterpenes (Fig. 2.4A) compared to controls. Five

individual monoterpenes (α-pinene, β-pinene, β-phellandrene, camphene, and myrcene) were also greatest in the MJ treated trees relative to other induction treatments and control. There was no effect of watering treatment on monoterpene concentrations at the systemic level (Fig. 2.4B).

The monoterpene profile above the induction treatment site also varied with induction treatment (perManova $F_{3,74}$=3.27, P=0.004) and not with watering treatment ($F_{2,74}$=1.49, P=0.186). Most monoterpene compounds were positively associated with MJ treated trees (Fig. 2.3B). Individual monoterpenes did not proportionally vary with induction or watering treatment.

2.3.5 Effect of water availability and induction treatment on systemic response to fungal challenge

In the defensive area surrounding the lesion caused by the fungal challenge inoculation, there was not an effect of initial induction treatment on concentration of total monoterpenes (Fig. 2.4A), but instead an effect of watering treatment (Fig. 2.4B). There was no interaction effect between induction and watering treatments on monoterpene responses. From Tukey's HSD tests (α=0.10), there was 22% more total monoterpenes (Fig. 2.4B) and 25% more camphene in seedlings in the lowest watering treatment compared to normal watering treatment.

In contrast, the relative proportions of individual monoterpenes were not related to watering treatment, but instead the relative proportion of two monoterpenes varied with initial induction treatment. Methyl jasmonate treated trees had greater percent composition of β-pinene (42.5%, $CI_{95\%}$=38.5 – 46.4%) compared to fungal treatment (34.8%, $CI_{95\%}$=31.6 – 38.0%; $F_{3,76}$=3.12, P=0.031) and greater β-phellandrene (1.4%, $CI_{95\%}$=1.3 – 1.5%) than control seedlings (1.2%, $CI_{95\%}$=1.1 – 1.3%; $F_{3,76}$=2.88, P=0.041).

Furthermore, the profile of the concentration of all monoterpenes did not differ with induction treatments but was affected by watering treatments in the defensive zone around the fungal challenge lesion (perManova $F_{2,88}$=2.22, P=0.039). Concentrations of some compounds (i.e., β-phellandrene, camphene, β-pinene, α-pinene, 4-allylanisole) were positively correlated with seedlings in the low watering treatment.

2.3.6 Effect of water availability and induction treatment on resistance to fungal challenge

The effect of induction treatment on lesion lengths depended on the watering treatment ($F_{6,67}$=2.72, P=0.020; Fig. 2.5). Control seedlings in the normal watering treatment had significantly greater lesion length compared to control seedlings in the low watering treatment from a Tukey's HSD test (P=0.017), while lesion length for seedlings with induction treatments did not vary with watering treatment. From partial models testing the effect of induction treatment for each watering treatment separately, seedlings initially treated with fungal inoculation in the normal watering treatment weakly had shorter lesions from subsequent fungal challenge compared to control seedlings (Fig. 2.5A). Furthermore, seedlings in the normal watering treatment and treated with the signaling hormones had slightly shorter lesions than control seedlings ($F_{1,18}$=3.68, P=0.071). Lesion lengths did not vary with induction treatment for seedlings under the moderate watering treatment (Fig. 2.5B). In contrast, seedlings in the low watering treatment and treated with MJ had significantly longer lesions than control from Tukey's HSD test (Fig. 2.5C). Similarly, seedlings treated with signaling hormone treatments had longer lesions than control trees ($F_{1,17}$=5.16, P=0.036).

2.4 Discussion

Our results demonstrate that water availability affects expression of SIR in jack pine to a bark beetle-associated necrotrophic fungus. Low water availability had no effect on monoterpene accumulation in jack pine induced defenses after initial induction from fungal inoculation or phytohormone application. However, SIR and SIS for *G. clavigera* infection showed some dependence on water availability with jack pine seedlings expressing resistance to subsequent inoculation with *G. clavigera* under normal watering treatment, but susceptibility under low watering treatment. These results extend the SIR hypothesis to include environmental stress and carbon resource availability as conditions altering plant induced responses to subsequent attack.

Furthermore, the type of initial attacker had an impact on tree response and the subsequent expression of SIR or SIS. At initial induction, the local and systemic response to MJ and MS differed, with a greater response in monoterpene concentration to MJ treatment. This is in contrast to Erbilgin and Colgan (2012), who did not find a difference in monoterpene response to MJ and MS application in jack pine seedlings. However, after challenge with *G. clavigera*, monoterpene levels of seedlings initially treated with MJ or MS were the same as control seedlings in this study. Initial inoculation by the fungus resulted in resistance to *G. clavigera* under normal watering treatment, but there was no effect under the low water. In contrast, initial application of the phytohormones resulted in induced resistance under normal watering treatment and induced susceptibility under low water. Even though the monoterpene concentrations induced from the *G. clavigera* challenge were the same among the fungal, MJ, MS and control initial induction treatments, the initial induction treatment systemically induced resistance in trees in the normal watering treatment and susceptibility in the low watering treatment. These results show that tree responses shortly after an initial induction may be driven by the availability of water.

In model plant systems, MS has been shown to interfere with jasmonic acid accumulation (Stout et al. 2006; Thaler et al. 2012). Therefore, the MS treatment in our study should have led to increased susceptibility to the nectrotrophic pathogen *G. clavigera*, which is sensitive to jasmonic acid-dependent defenses (Arango-Velez et al. 2016). However, we found no evidence of antagonistic cross-talk between the salicylic pathway and the potential jasmonic acid pathway induced from fungal inoculation, supporting earlier results in this system (Klutsch et al. In press) and other plant systems (Bostock et al. 2001; Beckers and Spoel 2006). If negative cross-talk does not occur in jack pine, then the attack by a biotrophic organism or the accumulation of endogenous salicylic acid found in jack pine under water stress (Arango-Velez et al. 2016) may not negatively impact the defensive response against *G. clavigera* or mountain pine beetle. Differential regulation of these and other defense pathways and signal synergy between defense responses dependent on particular pathways may also be in play in fine-tuning induced defenses and cross-induction of resistance to multiple organisms (Bostock et al. 2001; Beckers and Spoel 2006; Stout et al. 2006; Klutsch et al. In press).

We found no evidence of water treatment effect on monoterpene concentrations induced from initial induction treatment. Similarly, Erbilgin et al. (2017) found no effect of reduced water conditions on monoterpene responses to *G. clavigera* inoculation in mature jack pine. This result shows that even trees in resource limited environments invest in defenses when they are initially attacked by organisms. López-Goldar et al. (2016) found that feeding on bark tissue by weevils induced the same host defense response in carbon-starved young pines as trees grown in full light. Furthermore, Arango-Velez et al. (2014) found that drought stressed lodgepole pine x jack pine hybrids up-regulated the expression of genes associated with defense. However, if biotic stress continues, only plants with available resources may be able to continue producing defense chemicals, while plants with limited resources may not be able to produce more

secondary metabolites, including monoterpenes, as they are metabolically costly (Croteau and Loomis 1975; Bonello et al. 2006; Walters and Heil 2007). We found that after the initial allocation of resources by seedlings to defense responses, the seedlings in the low and moderate watering treatments had lower monoterpene responses in the lesion from the challenge by *G. clavigera* than seedlings in the normal watering treatment at the local level. We suspect that the initial response is perhaps driven by the non-structural carbohydrates stored in plant tissues; however, continued production of defenses may require allocation of newly synthesized carbohydrates (Teskey et al. 1987; Pallardy 2008; Erb et al. 2011; Lusebrink et al. 2011, 2016; Goodsman et al. 2013). In contrast, at the systemic level, tree response to fungal challenge in the lower watering treatment resulted in higher monoterpene concentrations than in seedlings in the normal or moderate watering treatments, demonstrating that the effect of water availability on the cross-induction of defense responses was dependent on tissue type. Monoterpene concentrations in lesions formed from *G. clavigera* challenge were more than three times greater than in the defensive zone, which suggests that lesions are more energetically costly in terms of secondary metabolism than surrounding tissue (Goodsman et al. 2013). In the energetically costly fungal challenge lesions, cross-induced host responses are potentially limited by the compromised ability to acquire additional resources in drought impacted plants.

While we found that the induced response to *G. clavigera* challenge in jack pine, as measured by lesion length, was positively impacted by water availability (i.e., seedlings in normal watering treatment had longer lesions than trees in the low watering treatment), this does not necessarily mean that reduced water availability made seedlings more resistant to fungal challenge. Others have documented a similar pattern of shorter lesion lengths after an initial inoculation of a bark beetle-associated fungus on drought stressed pine seedlings (Croisé and Lieutier 1993; Arango-Velez et al. 2014, 2016). This pattern may be due to slower *G. clavigera*

growth and lesion development in low water conditions compared to normal water (Bleiker and Six 2009; Arango-Velez et al. 2016). Furthermore, drought stressed lodgepole pine x jack pine hybrids down-regulated a subset of defense-associated genes when infected by *G. clavigera* (Arango-Velez et al. 2014). Therefore, in comparison with the control induction treatment within each watering treatment, the application of the initial phytohormone stress elicitors in our study resulted in the cross-induction of susceptibility to *G. clavigera* in the low watering treatment compared to seedlings with normal water availability.

2.4.1 Conclusion

In combination with the projected changes in drought patterns in western North American conifer forests, host tree responses to limited resources may potentially contribute to their susceptibility to MPB infestation. While low-level drought conditions have been associated with the potential to induce slight resistance to bark beetle outbreaks in other systems (Kolb et al. 2016), we were unable to detect any resistance response to *G. clavigera* in jack pine seedlings under reduced water availability, as were others (Lusebrink et al. 2011; Arango-Velez et al. 2014). This may be due to the use of seedlings instead of mature trees (Erbilgin and Colgan 2012), however, Erbilgin et al. (2017) also did not find a resistant response of mature jack pine trees to *G. clavigera* in low water conditions. However, because multiple organisms can also attack trees during drought conditions, low water availability can also potentially affect inter-species interactions among tree-infesting organisms. Our results demonstrate that jack pine seedling response to multiple attackers is drought dependent. These interactions may impact jack pine susceptibility to the expansion of MPB. Furthermore, information on drought's effect on induced resistance in conifers will be important to integrate into phenotype selection and tree breeding programs to sustain forest ecosystems.

Table 2.1. Sample location on tree stem for monoterpene concentrations. The initial induction treatments were applied on lower third of trees as follows: No application of induction agent (Control), inoculation with *Grosmannia clavigera* (Fungus), application of methyl jasmonate (MJ), and methyl salicylate (MS). Fungal challenge treatment involved the inoculation of *G. clavigera* on middle third of trees at two weeks after initial induction. Local tree response at the site of application of initial induction or fungal challenge treatments. Systemic tree responses are distal to the site of application of initial induction or fungal challenge treatments.

		Water Treatment					
		Normal		Moderate		Low	
Initial Induction	Fungal Challenge	Local	Systemic	Local	Systemic	Local	Systemic
Control Fungus MJ MS	Non-challenged Non-challenged Non-challenged Non-challenged	Phloem and lesion from lower third of tree	Phloem from middle third of tree	Phloem and lesion from lower third of tree	Phloem from middle third of tree	Phloem and lesion from lower third of tree	Phloem from middle third of tree
Control Fungus MJ MS	Challenged Challenged Challenged Challenged	Lesion from middle third of tree		Lesion from middle third of tree		Lesion from middle third of tree	

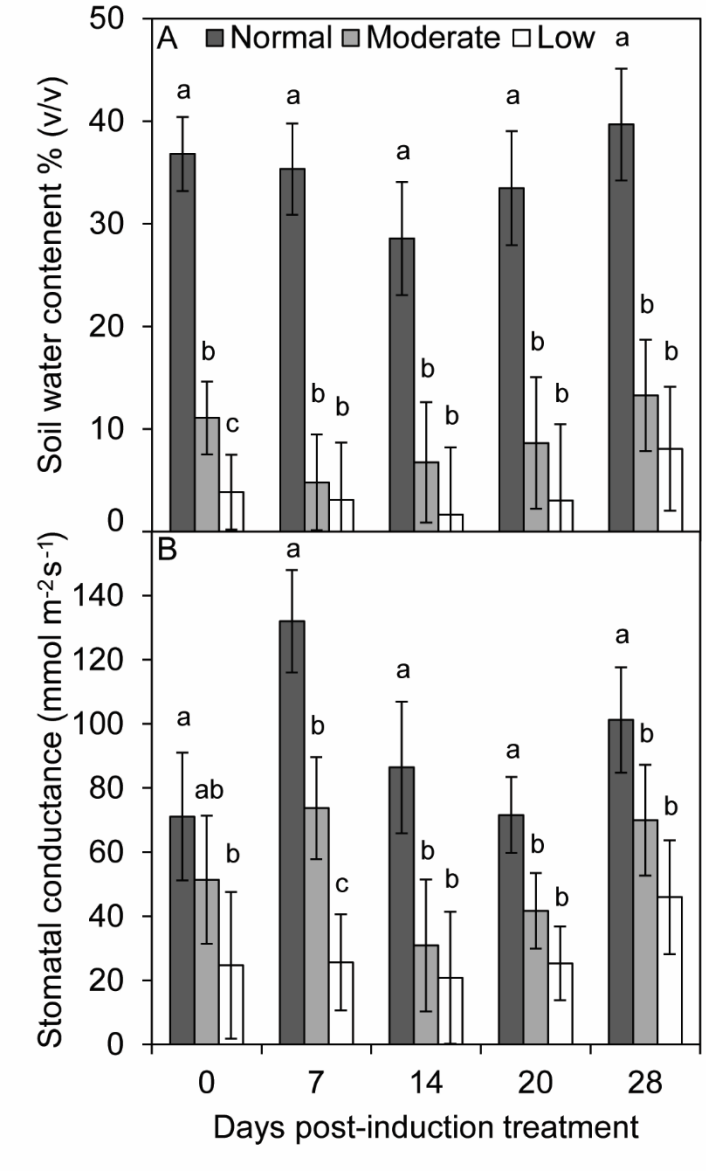


Figure 2.1. Mean (A) soil water content and (B) stomatal conductance of *Pinus banksiana* seedlings at three levels of water treatment (normal, moderate, and low) after application of induction treatment to time of tissue sampling. Error bars are 95% confidence intervals. Within sampling date, different letters denote significant difference among treatments using Tukey's HSD test (α=0.05).

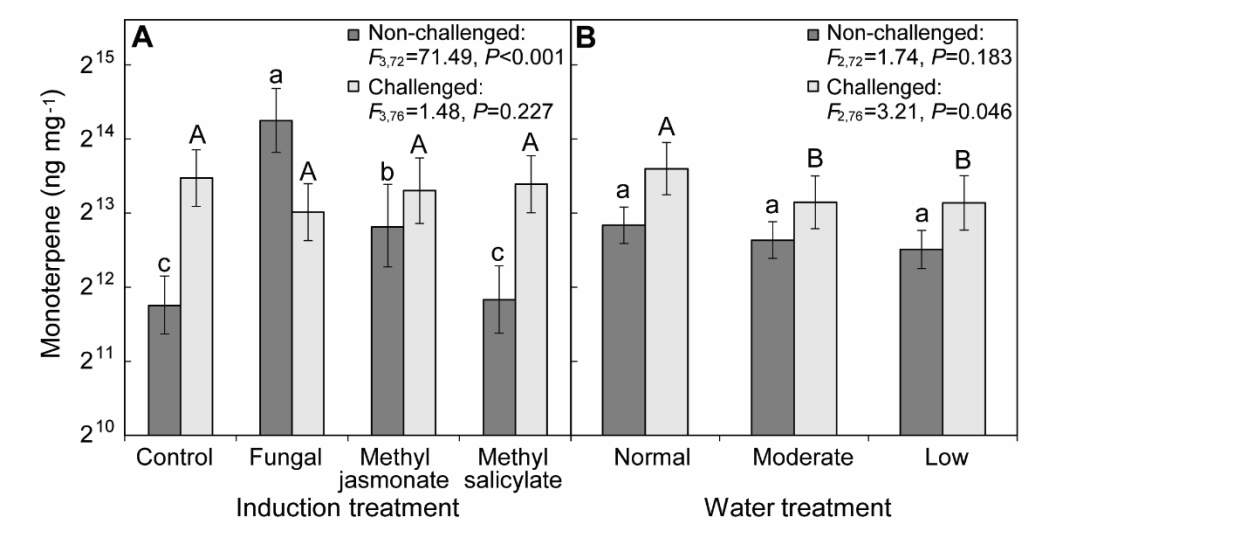


Figure 2.2. Effect of (A) initial induction (averaged across all watering treatments) and (B) watering treatment (averaged across all induction treatments) on monoterpene concentrations **local** to the site of induction treatment (dark bars) and fungal challenge (light bars) application in *Pinus banksiana*. There was not a significant interaction between induction and water treatment. Induction treatment monoterpene concentrations are from the lower third of non-challenged seedlings at the site of initial induction treatment application for methyl jasmonate and methyl salicylate and within the lesion caused by inoculation by *Grosmannia clavigera*. In non-challenged seedlings (dark bars), differences among induction treatments are indicated by lowercase letters from a Tukey's HSD test (α=0.05). Fungal challenge monoterpene concentrations (light bars) are from the lesion tissue in the middle third of trees and differences among induction treatments are denoted with uppercase letters (α=0.10). Error bars are 95% confidence intervals. The y-axis is shown in log scale.

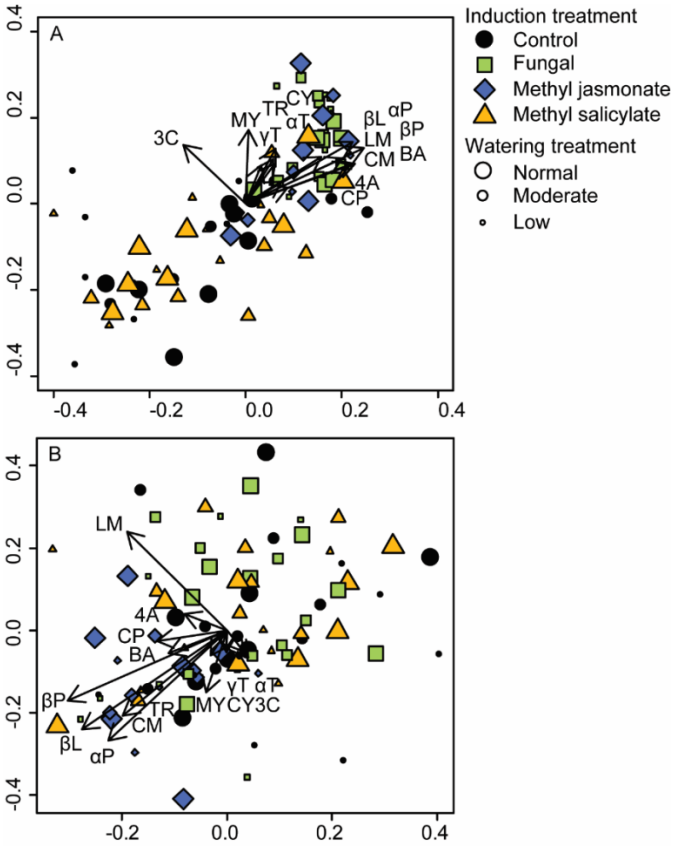


Figure 2.3. Effect of induction and watering treatments on the profile of monoterpene accumulations in *Pinus banksiana* bark (A) at the site of the initial induction treatment application (i.e., the lower third of tree for control, methyl jasmonate and methyl salicylate treated trees and lesion tissue where *Grosmannia clavigera* was inoculated) and (B) above the induction treatment. Non-metric multidimensional scaling with Bray-Curtis distance ordination was used to analyze relationships. Monoterpene compounds are represented by overlaid vectors and indicate the correlation with induction and watering treatments. Longer monoterpene vectors show stronger relationships with the ordination configuration. The minimum stress was: (A) 0.04 and (B) 0.17. Abbreviations for monoterpenes: αP = α-pinene, CM = camphene, βP = β-pinene, 3C = 3-carene, MY = myrcene, αT = α-terpinene, LM = limonene, βL = β-phellandrene, γT = γ-terpinene, CY = *p*-cymene, TR = terpinolene, CP = camphor, BA = bornyl acetate, and 4A = 4-allylanisole.

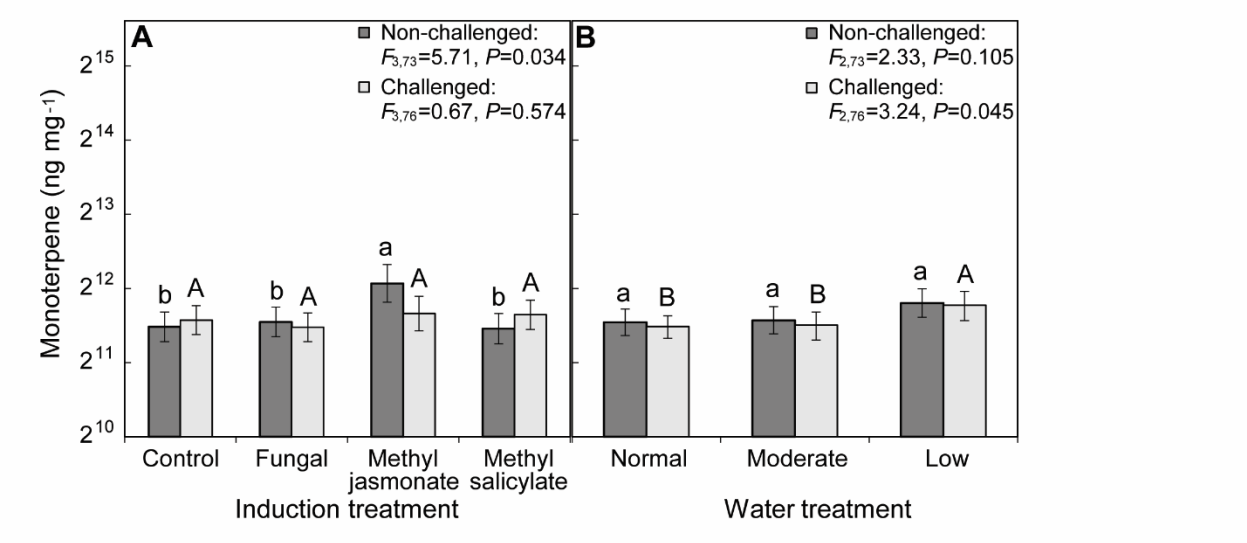


Figure 2.4. Effect of (A) initial induction and (B) watering treatment on monoterpene concentrations **systemic** to the site of induction treatment (dark bars) and fungal challenge (light bars) application in *Pinus banksiana*. There was not a significant interaction between induction and water treatment. Induction treatment monoterpene concentrations are from the middle third of non-challenged seedlings above the site of initial induction treatment application. In non-challenged seedlings (dark bars), differences among induction treatments are denoted by lowercase letters from a Tukey's HSD test (α=0.05). Fungal challenge monoterpene concentrations are from the defensive zone surrounding the lesion tissue in the middle third of seedlings (light bars) and differences among induction treatments are denoted with uppercase letters (α=0.10). Error bars are 95% confidence intervals. The y-axis is shown in log scale.

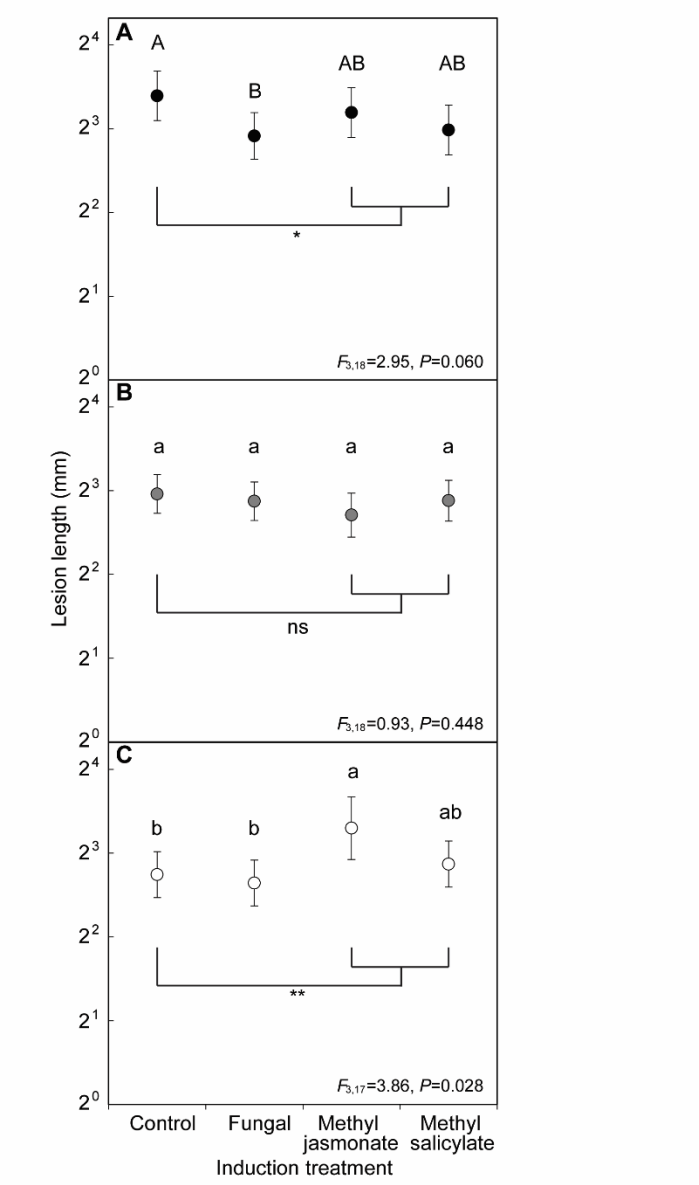


Figure 2.5. Differences in lesion lengths induced from *Grosmannia clavigera* challenge among induction treatments in *Pinus banksiana* treated with different watering treatments: (A) normal, (B) moderate, and (C) low. Different lower case letters (α=0.05) and upper case letters (α=0.10) denote significant difference among induction treatments using Tukey's HSD test. * and ** denote where control is significantly different from signaling hormone induction treatments at α=0.10 and α=0.05, respectively. Error bars are 95% confidence intervals. The y-axis is shown in log scale.

Chapter 3

A native parasitic plant systemically induces resistance in jack pine to a fungal symbiont of invasive mountain pine beetle

3.1 Introduction

Conifers deploy physical and chemical defenses that protect them from a multitude of attacking organisms (Franceschi et al. 2005; Keeling and Bohlmann 2006; Eyles et al. 2010). These defenses can be constitutive and induced, with induced responses occurring both locally, near the site of attack, and systemically in distal parts of the plant (Eyles et al. 2010). Local responses can be expressed in the form of resinous lesions saturated with defense compounds that can compartmentalize both insect and disease spread (Franceschi et al. 2005). Prior insect or pathogen attacks can systemically alter host tree relative susceptibility to subsequent attacks by the same or different organisms in different parts of the tree (Bonello et al. 2006; Eyles et al. 2010; Colgan and Erbilgin 2011; Klutsch et al. 2016). Although the systemic induced resistance (SIR) mechanism is reported for a number of conifer-necrotrophic pathogen systems (Wallis et al. 2008; Villari et al. 2014), the mechanism underlying SIR from biotrophic pathogens and particularly the role of different defense chemicals regulating the SIR is less clear. Furthermore, what is termed here as a resistant response is only relative to the tree response to attack without a prior induction event (i.e., prior insect or pathogen attack). Therefore, a tree that expresses SIR to an attack by an organism could still be overcome by that organism, but just to a lesser extent than trees without the initial induction event.

Conifers use multiple classes of chemicals for defense (Raffa and Berryman 1987; Franceschi et al. 2005; Raffa et al. 2005) and such diversity in defense compounds is considered a result of co-evolutionary interactions between trees and their multispecies enemy complex (Keeling and Bohlmann 2006; Bohlmann 2012; Moore et al. 2014). For example, in conifer-bark beetle (Coleoptera: Curculionidae, Scolytinae) interactions, monoterpenes play broad ecological roles from being both toxic to beetles and their associated fungi to being a precursor to pheromone production (Raffa and Berryman 1983b; Hunt et al. 1989). Although phenolic compounds are generally considered antimicrobial (Bennett and Wallsgrove 1994; Lattanzio et al. 2006) and induced in response to pathogen infection (e.g., Shrimpton 1973; Klepzig et al. 1995), their role in conifer defense is still ambiguous (Witzell and Martin 2008; Erbilgin et al. 2017). Studies are particularly needed to understand the roles of multiple classes of defense chemicals in tree responses to multiple attacking organisms on the same host plants (Stout et al. 2006; Erbilgin et al. 2017).

Production of defense chemicals is metabolically costly and relies on carbohydrates that also support other tree functions such as growth and reproduction, leading to a potential allocation trade-off within a tree (Herms and Mattson 1992; Gershenzon 1994; Wallis et al. 2011). While constitutive defenses are a 'fixed cost' for the tree, production of induced defenses may require both local and translocated carbohydrates (Koricheva et al. 1998; Goodsman et al. 2013). Trade-offs may lead to induced susceptibility or resistance depending on the availability of resources. For example, plants can be more susceptible to further insect or pathogen attacks if they are limited in acquiring additional resources, such as the reduced ability to photosynthesize due to long-lasting drought or severe pathogen infection (Arnold et al. 2004; Sherwood et al. 2015). Thus, how carbohydrates are allocated to multiple classes of defense chemicals can be important for determining tree susceptibility to multiple attacking organisms.

We utilized a tripartite system involving jack pine (*Pinus banskiana* Lamb.), dwarf mistletoe (*Arceuthobium americanum* Nutt. ex Engelm.), and a necrotrophic fungus (*Grosmannia clavigera* Robinson-Jeffrey & Davidson) associated with the mountain pine beetle (*Dendroctonus ponderosae* Hopkins, MPB) to investigate jack pine chemical responses. After killing millions of lodgepole pine (*P. contorta* Dougl. ex Loud.) trees in western Canada, MPB has invaded east into novel jack pine forests (Cullingham et al. 2011; Erbilgin et al. 2014a). Mountain pine beetle employs two important strategies to overwhelm tree defenses, which is required for successful reproduction. Beetles produce pheromones that trigger mass aggregation on host trees (Wood 1982). At the same time, trees are inoculated with beetle-associated necrotrophic fungi, such as *G. clavigera,* that deplete tree defenses and restrict water and nutrient flow between foliage and roots (Raffa and Berryman 1983a, b). While the separate effects of beetle damage to phloem and fungal inoculation do not cause the rapid tree mortality necessary for beetle establishment (Six and Wingfield 2011), it is the combined impacts of both that are integral for beetle success and reproduction (Raffa and Berryman 1983b). Furthermore, these pathogenic fungal associates provide nutrition to developing beetles (Klepzig and Six 2004; Goodsman et al. 2012). In its historical range, MPB displays density dependent host selection, whereby, beetles can successfully attack healthy trees at high population levels, but require stressed trees with limited chemical defenses at low populations (Safranyik et al. 2010). Currently, jack pine forests in Alberta are experiencing low MPB population levels.

A number of biotic disturbances can alter jack pine defense chemistry (Lusebrink et al. 2011, 2016; Colgan and Erbilgin 2011; Klutsch et al. 2016; Erbilgin et al. 2017), potentially impacting its susceptibility to MPB. For example, dwarf mistletoe (*Arceuthobium americanum*) is a biotrophic pathogen and the most widespread stressor of jack pine in western Canada (Hawksworth and Wiens 1996). This parasitic plant infects jack pine branches and slowly

intensifies over several years, causing reduced tree growth and vigor, and increased water stress (Hawksworth and Wiens 1996). Trees stressed from dwarf mistletoe infection are more likely to be colonized by the arriving MPB, however, infection by dwarf mistletoe could also trigger a SIR response to MPB (Bonello et al. 2006; Sherwood and Bonello 2016). In a companion study, monoterpene concentrations in jack pine phloem were altered by dwarf mistletoe infection, with the greatest concentration found in trees with moderate severity (Klutsch et al. 2016). Furthermore, dwarf mistletoe infection mediated interspecies interactions between MPB and woodboring beetles by reducing the competitive advantage of woodboring beetles on MPB (Klutsch et al. 2016).

In this study, we examined whether dwarf mistletoe can trigger SIR in jack pine to *G. clavigera* as a proxy for MPB, as the fungus is essential to successful beetle host colonization and reproduction (Raffa and Berryman 1983b). As reallocation of carbohydrates to defense chemical production may come from beyond the local area around the site of fungus inoculation (Goodsman et al. 2013), we also examined the effect of proximity to the source of photosynthates (i.e., tree crown) on defense chemical induction and carbohydrate allocation. Our objectives were to 1) identify the chemical defense mechanisms (i.e., constitutive and induced defense chemicals) that mediate the interaction between dwarf mistletoe and *G. clavigera*, and 2) examine whether there are patterns of coordination or trade-off among two classes of defensive chemicals based on the allocation of non-structural carbohydrates in jack pine.

3.2 Methods and materials

3.2.1 Site Description and Sampling

We selected 45 mature jack pine trees (> 20 cm in dia. measured at 1.4 m above soil line) in a naturally seeded site in central Alberta, Canada (54°05.2' N, 112°14.4' W). The dwarf mistletoe infection severity on the selected trees was rated using the Hawksworth Dwarf Mistletoe Rating (DMR) system with a scale of 0 (non-infested) to 6 (more than 50% of branches infested throughout the crown) (Hawksworth and Wiens 1996). Trees were chosen according to their DMR (0, 2, 3, 5, or 6) and measured for the following: tree height, height to bottom of live crown, and stem diameter at 1.4 m. Twenty-one of these trees were kept as controls (not inoculated), while the remaining 24 trees were inoculated with *G. clavigera* as described below. The *G. clavigera* used in this study was originally isolated from MPB in Fox Creek, AB (54°24'N, 116°48'W) by AV Rice at Northern Forestry Centre, Canadian Forest Service, Edmonton, AB and is housed and maintained by N Erbilgin at University of Alberta, Edmonton, AB. In trees with dwarf mistletoe infection, phloem and wood sample sites were at least 2 m below the closest infected branch.

Trees were inoculated with *G. clavigera* on 13-14 July 2011 (n=8, 5, 3, 5, and 3 trees with DMR=0, 2, 3, 5, and 6, respectively) (Table Appendix B.1). Eight equidistant phloem samples were taken at 1.4 m above soil line around the stem circumference for monoterpene and carbohydrate analyses as described below. A sterilized cork borer (1.2 cm dia.) was used to remove phloem and each hole was inoculated with an agar plug of actively sporulating *G. clavigera* strain grown on malt extract agar medium. Inoculation points were covered with Parafilm to prevent desiccation.

At six weeks after inoculation (24-25 August 2011), we removed the outer bark of inoculated trees and sampled phloem from within the lesion and 5-10 cm above the lesion (defensive zone tissue hereafter) for monoterpene, phenolic, and carbohydrate analyses (Figure Appendix B.1). We also took sapwood samples to the depth of about 5 cm next to every other

phloem sample position using an increment borer for phenolic analysis as described below. Furthermore, to identify systemic effects, phloem and wood samples from the north and south facing sides of each inoculated tree were taken at 0 and 2.4 m above soil line for monoterpene and carbohydrate analyses. Also, we hypothesized that concentrations of defense chemicals and non-structural carbohydrates would be greater with closer proximity to the canopy (i.e., 2.4 m compared to 0 m), which is the source of photosynthates needed for defense chemistry production (Goodsman et al. 2013). At the time of post-inoculation sampling, the control trees (n=7, 2, 5, 3, and 4 trees with DMR=0, 2, 3, 5, and 6, respectively) were also sampled for phloem and sapwood at 0, 1.4, and 2.4 m above soil line in the same manner as inoculated trees. All samples were transported in dry ice and stored at -40°C and then ground to a fine powder using a mortar and pestle in liquid nitrogen.

3.2.2 Analysis of Monoterpenes

Dichloromethane-extractable compounds were measured using established methods (Klutsch et al. 2016). Briefly, 100 mg of phloem ground sample were extracted twice with 0.5 ml of dichloromethane and 0.01% tridecane (internal standard). Samples were vortexed for 30 s, sonicated for 10 min, and centrifuged at 16,100 rcf at 2°C for 15 min for each extraction, before the two extracts were pooled. Sample extract (2 µl) was injected into a Gas Chromatograph/Mass Spectrometer (GC/MS, Agilent 7890A/5062C, Agilent Tech., Santa Clara, CA, USA) equipped with a DB-Wax column (I.D. 0.25 mm, length 30 m) (Agilent Tech.) with helium carrier gas flow at 1 ml min^{-1}, and a temperature of 50°C for 2 min, increased to 120°C by 10°C min^{-1}, and then to 250°C by 20°C min^{-1}. To quantify individual and total compounds (mainly monoterpenes) (ng mg^{-1} of fresh tissue, hereafter concentration), the following standards were used: borneol, pulegone, α-terpinene, γ-terpinene, α-terpineol, camphor (Sigma-Aldrich, St.

Louis, MO, USA), 3-carene, terpinolene, α-pinene, β-pinene, limonene, sabinene hydrate, myrcene, camphene, *p*-cymene, 4-allylanisole (Fluka, Sigma-Aldrich, Buchs, Switzerland), bornyl acetate, *cis*-ocimene (SAFC Supply Solutions, St. Louis, MO, USA), and β-phellandrene (Glidco Inc., Jacksonville, FL, USA).

3.2.3 Analysis of Phenolics

The identification and quantification of methanol-extractable compounds, comprisied mostly of phenolics and a diterpene resin acid, were conducted on samples from only a subset of trees and tissues (n=5, 2, 6, 4, and 4 trees with DMR=0, 2, 3, 5, and 6, respectively) due to limited amounts of ground samples available for different analyses. Samples from 1.4 m were used to determine phenolic compound content for control trees (phloem and sapwood) and post-inoculation trees (phloem, lesion, and sapwood). Phenolic compounds were extracted from 50 mg of freeze dried sample using 1 ml of methanol using methods modified from Najar et al. (2014). To prevent damage to the instrumentation from the high levels of non-polar compounds in the samples, we diluted methanol extracts to a 1:1 ratio with high-performance liquid chromatography (HPLC) grade water. Diluted samples were then centrifuged at 12,000 rcf for 5 min to pellet the insoluble compounds that precipitated out of solution. The resulting supernatants were used for all subsequent analyses. The peak area lost after the 1:1 dilution was near the expected 50% for most compounds. However, since the dilution effects for each compound are shared across all treatments, they can be considered negligible in the treatment comparisons.

Methods modified from Sherwood and Bonello (2016) were used to quantify and identify compounds. Compounds were first quantified in relative terms using ultra-high pressure liquid chromatography (UPLC) based on peak area at 280 nm using a photo diode array (PDA)

detector. Then, these peaks were assigned tentative identities using HPLC-MS. We injected 0.7 µl of extract into a Waters (Milford, MA, USA) Acquity H-Class UPLC equipped with a Waters Acquity UPLC BEH C18 (2.1 x 100 mm, 1.7 µm particle size) column heated to 50°C with a constant flow rate of 0.42 ml min^{-1} and a Waters Acquity PDA detector scanning all wavelengths between 230 and 400 nm. See Supplement for solvent gradient information.

To identify peaks detected via the UPLC analysis, pooled samples were run on an HPLC coupled with a PDA and MS. Extracts of the same tissue type were pooled in equal amounts and then diluted with water, as explained above. We injected 10 µl of extract into a Varian 212-LC pump system equipped and a Waters XBridge BEH C18 (4.6 x 100 mm, 2.5 µm particle size) column at room temperature with a constant flow rate of 0.6 mL min^{-1} and a post column flow split evenly between 1) Varian 500 IT ion trap MS, scanning for masses between 60 and 800 m/z, and 2) and Varian ProStar 335 PDA detector, scanning at all wavelengths between 230 and 400 nm. See Supplement for solvent gradient and MS parameter information. HPLC-PDA peaks were matched to corresponding UPLC peaks using UV profiles, relative retention times and elution orders. Peaks detected at 280 nm by the HPLC-PDA were matched to masses detected in the full scan mode based on retention time and analyzed in Turbo DDS mode to find their unique fragmentation patterns. UV patterns, full scan and Turbo DDS data were used to assign tentative identities to the matched UPLC peaks based on matches to external standards and relevant literature. The following standards were used: catechin, *trans*-4-coumaric acid and taxifolin (Apin Chemicals, UK); ferulic acid and vanillic acid (Sigma-Aldrich); levopimaric acid (Orchid Cellmark Inc, Princeton NJ, USA).

The level of total phenolics was calculated by summing all identified and unidentified phenolic peak areas (Bonello and Blodgett 2003). The unidentified peaks were also used in this analysis because our extraction method separated and removed many of the non-polar

compounds and the remaining polar compounds were predominantly phenolic glycosides or lignan derivatives.

3.2.4 Non-structural Carbohydrate Analysis

Water-soluble sugar and total starch concentration were quantified following protocols from Chow and Landhausser (2004). Briefly, frozen and ground phloem tissue from the north and south side of each tree were combined and oven dried (70 °C) for 3 d. Water-soluble sugar was extracted from 50 mg tissue in 80% hot ethanol and measured colormetrically using a spectrophotometer (Pharmacia LKB Ultrospec III, Sparta, NJ, USA) at a wavelength of 490 nm after reaction with phenol-sulfuric acid. Starches in the remaining solid residue were solubilized by sodium hydroxide and enzymatically digested by a mixture of α-amylase and amyloglucosidase. The resultant glucose hydrolyzate was combined with the coloring reagent peroxidase-glucose oxidase/*o*-dianisidine and total starch concentration was measured at a wavelength of 525 nm.

3.2.5 Data Analysis

In all parametric tests, the concentrations of all dichloromethane-extractable compounds and peak areas of methanol extractable compounds were natural log transformed to meet assumptions of normality and homogeneity of variance. A repeated-measures ANOVA using first order auto-regressive covariance structure (PROC GLIMMIX in SAS, ver. 9.3) was performed to identify the pattern of constitutive accumulation of defense compounds and non-structural carbohydrates along the tree stem. To identify whether there was a systemic induction of individual and total compounds due to inoculation with *G. clavigera* as compared to control trees, an ANOVA was

performed separately for sample heights above (2.4 m) and below (0 m, i.e., base of tree at soil line) the inoculation point and a mixed model was performed for the inoculation point sample height (1.4 m) to take into account that two tissues were sampled for each inoculated tree (defensive zone and lesion) (PROC GLIMMIX in SAS). A mixed model was performed to determine whether defense chemical concentration and non-structural carbohydrates varied with tissue type and DMR at the inoculation height (PROC GLIMMIX in SAS). Means (± SE) are presented in text. Non-metric multidimensional scaling (NMDS) with Bray-Curtis distance and perManova were used to see whether the profile of defense chemicals varied with DMR and tissue types.

3.3 Results

3.3.1 Pattern of Constitutive Defense Chemistry and Carbohydrates

Of the 19 monoterpene compounds with standards, 16 compounds were detected in the phloem of jack pine (Table Appendix B.2). Furthermore, there were 47 methanol-extractable compounds detected in phloem and wood tissues (Table Appendix B.3, Appendix B.4). Sample height had a significant effect on the constitutive concentration of monoterpenes and non-structural carbohydrates. The concentration of total monoterpenes, α-pinene, and limonene decreased as sample height increased in trees without dwarf mistletoe infection (Table 3.1). The concentration of starch decreased with sample height, while the concentration of soluble sugar had a weakly positive relationship with sample height.

3.3.2 Systemic and Local Responses to *G. clavigera*

There was a systemic monoterpene response to inoculation with *G. clavigera* in trees without dwarf mistletoe infection, but the response was stronger above the inoculation point than below. Above the inoculation point, inoculated trees had more than three times greater β-pinene concentration than control trees (Table 3.2), while below the inoculation point, β-pinene was double (t=-1.81, P=0.093). Furthermore, only above the inoculation point did trees have up to five times higher concentrations of total monoterpenes, α-pinene and camphene than control trees, though this increase was only marginally significant (P<0.1). Similarly, the percent composition of the phenylpropanoid 4-allylanisole above the inoculation point increased 6-fold in inoculated trees (0.5% ± 0.2, t=-2.14, P=0.052) compared to control trees (0.07% ± 0.01). Starch concentration was lower in inoculated trees than control trees (P<0.1) but only above the inoculation point (Table 3.2). There was no systemic change in sugar concentration from inoculation with *G. clavigera*.

At the inoculation point, the concentrations of total monoterpenes and 8 individual monoterpenes were up to 164 times higher in the lesion compared to the control trees (Table 3.3). Furthermore, camphene and β-pinene concentrations were highest in both the lesion tissue and the defensive zone compared to control trees. The only change in percent composition of dichloromethane-extractable compounds was greater 4-allylanisole in the defensive zone (0.4 ± 0.1%, t=-1.89, P=0.081) compared to the control trees (0.07 ± 0.01%).

The level of total phenolic compounds in the lesion tissue was 61% lower than control trees (Table 3.3). Only in trees without dwarf mistletoe infection was the level of levopimaric acid in the lesion similar to control trees (t=-1.75, P=0.178). Likewise, levels of total and individual phenolics (including both phloem and wood tissues) found in the defensive zone did not differ from control trees (P>0.05). Furthermore, 26 of the 46 individual phenolic compounds found in control and defensive zone tissues were absent in lesion tissue. In contrast, 12 phenolic

compounds were present only in lesion tissue and absent in control and defensive zone tissues. Sugar and starch concentrations were both four times lower in the lesion compared to the defensive zone and control trees (Table 3.3).

3.3.3 Systemic Responses to Dwarf Mistletoe

Because dwarf mistletoe infections were on branches above the sampling points on the main stem of trees, their effects on tree chemistry were considered as systemic. At 2.4 m, in control trees (i.e., not inoculated with *G. clavigera*), only 4-allylanisole varied with dwarf mistletoe severity, which had a marginally significant negative relationship with DMR (R^2=0.16, $F_{(1,20)}$=3.68, P=0.070). The concentration and profile of monoterpenes (Fig. 3.1D, Figure Appendix B.2B) and phenolics at the sample point 1.4 m above the soil line did not vary with DMR. However, before *G. clavigera* was inoculated onto the 24 trees chosen to test multi-species responses, the monoterpene concentrations (total, α-pinene, and camphene) at 1.4 m were greatest in trees with moderate DMR compared to trees with lower or higher DMR (Fig. 3.2). Monoterpene concentrations also had a similar quadratic relationship with dwarf mistletoe severity at the soil line in control trees (R^2=0.39, $F_{(2,20)}$=5.68, P=0.012) (Fig. 3.1G). Furthermore, α-pinene and β-pinene, camphene and myrcene along with the profile of monoterpenes at the soil line varied with DMR (perManova $F_{(4,20)}$=2.22, P=0.028). The NMDS analysis illustrates the positive association between concentrations of most monoterpenes (represented by arrow vectors) and trees with moderate DMR, and conversely, the negative association with trees not infected or with a high severity infection (Figure Appendix B.2C). There was not a relationship between DMR and concentrations of sugar and starch at any sampling heights.

3.3.4 Systemic Responses to *G. clavigera* and Dwarf Mistletoe

Above the inoculation point, the pattern of systemic response to *G. clavigera* inoculation changed with dwarf mistletoe infection. Trees without dwarf mistletoe had at least a weak systemic increase of monoterpene concentration following *G. clavigera* inoculations (Table 3.2); in contrast, there was no difference between control trees and trees with dwarf mistletoe plus *G. clavigera* (Fig. 3.1A). There was a 42% decrease in 4-allylanisole for every unit increase in DMR in control and *G. clavigera* inoculated trees (R^2=0.20, $F_{(2,44)}$=5.21, P=0.010). Dwarf mistletoe plus *G. clavigera* had no effect on sugar or starch concentrations above the *G. clavigera* inoculation point (Fig. 3.1B, C).

Control and *G. clavigera* inoculated trees did not differ in total and individual monoterpene concentrations at the soil line across all DMR (Fig. 3.1G, Table 3.S4). However, the concentration of total monoterpenes and α-pinene varied quadratically with DMR across control and *G. clavigera* inoculated trees. Sugar and starch concentrations at soil line were not affected by dwarf mistletoe severity or *G. clavigera* inoculation (Fig. 3.1H, I).

3.3.5 Local Responses to *G. clavigera* and Dwarf Mistletoe

The difference in secondary metabolites between control and *G. clavigera* inoculated trees local to the inoculation point did not change with dwarf mistletoe infection (Figs. 3.1D and 3.3A). Across all DMR, the concentration of total monoterpenes in lesions was 33 times greater than in control trees (Fig. 3.1D). Additionally, nine of the 12 individual monoterpenes that were greater in the lesion than in control trees were also greater than in the defensive zone. The NMDS analysis of the profile of monoterpene compounds illustrates that most monoterpenes (represented by arrow vectors) were positively associated with lesion tissue and negatively associated with control, defensive zone, and pre-inoculation phloem tissues (Fig. 3.4).

There was no interaction between dwarf mistletoe severity and *G. clavigera* on levels of phenolic compounds; the level of total phenolics was about half the amount in the lesion compared to the defensive zone and control trees across all DMR (Fig. 3.3A). The level of levopimaric acid detected in lesion tissue was almost five times greater than in control trees across all DMR (Fig. 3.3B). Additionally, the profile of phenolic compounds differed between tissue types, with lesions containing nine unique phenolic compounds not found in other tissues and missing 27 phenolic compounds that were present in control and defensive zone tissues (Fig. 3.5, Table Appendix B.3). In the wood, levels of total and individual phenolic compounds did not vary between control and *G. clavigera* inoculated trees (Fig. 3.3C and D).

There were no interactions between the severity of dwarf mistletoe infection and *G. clavigera* on local chemical defense induction or non-structural carbohydrate accumulations, but there was a significant effect of dwarf mistletoe severity on several defense compounds. Bornyl acetate concentration had a quadratic pattern of induction with dwarf mistletoe severity across all tissue types, with the greatest concentration in trees with moderate DMR. Furthermore, there was a significant positive effect of dwarf mistletoe severity on the level of total phenolics and a negative effect on levopimaric acid in the phloem of control and *G. clavigera*-inoculated trees (Fig. 3.3A, B, Table 3.S4). Similarly, the level of total phenolic compounds in wood tissue was positively related to dwarf mistletoe severity in both control and *G. clavigera* inoculated trees (Fig. 3.3C, Table 3.S4).

The concentration of *G. clavigera*-induced monoterpenes depended on pre-inoculation concentrations, where trees with initially higher total monoterpene concentration subsequently had higher concentration in the defensive zone tissue (R^2=0.42, F=7.56, P=0.003). However, the change in individual monoterpenes from pre-inoculation tissue to the defensive zone was related to DMR (Fig. 3.6). The increase in the concentration of α-pinene and 3-carene was significantly

lower in the defensive zone tissue in trees with moderate DMR compared with trees with no dwarf mistletoe infection or a more severe infection (Fig. 3.6). Also, β-pinene in the defensive tissue and α-pinene, camphene, and 3-carene in the lesion tissue had the same but weaker relationship between induction by *G. clavigera* and dwarf mistletoe ($P<0.1$).

Accumulation of non-structural carbohydrates at the *G. clavigera* inoculation point was significantly lower in the lesion compared to control and defensive zone tissue (Fig. 3.1E, F). However, there was no effect of dwarf mistletoe on non-structural carbohydrates for any tissue type.

3.3.6 Cross-resistance to *G. clavigera* Due to Dwarf Mistletoe Infection

Lesion length caused by infection by *G. clavigera* inoculation was dependent on dwarf mistletoe infection severity (Fig. 3.7). The lesion length was shortest at moderate DMR compared to trees with lower or higher DMR. Furthermore, lesion length had a negative relationship with total monoterpene concentration before inoculation with *G. clavigera* ($R^2=0.29$, $F=9.07$, $P=0.006$).

3.4 Discussion

Dwarf mistletoe infection triggered an expression of SIR at moderate severities, with a 17% decrease in lesion length from control, and induced susceptibility at high severities against *G. clavigera*. Similar or even stronger SIR has been demonstrated in other study systems (Blodgett et al. 2007; Eyles et al. 2007; Wallis et al. 2008). This response supports the SIR hypothesis (Bonello et al. 2006) and further suggests that SIR can also be induced by a biotrophic pathogen against a nectrotrophic pathogen (Eyles et al. 2010). However, each class of defense chemical responded to the infection severity differently. Monoterpene levels increased at moderate

severity infections but decreased at higher severities. In contrast, total phenolics had a positive relationship with dwarf mistletoe severity.

3.4.1 Cross-induction of Resistance

In general, the induction of defense chemicals is triggered through accumulation of several phytohormones that act as signaling pathways (Stout et al. 2006). For example, biotrophic pathogens trigger the salicylic acid pathway, while necrotrophic pathogens activate the jasmonic acid pathway (Stout et al. 2006). However, salicylic acid can be antagonistic to the accumulation of jasmonic acid (Stout et al. 2006), resulting in a systemic induced susceptibility to necrotrophic pathogens (Thaler et al. 2012). This pattern is in contrast with our findings in jack pine with moderate severity dwarf mistletoe infections, but consistent with the effect of severe infections. Therefore, our results demonstrate that cross-talk between different signaling pathways also occurs in conifers (Bostock et al. 2001; Stout et al. 2006) and is regulated by the level of prior infection.

Monoterpenes and phenolic compounds apparently play different ecological roles in the tripartite interactions among jack pine, dwarf mistletoe and *G. clavigera*, as monoterpene concentrations explained the interactions while phenolics did not. At high concentrations, monoterpenoids 3-carene, α-pinene, limonene, and the phenylpropanoid 4-allylanisole are commonly associated with tree defenses against MPB and its associated fungi (Raffa and Berryman 1983b; Raffa et al. 2005, 2013; Emerick et al. 2008). Furthermore, diterpenes, such as levopimaric acid, are important components of resin and act primarily as defense against pathogens and insects (Keeling and Bohlmann 2006). These compounds listed above along with others (e.g., β-pinene and camphene) were induced by *G. clavigera* or dwarf mistletoe in jack pine in the current study. However, as the infection severity of dwarf mistletoe increased, levels

of monoterpenes as well as 4-allylanisole and levopimaric acid decreased and jack pine trees seemed to be more susceptible to *G. clavigera* than healthy trees. Furthermore, monoterpenes, can be detoxified and used as a carbon source by *G. clavigera* (DiGuistini et al. 2011; Wang et al. 2013, 2014). Together, all of these mechanisms may can be underlying the observation of preferential selection of dwarf mistletoe infested trees by MPB in the beetle's historical range (Johnson et al. 1976; Smith et al. 2011).

Trees with high severity dwarf mistletoe infection had higher accumulations of phenolics in the phloem, but *G. clavigera* lesion lengths were longest in these trees, suggesting that phenolics did not interfere with the growth of *G. clavigera* or possibly they were detoxified, biotransformed, or even used as a carbon source (Hammerbacher et al. 2013). These results were in agreement with Erbilgin et al. (2017), but in disagreement with others who showed that phenolics can be an important component of tree defenses or precursors to compounds that act as antioxidants in defense against other fungal pathogens (Cheynier et al. 2013; Sherwood and Bonello 2013). Interestingly, earlier studies showed that the toxicity of some phenolics can be negated by fungal detoxification and degradation (Hammerbacher et al. 2013; Wadka et al. 2016). If the same fungal detoxification and degradation processes take place in our study system, this can explain why trees with higher levels of phenolic compounds at the higher dwarf mistletoe infection were more susceptible to *G. clavigera*. However, since Hesse-Orce et al. (2010) reported that several genes associated with detoxification of phenolics were not induced in *G. clavigera*, the above mechanism may not be likely. Currently, we do not have a clear understanding of the roles of phenolics in jack pine defenses, as some phenolics may be more a symptom of disease than a determinant of resistance (Bonello and Blodgett 2003; Wallis et al. 2008). Alternatively, *G. clavigera* inoculations may be associated with increases in more

complex phenolics, such as high molecular weight condensed tannins (Hammerbacher et al. 2014), but this remains to be tested in *Pinus* species.

We found that dwarf mistletoe infection systemically induced defenses at the two lowest sample heights on the tree (0 and 1.4 m), which were also the sites of the greatest concentration of monoterpenes and sugar the carbohydrate resource required for monoterpene production. In contrast, there was only a limited systemic effect of *G. clavigera* inoculation with a slight increase in monoterpenes at 1 m above inoculation point, supporting what others have found with bark-beetle associated fungi having limited potential to induce systemic resistance to subsequent attack (Krokene et al. 1999; Klepzig et al. 2005).

3.4.2 Coordination and Regulation of Multi-defense Compounds

Our results reinforce the notion that *Pinus* spp. generally do not undergo a pronounced qualitative change in induced monoterpenoid defenses (Shrimpton and Watson 1971; Raffa 1991; Lusebrink et al. 2011). Apparently, phenolics do not follow this pattern as this study and others (e.g., Erbilgin et al. 2006, 2017) found that the phenolic profile differed greatly between constitutive and induced tissues, as well as between tissue types. In the current study, for example, lesion tissue contained five unique phenolic compounds that were absent in the defensive zone surrounding the lesion and in control tissues. Possibly, fungal biotransformation of phenolic compounds can explain the increased number of phenolic compounds in the lesion (Hammerbacher et al 2013; Wadka et al. 2016). Although the mechanism is not clear, the broad induction of most monoterpene compounds and the more diverse or tissue restricted pathogen-induced response of phenolics by jack pine could be an evolutionary response to multiple pests (Rowe et al. 1969; Lieutier et al. 1996; Wallis et al. 2008) or reflect the ability of fungi to

metabolize and biotransform certain defense compounds but not others (Hammerbacher et al. 2013).

Our study also provides evidence of a potential trade-off between systemically and locally induced defense compounds in jack pine. Moderate dwarf mistletoe severity induced the highest concentrations of α-pinene, however, after *G. clavigera* inoculation these trees also had the lowest increase in α-pinene in tissue outside the lesion. Camphene and β-pinene generally followed this same pattern with dwarf mistletoe severity. Furthermore, the concentration of 3-carene, which was not influenced by dwarf mistletoe severity, actually decreased at moderate severity after *G. clavigera* inoculation. These results suggest that trees with high levels of systemically induced defensive compounds do not necessarily allocate more of the same compounds in the defensive zone locally after subsequent attack by the second pathogen. This differential allocation strategy between two subsequent pathogen infections was also demonstrated between constitutive and induced defenses in other perennial systems (Lewinsohn et al. 1991, Koricheva et al. 2004; Villari et al. 2014).

There is also evidence of a potential trade-off between monoterpenes and phenolics, similarly to what was observed in Austrian pine (*Pinus nigra*) (Wallis et al. 2011). Within lesions, the increase in the concentration of monoterpenes corresponded with decreased levels of total and most individual phenolics. While detoxification and metabolism of phenolics could result in reduced levels of phenolic compounds (Hammerbacher et al 2013; Wadka et al. 2016), biosynthetic trade-offs between classes of secondary compounds could also contribute to the divergent accumulation patterns. Monoterpenes and phenolics are biosythesized by different pathways: monoterpenes are produced in plants *via* mevalonic acid and 2*C*-methyl-D-erythritol-4-phosphate pathways (Bohlmann et al. 1998) while phenolics are produced *via* the phenylpropanoid pathway (Boudet et al. 1995). Since monoterpenoids and phenolics are both

carbon-based defenses, differences in allocation of carbohydrates between these two classes may affect their production. In fact, the increase of non-structural carbohydrates in the defensive zone and the decrease in the lesion tissue suggests that they were likely mobilized to the defensive zone and used for biosynthesis of monoterpenes at the expense of phenolics (Koricheva et al. 1998; Goodsman et al. 2013). Alternatively, physical displacement may also describe the relationship between these two defense classes. Björkman et al. (1998) found a negative relationship between the amounts of terpene and phenolics in *Pinus sylvestris* needles that was attributed to the production of resin ducts and exclusion of phenolic storage due to lack of available space within the tissue.

3.4.3 Conclusions

Since MPB has invaded jack pine forests, a clear understanding of regulation of defense chemicals can help us to determine the factors involved in jack pine susceptibility for further MPB spread in western Canada. We present evidence that dwarf mistletoe has a slight negative impact on a MPB-associated fungus through SIR in jack pine. Furthermore, this SIR switches to susceptibility in jack pine with severe dwarf mistletoe infection. An increase in susceptible response to *G. clavigera* with severe dwarf mistletoe infection may benefit MPB maintenance in the jack pine boreal forests (Arango-Velez et al. 2016; Erbilgin et al. 2017). The range expansion of MPB into jack pine could have many serious ecological and economic impacts on the boreal forest through causing changes in stand structure, potential fire behavior, and species succession (Safranyik et al. 2010). Furthermore, this novel interaction in jack pine can also impact other insects and pathogens native to jack pine forests and thus alter their interactions with jack pine trees (Klutsch et al. 2016).

Table 3.1. Constitutive concentration of phloem monoterpenes (ng mg^{-1}) and non-structural carbohydrates (w/w) sampled at three heights on the main stem of jack pine (*Pinus banksiana*) trees. Least squares mean (95% confidence interval) are presented.

	Sample height						Model Statistics	
	0 m		1.4 m		2.4 m		*F*	*P*
	Mean	$CI_{95\%}$	Mean	$CI_{95\%}$	Mean	$CI_{95\%}$		
Total monoterpene	1536.3 a	(835.0-2826.2)	762.6 b	(414.5-1403.0)	718.4 b	(390.5-1321.7)	4.68	0.032
α-Pinene	852.1 a	(469.4-1547.0)	415.3 b	(228.7-754.0)	380.2 b	(209.4-690.1)	5.48	0.020
Limonene	33.7 a	(8.6-131.1)	14.0 b	(3.6-54.4)	15.6 ab	(4.0-60.7)	2.92	0.090
Percent sugar	15.5 a	(13.8-17.3)	17.7 ab	(15.8-19.7)	18.5 b	(16.6-20.5)	3.65	0.065
Percent starch	3.0 a	(2.3-3.7)	2.2 b	(1.5-2.9)	2.2 b	(1.5-2.9)	7.74	0.009

Only monoterpenes that vary statistically with sample height are presented (α=0.10). All monoterpenes were natural log transformed for analysis. Back-transformed values are presented. Different letters on least squares means in a row indicate significant differences among sample heights. Only compounds determined to be statistically significant at α=0.1 with Tukey's adjusted *P* (n=7 trees) are shown.

Table 3.2. Concentrations of monoterpenes (ng mg^{-1}) and non-structural carbohydrates (w/w) in the phloem of control jack pine (*Pinus banksiana*) trees and trees inoculated with *Grosmannia clavigera,* 1 m above the point of inoculation (sample height of 2.4 m), in the absence of *Arceuthobium americanum* infection. Least squares mean (95% confidence intervals) are presented.

	Tissue types				Model statistics	
	Control (n=7)		*G. clavigera* inoculated trees (n=8)		*t*-value	*P*
	Mean	$CI_{95\%}$	Mean	$CI_{95\%}$		
Total monoterpene	872.2	(381.0 – 1354.8)	1400.7	(897.6 – 2185.5)	-2.12	0.054
α-Pinene	380.2	(207.7 – 695.8)	766.9	(466.8 – 1316.3)	-2.09	0.057
Camphene	1.6	(0.2 – 14.0)	8.2	(4.7 – 14.5)	-1.89	0.081
β-Pinene	29.3	(10.9 – 78.8)	94.5	(47.8 – 187.1)	-2.40	0.032
Percent sugar	18.6	(16.8 – 20.4)	17.0	(15.0 – 19.0)	1.36	0.198
Percent starch	2.1	(1.5 – 2.7)	1.6	(1.3 – 1.9)	1.84	0.090

Only monoterpenes that statistically vary with tissue type are presented (α=0.10). All monoterpenes were natural log transformed for analysis. Back-transformed values are presented.

Table 3.3. Concentrations of monoterpenes (ng mg^{-1}), phenolics (AU) and non-structural carbohydrates (w/w) in the phloem of control trees (*Pinus banksiana*) and trees inoculated with *Grosmannia clavigera,* at the point of inoculation (sample height of 1.4 m), in the absence of *Arceuthobium americanum* infection.

	Tissue types						Model statistics	
	Control (n=7)		Defensive zone (n=8)		Lesion (n=8)		*F*	*P*
	Mean	$CI_{95\%}$	Mean	$CI_{95\%}$	Mean	$CI_{95\%}$		
Total monoterpene	762.6 a	(434.4 – 1295.3)	1761.3 a	(1040.4 – 2981.6)	26207 b	(15483 – 44364)	70.84	<0.001
α-Pinene	415.3 a	(246.5 – 699.6)	983.4 a	(603.8 – 1601.7)	14972 b	(9193 – 24384)	84.50	<0.001
Camphene	2.8 a	(1.4 – 5.6)	10.1 b	(5.3 – 19.0)	149.9 c	(79.2 – 283.7)	57.71	<0.001
β-Pinene	20.5 a	(6.7 – 62.6)	142.5 b	(50.2 – 404.4)	2361.1 c	(832.1 – 6700.2)	29.54	0.001
3-Carene	45.0 a	(4.6 – 443.0)	261.0 ab	(30.7 – 2218.5)	2512.4 b	(295.6 – 21352.0)	5.00	0.053
Myrcene	19.1 a	(8.3 – 44.0)	47.5 a	(21.8 – 103.5)	548.1 b	(251.4 – 1195.1)	28.39	0.001
α-Terpinene	0.1 a	(0.1 – 0.7)	0.6 ab	(0.1 – 4.4)	16.4 b	(2.7 – 139.4)	11.20	0.009
Limonene	14.0 a	(4.4 – 44.2)	38.0 ab	(12.9 – 111.6)	190.8 b	(64.9 – 560.6)	8.48	0.018
β-Phellandrene	3.6 a	(0.8 – 15.9)	13.8 a	(3.4 – 55.2)	235.7 b	(58.8 – 943.9)	13.48	0.006
p-Cymene	0.2 a	(0.1 – 0.8)	0.7 a	(0.2 – 2.9)	13.6 b	(3.1 – 59.8)	13.16	0.006

Table 3.3 (cont.)

	Tissue types						Model statistics	
	Control (n=7)		Defensive zone (n=8)		Lesion (n=8)		*F*	*P*
	Mean	$CI_{95\%}$	Mean	$CI_{95\%}$	Mean	$CI_{95\%}$		
Terpinolene	3.8 a	(0.5 – 30.8)	26.7 ab	(3.8 – 187.2)	443.8 b	(63.3 – 3110.1)	8.49	0.018
Total phenolics (x 10^5)	21.1	(15.2 – 29.3)	-		8.2	(5.5 – 12.2)	5.85*	0.010
Percent sugar	17.8 a	(14.3 – 21.4)	19.7 a	(16.6 – 22.8)	4.4 b	(1.3 – 7.5)	42.23	<0.001
Percent starch	2.0 a	(1.4 – 2.6)	2.1 a	(1.6 – 2.6)	0.5 b	(0.0 – 1.0)	18.39	0.003

Only monoterpenes that statistically vary with tissue type are presented (α=0.10). Different letters on least squares means (95% confidence intervals) in a row indicate significant differences among tissue types with a Tukey's adjusted *P* (α=0.05). All monoterpenes and phenolics were natural log transformed for analysis. Back-transformed values are presented. * t-value test statistic.

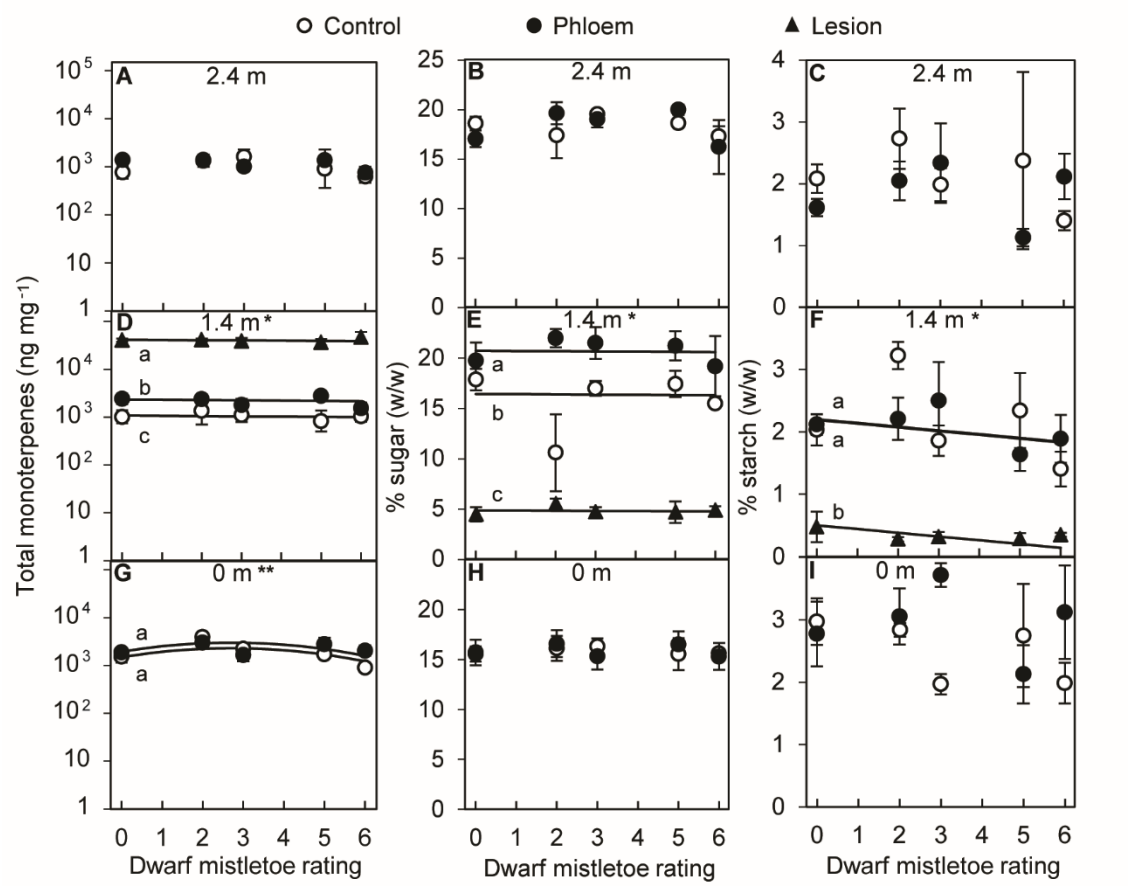


Figure 3.1. Concentrations of monoterpenes (A, D, G) and non-structural carbohydrates (B, C, E, F, H, I) at different sampling heights in the phloem on the main stems of *Pinus banksiana* over a gradient of infection severities of *Arceuthobium americanum* (dwarf mistletoe rating). Trees were either not inoculated with *Grosmannia clavigera* (control) or were inoculated (phloem or lesion) at the 1.4 m sample height. Error bars are standard errors. The y-axis is shown in natural log scale for monoterpenes. See Table Appendix B.5 for equations. Different lower case letters denote significant differences among control, phloem and lesion, controlling for the effect of dwarf mistletoe rating at α=0.05. * Denotes when monoterpene or non-structural carbohydrates concentrations differed significantly among tissue types (α=0.05), while dwarf mistletoe rating did not describe a significant amount of variance in the model. ** Denotes when there was a

significant effect of dwarf mistletoe rating (α=0.05) on monoterpene concentration across tissue type.

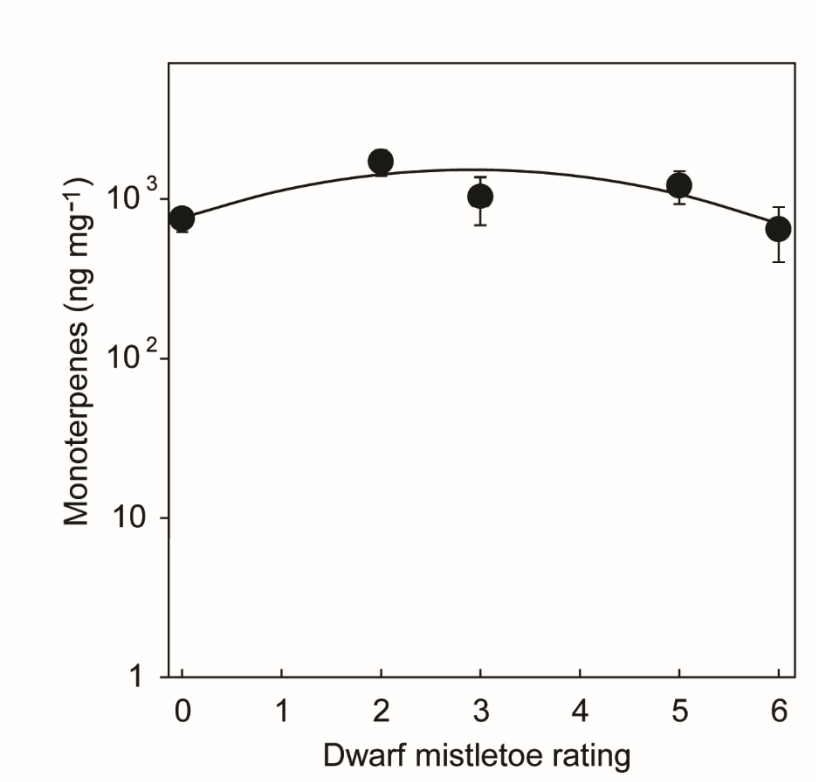


Figure 3.2. Mean concentration of total monoterpene in jack pine (*Pinus banksiana*) phloem with varying levels of *Arceuthobium americanum* (dwarf mistletoe) infection before inoculation with *Grosmannia clavigera* at 1.4 m sample height. The y-axis is shown in natural log scale for monoterpenes. Error bars are standard errors and n=24 trees. Total monoterpenes: R^2=0.26, $F_{(2,21)}$=3.40, P=0.056 (modified from Klutsch et al. 2016).

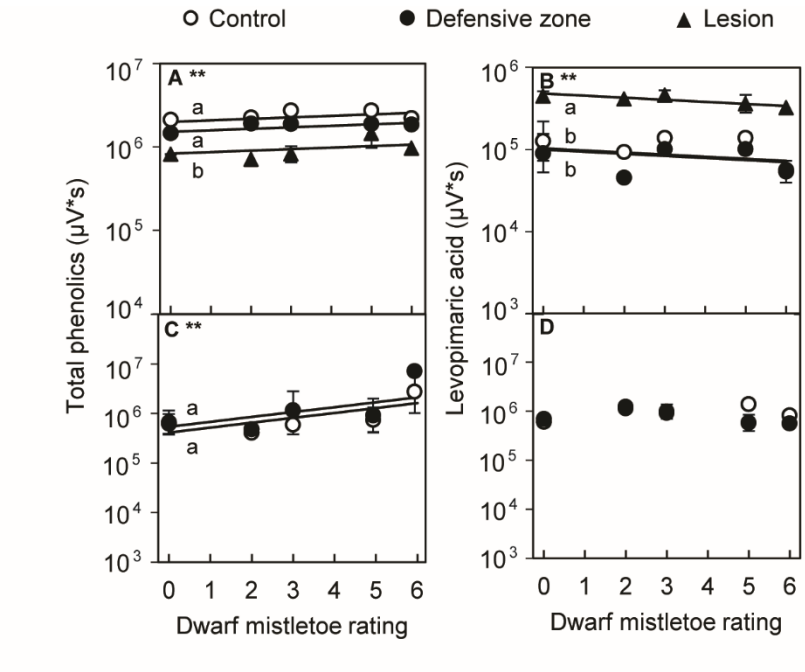


Figure 3.3. Peak areas of phenolics (A, C) and levopimaric acid (B, D) in the phloem (A, B) and wood (C, D) of the main stems of *Pinus banksiana* over a gradient of infection severities of *Arceuthobium americanum* (dwarf mistletoe rating). Trees were either not inoculated with *Grosmannia clavigera* (control) or were inoculated (defensive zone or lesion). Error bars are standard errors. The y-axis is shown in natural log scale. Different letters denote levels of phenolic and levopimaric acid that differed significantly among tissue types (α=0.05).

** Denotes a significant effect of dwarf mistletoe rating (α=0.05) on phenolic and levopimaric acid levels across tissue type.

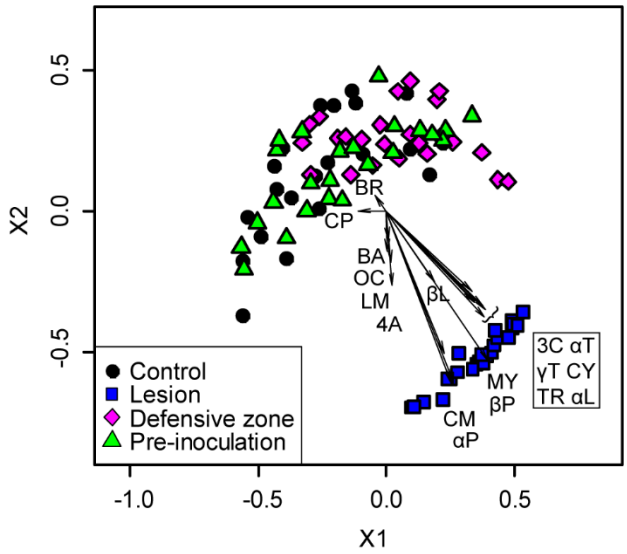


Figure 3.4. Effects of inoculation with *Grosmannia clavigera* on monoterpene concentrations in the phloem of *Pinus banksiana* representing control, pre-inoculated, defensive zone, and lesion tissue. Data were analyzed using non-metric multidimensional scaling with Bray-Curtis distance ordination (stress=0.12). Monoterpene compounds are represented by overlaid vectors and indicate the correlation with tissue type. Longer vectors show stronger relationships with the ordination configuration. Ellipses are 95% confidence intervals. Acronyms for monoterpenes: αP = α-pinene, CM = camphene, βP = β-pinene, 3C = 3-carene, MY = myrcene, αT = α-terpinene, LM = limonene, βL = β-phellandrene, OC = ocimene, γT = γ-terpinene, CY = p-cymene, TR = terpinolene, CP = camphor, BA = bornyl acetate, 4A = 4-allylanisole, αL = α-terpineol, BR = borneol.

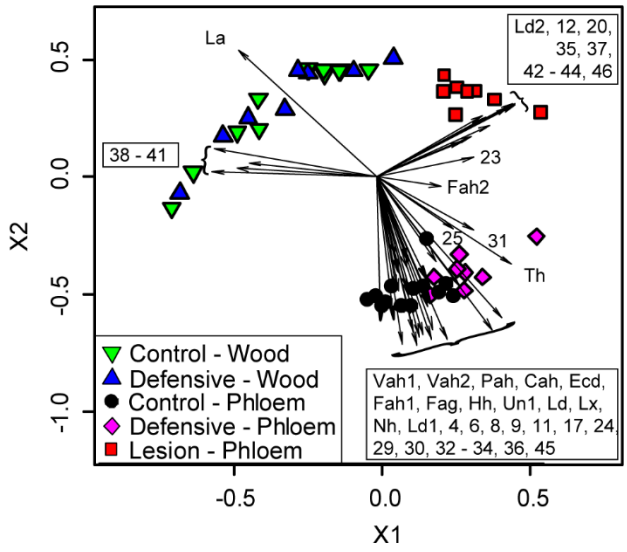


Figure 3.5. Effects of inoculation with *Grosmannia clavigera* on phenolic compounds (plus levopimaric acid [La]) in the phloem and sapwood of *Pinus banksiana* representing control, pre-inoculated, defensive zone, and lesion tissue. Data were analyzed using non-metric multidimensional scaling with Bray-Curtis distance ordination analysis (stress=0.14). Phenolic compounds are represented by overlaid vectors and indicate the correlation with tissue type. Longer vectors show stronger relationships with the ordination configuration. Ellipses are 95% confidence intervals. See Table Appendix B.3 for abbreviations.

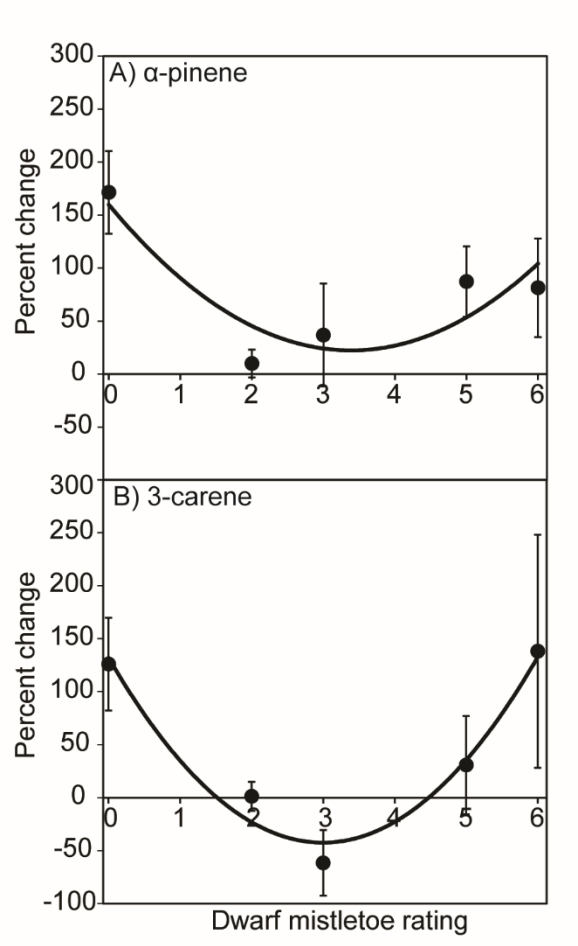


Figure 3.6. Percent change in concentration of A) α-pinene and B) 3-carene from pre-inoculation to post-inoculation with *Grosmannia clavigera* in the phloem defensive zone of *Pinus banksiana* infected with *Arceuthobium americanum* (dwarf mistletoe). Error bars are standard errors. A) Equation for the line: Percent change of α -pinene concentration =165.0+(DMR*-88.9)+(DMR^2*13.4), all parameter estimates are significant at α=0.05, n=24 trees, R^2=0.344, $F_{(2,23)}$=5.49, P=0.012. B) Equation for the line: Percent change of 3-carene concentration=129.2+(DMR*-110.9)+(DMR^2*18.5), all parameter estimates are significant at α=0.05, n=24 trees, R^2=0.326, $F_{(2,23)}$=5.08, P=0.016.

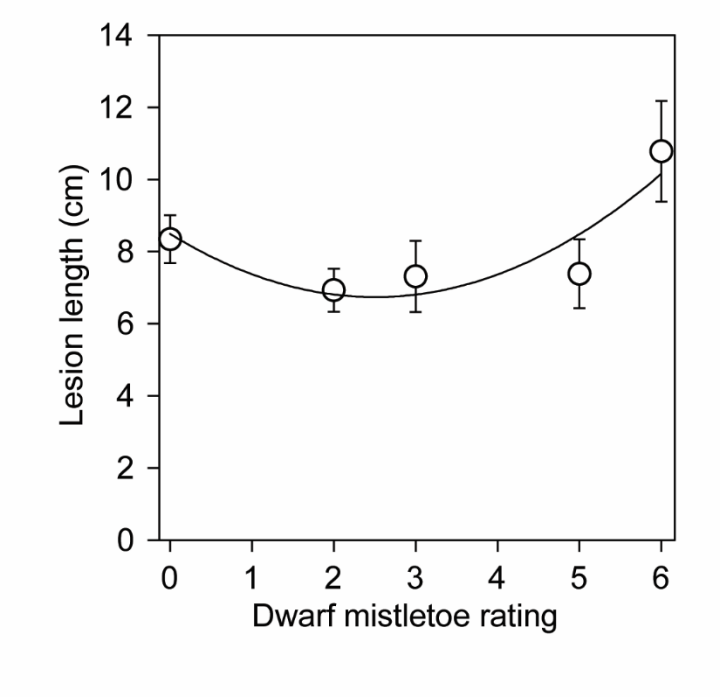


Figure 3.7. Mean *Grosmannia clavigera* lesion length in *Pinus banksiana* infected with *Arceuthobium americanum* (dwarf mistletoe). Error bars are standard errors. Equation for the line: Lesion Length = 8.43 + (DMR*-1.38) + (DMR^2*0.27), all parameter estimates are significant at α=0.05, n=24 trees, R^2=0.253, $F_{(2,23)}$=3.55, P=0.047.

Chapter 4

Direction of interaction between mountain pine beetle (*Dendroctonus ponderosae*) and resource-sharing woodboring beetles depends on plant parasite infection

4.1 Introduction

Plants are consumed by a myriad of organisms that compete for resources (Stout et al. 2006; Karban et al. 2012). Direct interactions among multiple plant feeding organisms on a single host can range for each species from positive (facilitation) to negative (competition) (Stout et al. 2006). However, herbivore interactions also can occur indirectly via plant-mediated interactions, where initial infestation of the plant by an attacker (e.g., pathogen) positively or negatively affect the performance of subsequent attacker(s) (e.g., insect herbivores) on the shared-host by inducing changes in the host plant characteristics (Stout et al. 2006; Karban et al. 2012; Tack and Dicke 2013). More recently, a plant-mediated interactions framework was applied to a wider community context (e.g., Tack et al. 2012) and such interactions can have consequences not only for the individual organisms but also to population and community dynamics. This suggests the need to identify how plant-mediated interactions can influence interactions among resource-sharing herbivores. Likewise, the effect of a full range of host plant conditions induced by an initial infestation of the plant by an organism should also be integrated into our understanding of how resource-sharing species interact and how these interactions organize and structure natural communities (Bertness and Callaway 1994; Barrio et al. 2013).

A recent synthesis paper by Tack and Dicke (2013) explored both direct and indirect interactions between a fungal plant pathogen and insect herbivores within a community context

in various host plant systems. They predicted that pathogen infection can: (1) impact herbivore performance on the infected plants by altering host plant quality, (2) affect herbivore preference for a particular host plant by modifying the attraction (or deterrence) of the infected plant, and therefore pathogen infection may affect the spatial and temporal distribution of herbivores within the plant, and (3) impact herbivore population dynamics by altering their growth rate on infected plants, and therefore herbivore densities may vary between infected and uninfected plants. The above predictions are based on the species-specific responses of herbivores to a given plant pathogen, which can affect spatial and temporal herbivore distribution and abundance. However, patterns of herbivore responses to host plant pathogens can vary among different study systems. Indeed, whether these predictions extend to interactions among subcortical insects sharing resources or whether plant traits altered by pathogen infection level (e.g., intensity) differentially affect insect herbivore responses remain unknown (Tack and Dicke 2013). A framework elucidating how the community of plant-feeding insects and pathogens interact though induced changes in host plant quality could improve our understanding of community interactions that can ultimately impact biodiversity, disturbance regimes, and invasion dynamics of a system.

The bark beetle mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (Coleoptera: Curculionidae) (hereafter MPB) has killed millions of lodgepole pine (*Pinus contorta* Douglas var. *latifolia* (Engelm.) Critchfield) during its recent outbreak in western Canada and has expanded its host range to jack pine forests (*P. banksiana* Lamb.) (Cullingham et al. 2011; Erbilgin et al. 2014a). As MPB establishes in the novel host system, it will interact with a large number of organisms associated with jack pine including pathogens and a community of subcortical herbivores that also consume and develop in host tree phloem resources. The impact of MPB on the biodiversity and sustainability of ecosystem services by jack pine in the boreal forest will depend on factors affecting MPB performance in this novel host system.

The widespread tree pathogen in the western extent of jack pine, the parasitic plant dwarf mistletoe (*Arceuthobium americanum* Nutt. ex Engelm.), has the potential to impact MPB as it establishes in jack pine. Dwarf mistletoe infects tree branches and slowly reduces host growth and vigor, increases water stress, and eventually contributes to tree mortality (Nebeker et al. 1995; Hawksworth and Wiens 1996). Given that dwarf mistletoe has been shown to impact host susceptibility to several bark beetle species in pine forests, including MPB (Johnson et al. 1976) and *Ips pini* (Say) (Kenaley et al. 2006), similar interactions may also occur in jack pine forests. Such interactions are critical for MPB because its survival in the invaded habitat will depend on trees stressed by widespread agents, like dwarf mistletoe. While *I. pini* has been studied as a common competitor to MPB in its historical range (e.g., Safranyik et al.1999), MPB interacts with a number of other subcortical insect herbivores, including woodboring beetles (Coleoptera: Curculioniade and Cerambyicidae) that also rely on stressed host trees to infest, resulting in competition (Safranyik et al. 1999), facilitation (Smith et al. 2011), or even predation (Dodds et al. 2001). We only captured woodboring beetles in this study, but *I. pini* and other bark beetles may also be important competitors to MPB in jack pine. Detailed biological and ecological descriptions of MPB and woodboring beetles are summarized in the Materials and Methods section.

In this study, our main objective was to investigate whether the severity of dwarf mistletoe infection can alter the direction of interaction between MPB and woodboring beetles on jack pine in a community context. Based on the reported interactions among MPB, dwarf mistletoe and the woodboring beetle community in other host systems, we hypothesize that dwarf mistletoe infection severity in jack pine can affect the competition between MPB and woodboring beetles due to differences in species-specific responses to the pathogen-altered plant quality (Bonello et al. 2006; Röder et al. 2007; Tack et al. 2012). In order to determine the

pattern of interactions between the plant pathogen and insect herbivores, we had four objectives which were to: 1) determine the indirect impact of dwarf mistletoe on MPB performance (arrow b1 in Fig. 4.1), 2) characterize the impact of feeding by woodboring beetles on MPB performance (arrow c in Fig. 4.1), 3) evaluate whether the interaction between MPB and woodboring beetles is influenced by infection severity of dwarf mistletoe, and 4) examine possible mechanisms that underlie the interspecies interactions investigated in Obj. 1 – 3 (arrow a in Fig. 4.1).

4.2 Materials and Methods

4.2.1 Biology and ecology of *D. ponderosae* and woodboring beetles

The general life cycle of MPB in the historical beetle range is well characterized (Safranyik et al. 2010). Briefly, MPB kills trees through pheromone-mediated mass attack and host inoculation with associated pathogenic fungi in order to reproduce. Once a female beetle tunnels into the phloem and mates, she constructs a cambial maternal gallery into which eggs are deposited. After hatching, larvae develop by feeding on phloem and constructing larval galleries where pupation occurs followed by development into adults that emerge the subsequent season. Populations of MPB move cyclically from low-density to outbreak levels and demonstrate density-dependent host selection behaviors. At low-density populations, MPBs are restricted to stressed or damaged trees, but as populations increase they can attack and kill large and healthy trees across the landscape. However, since MPB was recently established in the novel jack pine habitat, it is not known whether the population dynamics of MPB will be the same as it is in historical host habitats.

Three features of bark beetle interactions with woodboring beetles are particularly relevant to our investigations. First, bark and woodboring beetles share the same subcortical environment; however, they differ in their behaviors to utilize plant resources (Safranyik et al. 1999; Allison et al. 2001). While bark beetles primarily feed on the host phloem, woodboring beetle larvae begin their development by feeding on the phloem and migrate into the xylem as they develop. We assume that bark and woodboring beetles are potential competitors when both groups feed on the phloem. Second, there is a close association between bark and woodboring beetles in terms of host colonization as some woodboring beetles (particularly in the family Cerambycidae) use bark beetle pheromones to detect suitable host trees (Allison et al. 2001), which could result in competition. Finally, both beetle groups are affected by host plant secondary metabolites (Franceschi et al. 2005; Erbilgin et al. 2006; Sanchez-Husillos et al. 2013), creating the potential for both beetles to respond to changes in plant quality due to pathogen infection and feeding by the other beetle species.

4.2.2 Study site and parasitic plant infection

To test whether the plant pathogen infection alters bark and woodboring beetle performance, we used logs cut from jack pine infected with a range of intensities of dwarf mistletoe. Study trees were located in central Alberta, Canada (54°05.2' N, 112°14.4' W). One log (35 cm long) from the bottom 150 cm of each tree stem was cut from each felled tree and the number of trees cut for each objective is described below. All trees were bigger than 20 cm in diameter at breast height (measured at 1.37 m in height). The ends of each log were covered with paraffin wax to prevent desiccation.

Prior to cutting, the trees were assessed for their disease severity using the Hawksworth Dwarf Mistletoe Infection Rating (hereafter DMR) system with a scale of 0 (non-infected) to 6

(> 50% of crown infected) (Hawksworth and Weins 1996). We did not experimentally apply dwarf mistletoe infections because it can take over 20 yrs to develop a DMR of 6 (Hawksworth and Weins 1996). The potential for variation in susceptibility among jack pine trees in our study area seemed low because the pathogen spreads from an infection center and all trees within the center seemed to be infected. To identify whether the presence of the parasitic plant affected physical and chemical characteristics (arrow a in Fig. 4.1), we measured phloem thickness and tree chemical defenses in trees at the same site (See Obj. 4*)*. Although logs may not be the most ideal environment to test host-insect interactions, they are effectively used in the scientific literature and have been substantiated in field experiments (Dyer and Seabrook 1978; Erbilgin et al. 2006, 2014a; Raffa et al. 2013).

4.2.3 Obj. 1 – Determine the indirect impact of the plant pathogen on MPB performance

We used logs from 17 trees felled in 2012 (DMR=0, 2, 3, 5, and 6 had n=4, 2, 4, 4, and 3, respectively) and brought them to the lab for live MPB inoculations (Table Appendix C.1). To introduce MPB into logs, we used live adults caught in Lindgren funnel traps baited with MPB attractant (*trans*-verbenol, *exo*-brevicomin, terpinolene; Synergy Semiochemicals Corp.) that were surrounding the Weyerhaeuser yard, Grande Prairie, Alberta. Four mating pairs of MPB were introduced into 0.8 cm diameter holes, drilled through the bark and phloem and equally spaced around the lower section of each log (Erbilgin et al. 2014a) (Figure Appendix C.1A,B). We first introduced a female beetle, followed by a male 6 h later. After 4 wks in a growth chamber (23°C, 20% relative humidity, 23:1 light:dark), logs were moved to 4°C and were kept there for 4 wks. They were then returned to the growth chamber and monitored for MPB emergence.

When MPB emergence had ceased (two months after removal from cold room), we removed the outer bark and measured MPB brood production and survival via the following variables: percentage of total count of introductions resulting in maternal galleries, which gave us introduction success, length of maternal and larval galleries length, and count of larval galleries, pupal chambers and emerged broods (Figure Appendix C.2A, Figure Appendix C.3A). The count of larval galleries and pupal chambers equates to the number of larvae that hatched and the number of those larvae that pupated. The area of phloem consumed by MPB was quantified on two 15 × 15 cm sections on opposite sides of each log (Figure Appendix C.2C).

4.2.4 Obj. 2 – Characterize the impact of feeding by woodboring beetles on MPB performance

We felled 30 trees in 2011 with a range of dwarf mistletoe infection (DMR = 0, 2, 3, 5, and 6 had n=10, 5, 5, 6, and 4, respectively) (Table Appendix C.1). The logs infested with woodboring beetles but not infected with dwarf mistletoe were used to identify the competitive effect of woodboring beetles on the MPB performance (n=10 logs) (arrow c in Fig. 4.1), while all logs were used to evaluate the effect of dwarf mistletoe on the competitive effect of woodboring beetles on MPB (n=30 logs) (See Obj. 3). To infest logs with woodboring beetles, we transported the freshly cut logs to a forest west of Little Smoky, Alberta (54°33.9' N, 117°37.6' W) dominated by mature lodgepole pine - jack pine hybrids that had an active MPB population. Maps from the Alberta Government indicated moderate levels of recent attacks by MPB in the area and we found pine trees in the stand that had evidence of MPB attacks both current and older. The site also had white spruce (*Picea glauca* [Moench] Voss) in the overstory and an understory dominated by *Ledum groenlandicum* (Oeder), *Rosa woodsii* (Lindl.) and mosses. Lots were arranged on the forest floor in groups of three, one log of each level of dwarf mistletoe

infection (DMR = 0, 2-3, or 5-6) (Figure Appendix C.4). We set up a total of 10 groups that were spaced 10 m apart and logs within groups were equidistantly placed 1 m apart. Each group was baited with a commercially available MPB attractant in order to attract woodboring beetles (*trans*-verbenol, *exo*-brevicomin, terpinolene; Synergy Semiochemicals Corp.). After 10 d, all logs were colonized by woodboring beetles but not MPB, potentially due to interference by dense forest floor vegetation to MPB attraction. The woodboring beetle community in the lodgepole pine - jack pine hybrid forest where logs were placed would resemble the community reported in jack pine forests (Gardiner 1975; Furniss and Carolin 1977; Erbilgin and Raffa 2001).

Immediately after removing the logs from the field, we manually introduced MPB into all woodboring beetle infested logs in the laboratory as described under Obj. 1. We suspected that during MPB introduction, woodboring beetle developmental stages in the logs spanned eggs to first instar when these two groups of beetles are potentially competitors (Peddle et al. 2002). To minimize intraspecific competition and to maintain a low-density MPB population (14-55 MPB attacks per m^2; Smith et al. 2011), the number of mating pairs introduced into each log was scaled to the log diameter, with a mean introduction density of 16.9 ± 0.3 per m^2 (mean ± standard error). Five mating pairs were introduced into logs with woodboring beetles because the diameter of logs with woodboring beetles was greater (24.2 ± 0.4 cm) than logs with only MPB (22.4 ± 0.4 cm). Although diameter of logs slightly varied, logs were well within the size range of MPB attacks (Safranyik et al. 2010). The conditions in the growth chamber and cold room were the same as in Obj. 1. Two months after removal from cold room, the area of phloem consumed by MPB and woodboring beetles was quantified on two 15 × 15 cm sections on opposite sides of each log (Figure Appendix C.2C). Measurements of MPB performance were performed as described in Obj. 1. Woodboring beetle feeding damage was differentiated from

that of MPB based on the size and pattern of maternal and larval galleries, as well as the tissue consumed (Figure Appendix C.2B, Figure Appendix C.3B). Galleries excavated by MPB were identified by their characteristic pattern of larval gallery construction perpendicular to the vertical maternal galleries. Cerambycid beetle galleries were generally wider than those of MPB and were packed with frass (fecal material and boring dust) containing strips of xylem tissue.

4.2.5 Obj. 3 – Evaluate whether the interaction between MPB and woodboring beetles is influenced by the infection intensity of the plant pathogen

All logs from Obj. 2 were used to evaluate the effect of dwarf mistletoe infection on the interaction of MPB and woodboring beetles (arrow c in Fig. 4.1) (n=30 logs) and measurements of beetle performance were performed as described in Obj. 1 and 2.

A relative interaction index (RII) (Armas et al. 2004; Dangles et al. 2013) was calculated to further identify the effect of DMR on MPB and woodboring beetle interactions:

$$RII = \frac{(P_{+N} - P_{-N})}{(P_{+N} + P_{-N})}$$

where P_{+N} is a measure of MPB in the presence of woodboring beetles and P_{-N} is a measure of MPB without woodboring beetles. The RII ranges from -1 (competition) to +1 (facilitation).

4.2.6 Obj. 4 – Identification of potential mechanisms of plant-mediated interactions among plant pathogen, MPB, and woodboring beetles

Average phloem thickness of all logs used to rear MPB and woodboring beetles was calculated by taking three measurements on the top portion of each fresh log. We also quantified the concentration of individual monoterpenes of jack pine phloem because they play a number of

ecological roles, including being toxic to insects and fungi, and their concentration and composition can be altered due to abiotic and biotic stress agents (Mattson and Haack 1987; Langenheim 1994; Colgan and Erbilgin 2011; Erbilgin and Colgan 2012; Goodsman et al. 2013). In the same stand of jack pine used for all the beetle rearing experiments, we selected mature jack pine trees according to their DMR (DMR=0, 2, 3, 5, and 6 had n=8, 4, 3, 4, and 3 trees, respectively) in 2011. Phloem samples from both the north and south sides of each tree were sampled at breast height, transported to the University of Alberta in dry ice, and stored at -40°C until extraction and chemical analysis using established methods (Lusebrink et al. 2011). Briefly, 100 mg of ground phloem were extracted twice with 0.5 ml of dichloromethane and 0.01% tridecane (surrogate standard). Samples with solvent were vortexed for 30 s, sonicated for 10 min, and centrifuged at 13,200 rpm at 0°C for 15 min. Sample extract (2 µl) was injected into a Gas Chromatograph/Mass Spectrometer (Agilent 7890A/5062C, Agilent Tech., Santa Clara, CA, USA) equipped with a DB-Wax column (I.D. 0.25 mm, length 30 m) (Agilent Techn.) with helium carrier gas flow at 1 ml min^{-1}, and a temperature of 50°C for 2 min, increased to 120°C by 10°C min^{-1}, and then to 250°C by 20°C min^{-1}. To quantify individual and total monoterpene concentrations, the following standards were used: borneol, α-terpinene, γ-terpinene, α-terpineol (Sigma-Aldrich, St. Louis, MO, USA), 3-carene, terpinolene, α-pinene, β-pinene, limonene, myrcene, camphene, *p*-cymene, 4-allylanisole (Fluka, Sigma-Aldrich, Buchs, Switzerland), bornyl acetate, *cis*-ocimene (SAFC Supply Solutions, St. Louis, MO, USA), and β-phellandrene (Glidco Inc., Jacksonville, FL, USA).

4.2.7 Data analyses

To identify relationships among woodboring beetle activity, DMR and MPB brood production and survival, we used a Generalized Linear Model for count data (e.g., number of larval

chambers per maternal gallery) with a Poisson distribution (i.e., Poisson regression) and a log link function (PROC GLIMMIX in SAS, ver. 9.3, SAS Institute, 2010). Model fit was tested by goodness-of-fit chi-squared test and overdispersion by Pearson statistic divided by degrees of freedom. To determine whether tree size influenced the host plant-beetle interactions it was included as a potential covariate but was not included in any models because it was not a significant predictor (i.e., $P > 0.05$). We expressed Poisson regression results on count variables (e.g., number of larvae per maternal gallery) as incidence rate ratios. These are the exponentiated parameter estimates (β coefficient), interpreted as the rate change in the dependent variable with a unit increase of the independent variable, holding all other variables constant. A linear regression model (PROC GLM in SAS, ver. 9.3) was constructed for non-count variables (e.g., gallery lengths). Model assumptions of normality and homogeneity of variance were visually assessed using Q-Q normality and residual plots. Quadratic relationships between DMR and measures of beetle performance were assessed by comparing the Akaike's information criterion between linear models with and without a squared DMR variable and selecting the model with the lowest criterion. Linear regressions (PROC GLM in SAS, ver. 9.3) were also constructed to assess relationships between DMR and measures of tree physical and chemical characteristics after natural log transformation of monoterpene concentrations to meet model assumptions. To compare RII values among DMR, we performed a bootstrap analysis by sampling with replacement records by DMR within each year. RII was calculated for each of 10,000 resampling iterations, from which a sampling mean was calculated and used to infer interaction type (i.e., competition or facilitation). Pearson's correlations were performed on these mean RII values to identify relationships between interaction type and DMR.

4.3 Results

4.3.1 Impact of the plant pathogen on MPB performance

In the absence of woodboring beetles (Obj. 1), the dwarf mistletoe infection severity had a negative impact on the length of maternal gallery and phloem area consumed by MPB (Fig. 4.2), as well as on the number of larvae and pupae per maternal gallery (arrow b1 in Fig. 4.1). With every 1 unit increase in DMR, there was a 10% decrease in maternal gallery length and a 15% decrease in MPB percent area consumed. Furthermore, for every 1 unit increase of DMR, there was a 21, 30, and 22% decrease in the number of MPB larval galleries, pupal chambers and emerged brood, respectively (n= 17 trees, larvae, pupae, brood incidence rate ratio = 0.79 (± 0.02), 0.70 (± 0.03), 0.78 (± 0.04), respectively, $P < 0.0001$). The number of MPB larvae, pupae, and emerged brood in logs with dwarf mistletoe infection was 24.2 (± 6.1) larvae per maternal gallery, 9.0 (± 2.3) pupae per maternal gallery, and 6.0 (± 1.7) brood per log, respectively.

Dwarf mistletoe severity also related to woodboring beetle feeding activity (arrow b2 in Fig. 4.1). Phloem area consumed by woodboring beetles in trees with moderate DMR (DMR = 2-3) was, on average, 48 and 39% lower than in trees without infection (DMR = 0) and trees with a high intensity rating (DMR = 6), respectively (Fig. Appendix C.1).

4.3.2 Effect of woodboring beetles on MPB performance

The woodboring beetle community was the same across all logs and was primarily made up of immature stages of cerambycids, accounting for 92 (± 4)% of total woodboring beetle community. The introduction success, defined as the percent of MPB introductions that resulted in the construction of maternal galleries, and brood production of MPB were negatively

associated with feeding activities of the woodboring beetles in the absence of dwarf mistletoe (Obj. 2) (arrow c in Fig. 4.1). The percent phloem area consumed by MPB decreased by 1% and the introduction success decreased by 10% with every 10% increase in the phloem area consumed by woodboring beetles (Figs. 4.3 and 4.4a). Furthermore, there was a 1% decrease in the number of MPB larvae per maternal gallery with every 1% increase in the phloem area consumed by woodboring beetles (incidence rate ratio= 0.99 (± 0.01), $P = 0.029$). There was a greater impact of woodboring beetle activity on MPB pupae, with a 5% decrease in the number of pupae per maternal gallery with every 1% increase in the area consumed by woodboring beetles (incidence rate ratio = 0.95 (± 0.03), $P = 0.050$). The number of larvae and pupae per maternal gallery averaged 9.5 (± 1.5) larvae and 0.6 (± 0.3) pupae, respectively. Mortality of pupae was high (88%) and only two broods emerged from the ten logs.

4.3.3 Influence of a plant pathogen on the interaction between MPB and woodboring beetles

The severity of infection by dwarf mistletoe altered the competition between woodboring beetles and MPB (arrow c in Fig. 4.1). Introduction success of MPB was dependent on the interaction of phloem area consumed by woodboring beetles and DMR (Fig. 4.4) and was negatively associated with woodboring beetle activity at the low dwarf mistletoe severities, but was neutral at higher severities. In contrast, the percent of phloem area consumed by MPB was negatively impacted by woodboring beetles but not by dwarf mistletoe infection (n = 30, $R^2 = 0.36$, $F_{1, 28} = 7.86$, $P = 0.002$, regression equation: percent of phloem area consumed by MPB = 9.78 + (-0.12*percent area consumed by woodboring beetles) + (-0.14*DMR), $P < 0.05$ for $\beta_{woodborers}$).

In dwarf mistletoe infected host trees, there was a significant effect of woodboring beetles on the number of MPB larvae and pupae. There was a decrease of 1 and 2% in the number of

larvae and pupae per maternal gallery, respectively, with every increase of 1% in woodboring beetle phloem area consumed in the presence of dwarf mistletoe (larval incidence rate ratio = 0.99 (± 0.003), n = 30 trees, $P = 0.002$, pupal chamber incidence rate ratio = 0.98 (± 0.009), n = 30 trees, $P = 0.015$). The number of MPB larvae and pupae in logs with dwarf mistletoe and woodboring beetles was 10.6 (± 1.0) larvae per maternal gallery and 1.2 (± 0.3) pupae per maternal gallery.

There was an overall decline in the competitive effect of woodboring beetles on MPB as the DMR increased (Fig. 4.5). Further, as the DMR increased, the effect of woodboring beetles on MPB maternal gallery length (r = 0.97, $P = 0.007$), larvae count (r = 0.86, $P = 0.061$), and pupae count (r = 0.99, $P = 0.003$), shifted from negative to neutral or positive RII values. Among all developmental stages analyzed, MPB pupae were subjected to the greatest competitive effect from woodboring beetles (Fig. 4.5). However, MPB introduction success and maternal gallery length exhibited generally neutral interactions with woodboring beetles.

4.3.4 Potential mechanisms of plant-mediated interactions among plant pathogen, MPB, and woodboring beetles

Dwarf mistletoe infection severity was associated with changes in the quality of the feeding and reproductive environments of MPB and woodboring beetles (arrows a, b1 and b2 in Fig. 4.1). There was a negative relationship between the DMR and the phloem thickness (n = 57 trees, $R^2 = 0.62$, $F_{2,56} = 44.06$, $P < 0.0001$, regression equation: natural log (phloem thickness) = 0.413 + (-0.035*DMR) + (0.506*year of experiment), P for $\beta_{DMR} = 0.013$, $\beta_{year} < 0.0001$). The year the trees were sampled was a significant covariate, where trees sampled in the first year had thicker phloem than trees in the second year. Furthermore, there was a quadratic relationship between the concentrations of monoterpenes (natural log transformed) and DMR: in lightly infected trees

the concentrations of α-pinene ($R^2 = 0.33$, $F_{2,21} = 4.66$, $P = 0.023$), camphene ($R^2 = 0.36$, $F_{2,21} = 5.35$, $P = 0.011$), and total monoterpenes ($R^2 = 0.26$, $F_{2,21} = 3.40$, $P = 0.056$) increased with increasing DMR, in contrast, in severely infected trees, there was a negative relationship (Fig. 4.6). Other monoterpenes identified did not have any type of relationship with levels of dwarf mistletoe infection (see Table Appendix C.2).

4.4 Discussion

Our results demonstrate that gradients of infection by a parasitic plant can induce non-linear changes in host plant quality, which can in turn modify interactions among co-occurring insect taxa on the same host. These results support the results of Tack and Dicke (2013), which predicted that plant pathogens are critical in performance, preference and population dynamics of host-sharing herbivores, and extend their predictions that gradients of pathogen infection can also be important in altering competitive interactions among subcortical insect herbivores that share the same resources.

We found that in the absence of dwarf mistletoe, woodboring beetles negatively affected MPB performance (arrow c in Fig. 4.1). As Lawton and Strong (1981) speculated, competition among resource-sharing species should result in the spatial disruption (e.g., depletion of resources by a competitor prevents feeding by others) and reduced survival of one species when in the presence of others. In the current study, the reduction in both host colonization and establishment of MPB was strongly affected by the feeding activities of the woodboring beetles. There was also a cascading impact of woodboring beetles on MPB performance as MPB developmental stages progressed with the negative impact of competition most evident at the pupal stage. These results suggest that MPB and woodboring beetles are likely in competition in

the subcortical environment and the spatial distribution and performance of the former was most likely altered by the competition.

One of the most novel findings of our study is that the direction of interaction between MPB and woodboring beetles MPB was altered by the dwarf mistletoe infection intensity. The interactions between resource-sharing beetles resulted in a dampening of the competitive effect of woodboring beetles on MPB performance to the point of potential facilitation when the dwarf mistletoe infection was the most severe (arrow c in Fig. 4.1). With increasing infection severity, the effect of woodboring beetles on MPB introduction success, maternal gallery length, and numbers of larvae and pupae shifted from negative to neutral or positive. Although species interactions were tested in logs in the current study, there is evidence from live host trees that MPB performance can be facilitated by a competing bark beetle species, *Pseudips mexicanus*, on dwarf mistletoe infected trees (Smith et al. 2011). However, Smith et al. (2011) did not report the severity of dwarf mistletoe infection. In general, our study is the first to demonstrate a shift in interaction from competitive to potentially facilitative due to a parasitic plant infection. These results emphasize that the cascading effects of plant pathogen infection on host quality can lead to a wide range of consequences and outcomes among co-occurring herbivores (Bertness and Callaway 1994; Barrio et al. 2013).

We also found that a plant pathogen can differently affect members of the same feeding guild depending on the level of pathogen infection (arrows b1 and b2 in Fig. 4.1). The performance of MPB declined with the increasing infection severity in the absence of woodboring beetles, whereas in the hosts with the highest infection severity, the woodboring beetles consumed more subcortical tissues relative to their consumption rate at the moderate infection severity. The outcome of this study also bears similarities to interactions among a powdery-mildew fungal parasite, a free-feeding defoliator, and a leaf miner on oak (*Quercus*) in

that the plant parasite differentially affected the performance of insects, which had cascading effects on the insect community (Tack et al. 2012). In the earlier study, although the plant parasite reduced the fitness of the defoliator insect, it improved growth rate, decreased developmental time, and increased parasitism rate of the leaf miner. It appears that the outcome of plant-mediated interactions between plant pathogens and insect herbivores on the same host plant can lead to the improvement of performance of some organisms while impairment of performance of others depending on the intensity of pathogen infection (Bonello et al. 2006; Tack and Dicke 2013), which in turn can influence interactions among members of a community (Tack et al. 2012; Zargaran et al. 2012).

4.4.1 Possible mechanisms that mediated parasite-subcortical community interactions on jack pine

Although multiple mechanisms may be responsible for the observed interactions among the subcortical community on jack pine, our study provided two possible drivers: changes in phloem thickness and amount of defense chemicals in response to pathogen infection (arrow a in Fig. 4.1). Since the phloem represents the food and water source for beetles, phloem thickness has been shown to be positively associated with MPB brood production in lodgepole pine (Amman 1972). In the current study, when food quality was optimal (e.g., thick phloem), for example, we observed a 5% reduction in the number of pupae per maternal gallery with every 1% increase in the area consumed by woodboring beetles. However, when food quality diminished (e.g., thin phloem) the same rate was only 2% with every 1% increase in the area consumed by woodboring beetles in the presence of dwarf mistletoe. The difference of 3% in the rate of decrease of MPB pupae count likely released MPB from inter-species competition. Since the negative impact of dwarf mistletoe severity on thickness of jack pine phloem was evident in both years, beetle

reared in logs either year likely experienced the same reduced host quality. We suspect that changes in the phloem thickness accelerated the feeding by the woodboring beetles on the thin phloem, through compensatory feeding, which in turn fostered their developmental rates, as reported for other subcortical beetles (Amman and Cole 1983; Smith et al. 2011). This could have resulted in the migration of woodboring beetle larvae into the xylem sooner than in hosts with thicker phloem. This early migration would have reduced competition between species. Higher initial feeding rates in thin versus thick phloem are due to the need to consume a greater quantity of phloem substrate to obtain sufficient resources for development (Safranyik and Carroll 2006). Food quality-induced behavioral changes have been reported for other herbivorous insects in response to low resource quality (Berner et al. 2005; Colgan and Erbilgin 2011) and may limit the competitive ability of consumers (Levin 1970).

Furthermore, increased amounts of host defense chemicals may be another potential mechanism that drove the observed interactions. We found the greatest amount of monoterpenes in the jack pine with a moderate pathogen infection severity compared with the trees with no infection or high infection severity. Woodboring beetles and MPB were differentially affected by the pathogen-induced changes in monoterpene concentrations. While woodboring beetle performance was inversely related to the monoterpene concentrations, MPB performance was not affected. Some bark beetle species, including MPB, can detoxify host tree terpenes, reflecting both their ability to colonize and kill live trees during outbreaks and their life history strategy as near obligate parasites (Paine et al. 1997; Keeling and Bohlmann 2006; Adams et al. 2013). In contrast, woodboring beetles act as facultative parasites and are generally restricted to colonizing host trees that are weak or under attack by other organisms and do not tolerate high concentrations of host defense chemicals (Paine et al. 1997; Safranyik et al. 1999). Taken together, these results support that life history strategies play a role in the species-specific

responses to the pathogen induced defenses in plants (Tack et al. 2012), and thus pathogen infection does not necessarily lead to the same level of host suitability to all community members. Furthermore, though not examined in this study, defense chemicals induced by insect species can also be important for understanding community level interactions (Kant et al. 2015).

Likewise, the potential importance of beetle-associated fungi in mediating interactions among the phloem infesting insect community cannot be discounted (Paine et al. 1997). The symbiotic fungi that are introduced by beetles during host colonization increase the phloem nutritional value (Adams and Six 2007; Bleiker and Six 2007; Goodsman et al. 2012; Therrien et al. 2015). Furthermore, some bark beetle-associated fungi have been shown to metabolize terpenes, thus potentially contributing to detoxification of tree defenses (DiGuistini et al. 2007). Host conditions, such as moisture or changes in defense chemistry, can also affect the community of bark beetle-associated fungi, which could impact beetle performance (Bleiker and Six 2007; Adams et al. 2011). These complex subcortical interactions can create opportunities for further research to understand how fungal interactions with the host plant and insects may alter the resource quality for both MPB and resource-sharing woodboring beetles and may be one of the mechanisms affecting herbivore interactions.

4.4.2 Conclusions

Our results show that changes in the direction of interactions between beetles from competition to potential facilitation are driven by species-specific responses to pathogen-induced changes to host condition and quality. These responses are important in understanding community organization (Maestre et al. 2009), as species can differentially respond to plant parasite-induced changes in host condition (Röder et al. 2007; Tack et al. 2012).

We demonstrate that plant-mediated interactions driven by plant pathogens can affect insect community composition by altering interactions among insects. These changes to insect interactions may be important for our study system, where the maintenance of the eruptive bark beetle MPB could be influenced by its interactions with native organisms in the novel host jack pine forests. Furthermore, our results show that considering a range of interactions into a wider community context is critical to understand and predict invasive species establishment success and population dynamics in novel habitats. Positive effects of woodborer-plant pathogen interactions on MPB will be particularly important when MPB populations are low in jack pine forests, as they will be highly vulnerable to local extinction due to Allee effect (Liebhold and Tobin 2008; Altieri et al. 2010). Current wide-spread dwarf mistletoe infestations on jack pine forests in northern Alberta may in fact encourage establishment success of low-density MPB populations and lower the probability of 'extinction' of populations in this novel habitat.

Figure 4.1. Conceptual model showing the potential impact of increasing intensity of plant pathogen infection of a host plant on interactions among resource-sharing subcortical beetles and illustrating the interaction patterns tested in this study. Plant pathogen directly affects plant characteristics (a, solid line), for example, in our study jack pine (*Pinus banksiana*) defense chemicals and phloem thickness are altered by dwarf mistletoe infection (*Arceuthobium americanum*). Plant pathogen has an indirect (b, dotted line) negative effect on herbivore 1 (*Dendroctonus ponderosae*) (b1) and on herbivore 2 (woodboring beetles) (b2). Effects of herbivores 2 on 1 are mediated by plant pathogen infection intensity (c, dotted line), where at none to low, moderate, or high intensity leads to a negative, neutral, or potentially positive effect of herbivore 2 on herbivore 1, respectively.

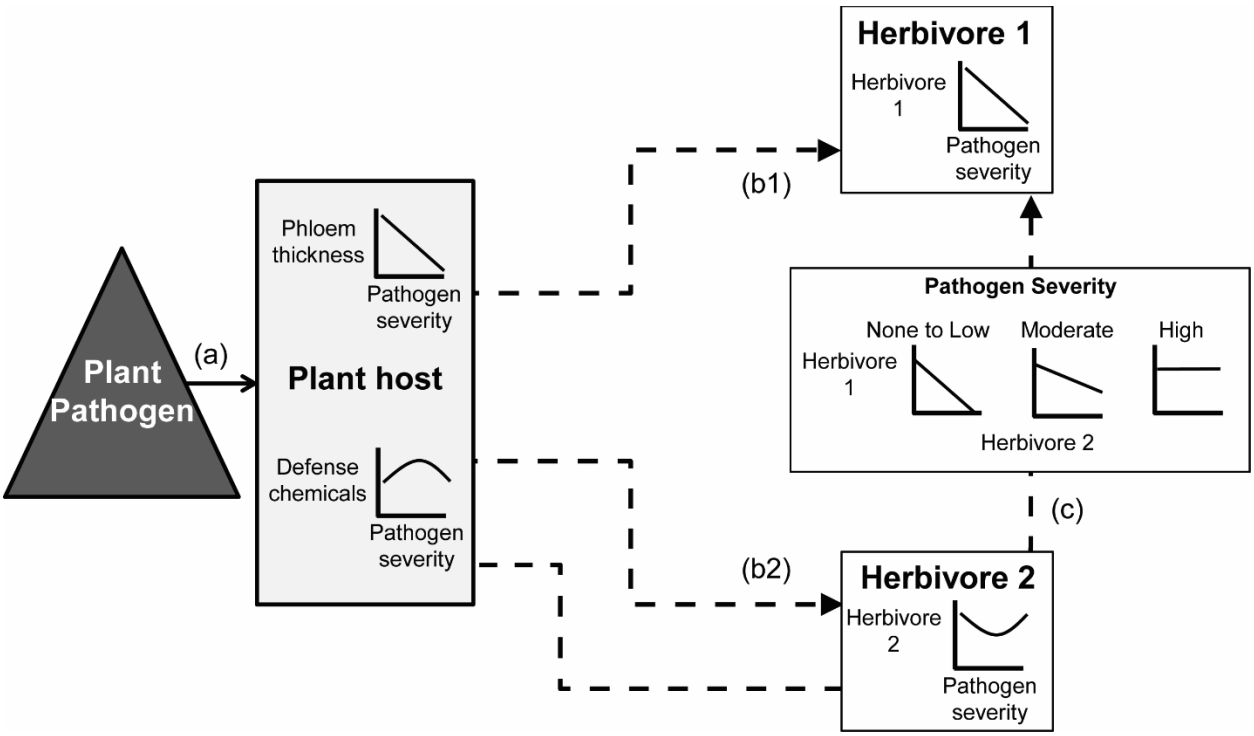


Figure 4.2. Relationship between the mountain pine beetle (MPB, *Dendroctonus ponderosae*) and dwarf mistletoe (*Arceuthobium americanum*) in the absence of woodboring beetle infestations in jack pine (*Pinus banksiana*) (n = 17 trees). Dwarf mistletoe infection severity quantified by dwarf mistletoe rating (DMR). (a) Maternal gallery length = 22.84 + (-2.34*DMR), $R^2 = 0.31$, $F_{1,15} = 6.61$, $P = 0.021$, $P < 0.05$ for $\beta_{intercept}$ and β_{DMR}. (b) Percent area consumed by MPB = 18.78 + (-2.85*DMR), $R^2 = 0.35$, $F_{1,15} = 8.02$, $P = 0.013$, $P < 0.05$ for $\beta_{intercept}$ and β_{DMR}.

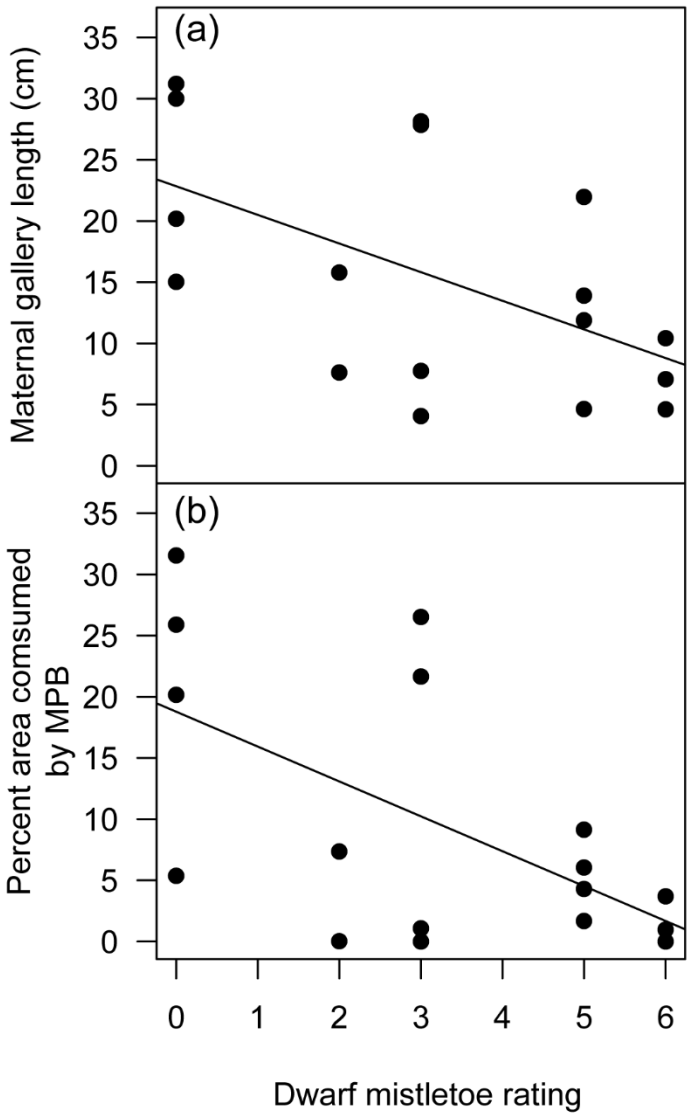


Figure 4.3. Relationship between woodboring beetle feeding activity and the mountain pine beetle (MPB, *Dendroctonus ponderosae*) feeding activity, in the absence of host jack pine (*Pinus banksiana*) infection by the parasitic plant dwarf mistletoe (*Arceuthobium americanum*) (n = 10 trees). Percent area consumed by MPB = 8.54 + (-0.08*woodboring beetles), $R^2 = 0.52$, $F_{1,8} = 8.74$, $P = 0.018$, $P < 0.05$ for $\beta_{intercept}$ and $\beta_{woodboring\ beetles}$.

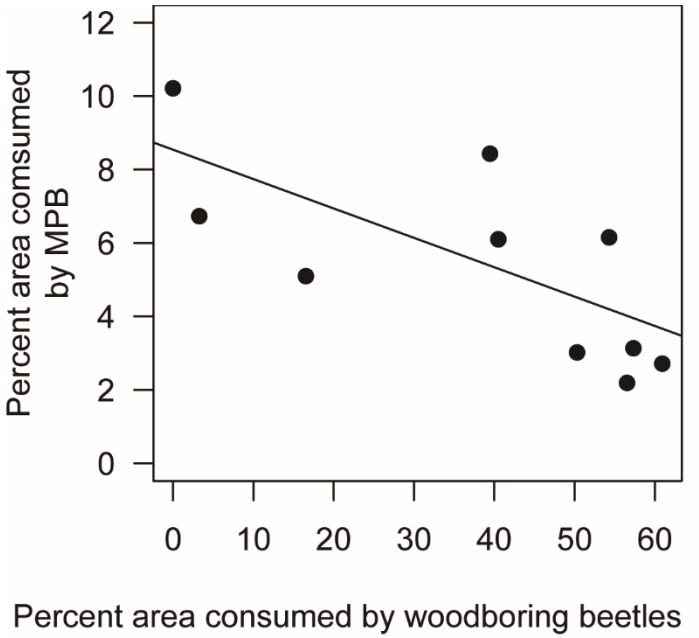


Figure 4.4. Relationship between introduction success of the mountain pine beetle (*Dendroctonus ponderosae*) and the interaction between phloem area consumed by woodboring beetles and dwarf mistletoe (*Arceuthobium americanum*) rating in jack pine (*Pinus banksiana*) (n = 30 trees). Dwarf mistletoe infection severity quantified by dwarf mistletoe rating (DMR). Introduction success = 105.20 + (-0.89*percent area consumed by woodboring beetles) + (-3.00*DMR) + (0.14*woodboring beetles*DMR), $R^2 = 0.43$, $F_{3,26} = 6.59$, $P = 0.002$. (a) through (e) are *D. ponderosae* introduction success in relation to percent area consumed by woodboring beetles at each DMR (0 through 6).

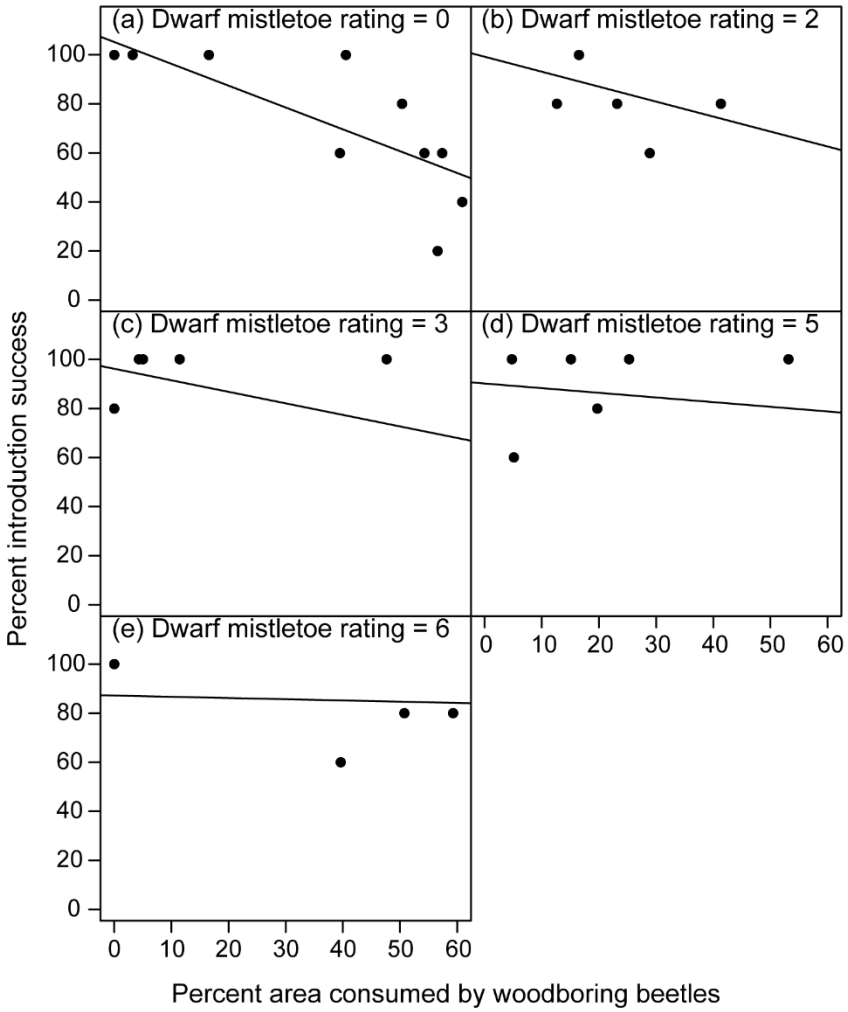


Figure 4.5. Relative interaction indexes for measures of the mountain pine beetle (*Dendroctonus ponderosae*) interaction with woodboring beetles over increasing habitat stress caused by dwarf mistletoe (*Arceuthobium americanum*) in jack pine (*Pinus banksiana*). (a) Introduction success, Pearson's r = 0.47, $P = 0.419$. (b) Maternal gallery length, Pearson's r = 0.97, $P = 0.007$. (c) Larval gallery counts per maternal gallery, Pearson's r = 0.86, $P = 0.061$. (d) Pupal chamber counts per maternal gallery, Pearson's r = 0.99, $P = 0.003$.

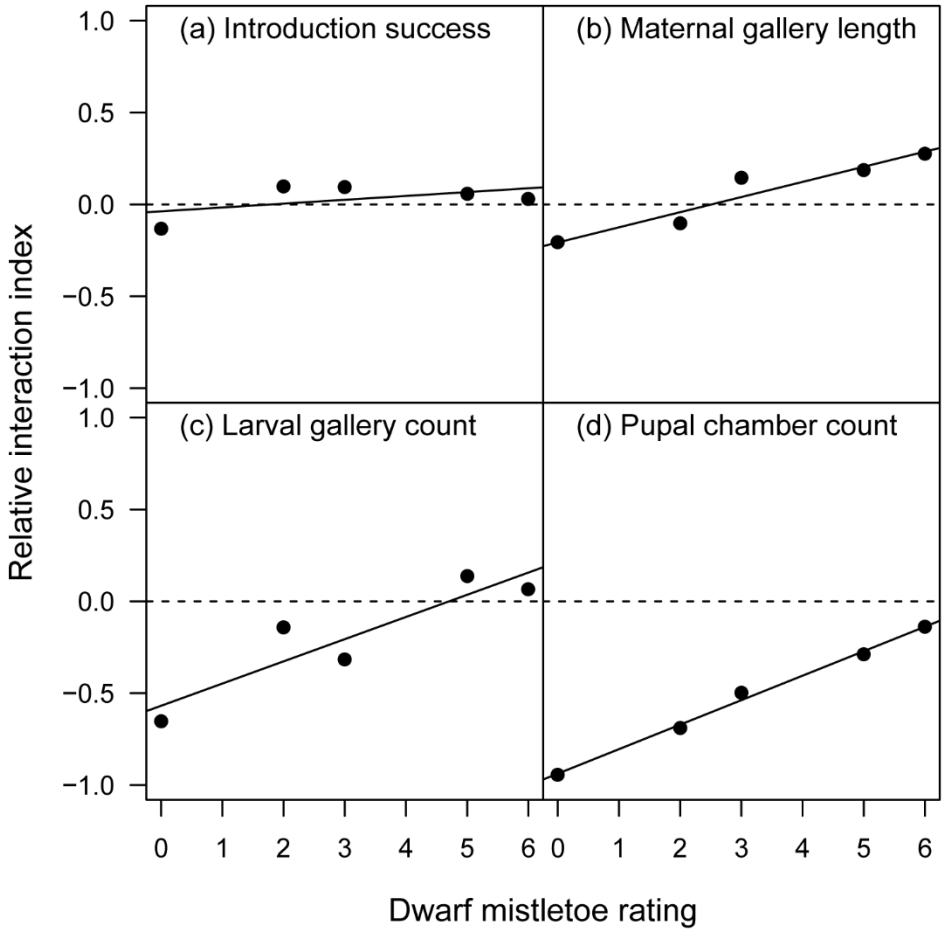
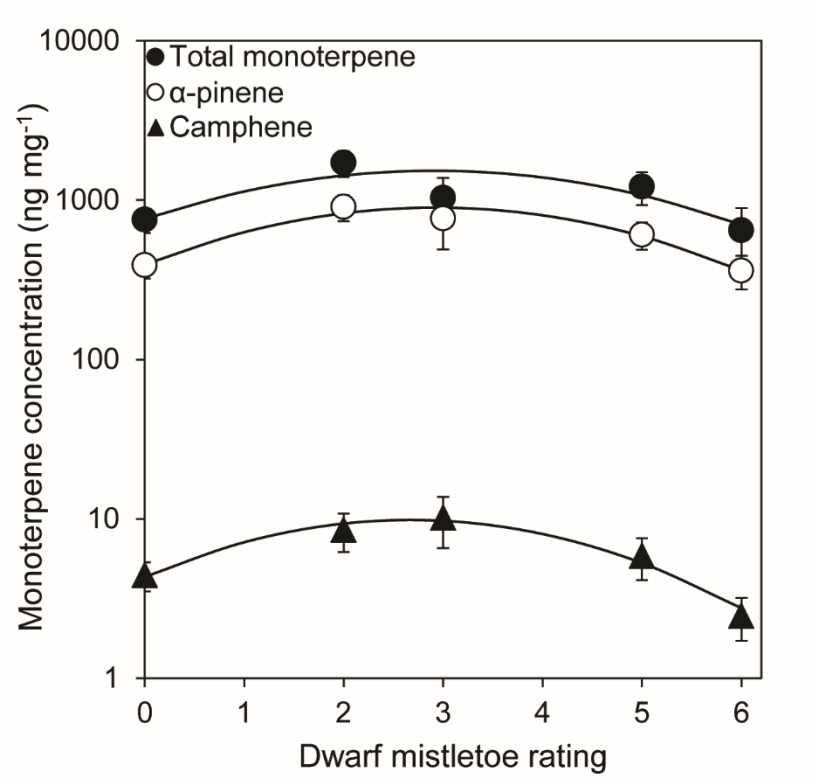


Figure 4.6. Relationship between severity of dwarf mistletoe (*Arceuthobium americanum*) infection on jack pine (*Pinus banskiana*) and monoterpene concentration. Points are mean natural log transformed concentrations of total monoterpenes ($R^2 = 0.26$, $F_{2,21} = 3.40$, $P = 0.056$), α-pinene ($R^2 = 0.33$, $F_{2,21} = 4.66$, $P = 0.023$), and camphene ($R^2 = 0.36$, $F_{2,21} = 5.35$, $P = 0.011$) at each dwarf mistletoe rating. Error bars are standard errors (n=24 trees). Note log scale used in panel.



Chapter 5

Thesis discussion

My research demonstrates that abiotic and biotic disturbances can affect interactions among jack pine (*Pinus banksiana* Lamb.)-infesting organisms through systemic cross-induction of resistance or susceptibility. These resistant and susceptible responses are only relative to trees without the initial induction event as discussed by Bonello et al. (2006), and does not infer that the tree is completely resistant or susceptible to attack by MPB. While drought did not impact induced responses of jack pine seedlings to fungal inoculation or phytohormone application, reduced water availability did lead to systemically induced susceptibility to subsequent inoculation by the beetle-associated fungus, *Grosmannia clavigera* Robinson-Jeffrey & Davidson, as measured by lesion length. Also, my research quantified the change in defensive chemistry, physical characteristics, defensive anatomy, and growth (Appendix C) in jack pine as a result of dwarf mistletoe (*Arceuthobium americanum* Nutt. ex Engelm.) infection. These dwarf mistletoe-induced changes also altered the relative resistant response by the tree to *G. clavigera* in a non-linear manner, with the greatest monoterpene concentrations and expression of systemic induced resistance (i.e., shortest lesion length) in trees with moderate dwarf mistletoe severity. Furthermore, dwarf mistletoe systemically mediated interactions between mountain pine beetle (*Dendroctonus ponderosae* Hopkins, MPB) and a community of resource-sharing insects through altering defensive chemistry and physical characteristics. These results identify the defense chemical classes and physical characteristics that are altered due to dwarf mistletoe infection of jack pine and whether these changes are associated with a relative resistant response to MPB. Furthermore, this research extends the systemic induced resistance hypothesis to

include environmental stress and biotrophic pathogens as factors that impact resistant responses to necrotrophic pathogens.

5.1 Jack pine response to multiple attackers is drought dependent

Jack pine seedlings in my study responded to drought conditions with a relative resistance response to *G. clavigera*, which supports other research that also found no difference in monoterpene concentrations at moderate or low water availability (Lusebrink et al. 2011, Arango-Velez et al. 2014, Erbilgin et al. 2017). However, low-level drought conditions have been shown to increase monoterpene concentrations in *P. contorta* (Dougl. ex Loud.) (lodgepole pine) (Lusebrink et al. 2011, Arango-Velez et al. 2014) and also potentially to induce slight resistance to bark beetle outbreaks in some pine systems (Kolb et al. 2016). In the jack pine study, an application of initial phytohormone stress elicitors as a proxy for an attack by biotic organisms resulted in the cross-induction of susceptibility to *G. clavigera* in the low watering treatment compared to seedlings with normal water availability. This suggests that jack pine may be more susceptible to *G. clavigera* and potentially MPB when under drought conditions and when under attack by multiple organisms than without other attacking organisms.

5.2 Differential defense induction from dwarf mistletoe impacts host resistance

Monoterpene levels in the lower stem of jack pine increased at moderate severity infections of dwarf mistletoe but decreased at higher severities. Furthermore, resin duct production in recent wood formation decreased with increasing dwarf mistletoe infection severity (Appendix C). In contrast, total induced phenolics had a slight positive relationship with dwarf mistletoe severity. Along with defensive traits, physical characteristics were altered in jack pine by dwarf mistletoe; both phloem thickness and moisture decreased with increasing dwarf mistletoe severity (Appendix C).

These defensive traits may play different ecological roles for jack pine. The differential induction of defenses from dwarf mistletoe impacts the subsequent relative resistance to *G. clavigera*. Monoterpene concentrations negatively correlated with susceptibility to *G. clavigera*, which supports other research identifying its importance in tree resistance to bark beetle fungi (Raffa and Berryman 1983b). In contrast, phenolics did not appear to interfere with the growth of *G. clavigera*; the highest accumulation of phenolics occurred in trees with the greatest expression of susceptibility, as measured by lesion length. Instead of acting as defensive compounds, phenolics could have been detoxified or used as a carbon source by *G. clavigera*. For example, Hammerbacher et al. (2013) and Wadka et al. (2016) found that the bark beetle-associated fungus *Endoconidiophora polonica* (Siemaszko) Z.W. de Beer, T.A. Duong & M.J. Wingf. (previously *Ceratocystis polonica*) reduced the concentration of toxic phenolics (stilbenes) in Norway spruce (*Picea abies* (L.) Karst.) through specific fungal enzyme activities and by metabolizing these phenolics into a source of carbon. Furthermore, decreased resin duct production is a predictor for susceptibility of pines to bark beetle attack (Kane and Kolb 2010, Gaylord et al. 2013, Ferrenberg et al. 2014). The negative impact of dwarf mistletoe on resin duct production along with concentrations of 4-allylanisole and levopimaric acid identifies that jack pine with severe dwarf mistletoe infections are more susceptible to bark beetle attack.

5.3 Coordination of defense chemicals and cross-talk of signaling pathways

My results demonstrate that cross-talk between different signaling pathways in conifers (Bostock et al. 2001, Stout et al. 2006) may be regulated by the level of prior infection. Biotrophic pathogens trigger the salicylic acid pathway, while infection by necrotrophic pathogens or chewing insects results in the induction of the jasmonic acid pathway (Stout et al. 2006). However, the salicylic acid has been shown in model plant-pathosystems to be antagonistic to the accumulation of jasmonic acid (Stout et al. 2006, Thaler et al. 2012). This negative cross-talk

between signaling pathways can induce systemic susceptibility to necrotrophic pathogens and chewing insects (Thaler et al. 2012). This pattern is in contrast with my findings in jack pine with dwarf mistletoe infections of moderate severity, but consistent with the effect of severe infections. Furthermore, I did not find induced susceptibility from application of methyl salicylate at 100 mM to inoculation by *G. clavigera*, a necrotrophic pathogen that should be susceptible to jasmonate-dependent defenses. Infection of tomato plants (*Lycopersicon esculentum* Mill.) by the bacterium *Pseudomonas syringae* pv. *tomato* (Okabe) Young, Dye & Wilkie also triggers salicylic acid-dependent responses, which do not counteract the induction of jasmonate-dependent resistance to chewing insects (Bostock et al. 2001). If negative cross-talk does not occur in jack pine (e.g., potentially at low dwarf mistletoe infection severity), then the attack by a biotrophic organism or the accumulation of endogenous salicylic acid found in jack pine under water stress (Arango-Velez et al. 2016) may not negatively impact the defensive response against *G. clavigera* or MPB. However, at higher severities of dwarf mistletoe infection, there is induced susceptibility and the potential for negative cross-talk between defense pathways. Therefore, other factors such as differential regulation of these defense pathways depending on host stress levels, infection severity, and/or specific type of biotrophic pathogen (e.g., fungal, bacterial, or parasitic plant) along with cross-talk between these and other signaling pathways may also be occurring in both model plants and in conifers (Bostock et al. 2001; Stout et al. 2006).

5.4 Plant pathogens can mediate insect interactions

The study system I used to research tripartite interactions was the interaction of MPB and woodboring beetles in jack pine with differing levels of dwarf mistletoe infection. Bark beetles and the community of resource sharing insects also interact in many other systems, such as MPB and *Ips* spp. in lodgepole pine (Moeck and Safranyik 1984, Rankin and Borden 1991, Safranyik

et al. 1999), and *D. rufipennis* Kirby and *Ips tridens* Mannerheim and *Dryocoetes affaber* Mannerheim in spruce (Engelmann spruce, *Picea engelmannii* Parry) (Poland and Borden 1998) as two examples. These interactions tend to result in competition, and usually this competition can be asymmetric by favoring one insect over the other (Safranyik et al. 1999, Allison et al. 2004). However, beetles can use semiochemicals to either take advantage of hosts initially attacked by one species (Wood 1982, Borden 1989, Allison et al. 2001, 2004) or to avoid competition with other species (Birch and Wood 1975, Borden 1989, Poland and Borden 1998). For example, MPB was less attracted to traps baited with the aggregation pheromone of *I. pini*, ipsdienol, in combination with MPB aggregation pheromones (Hunt and Borden 1988). Furthermore, Hunt and Borden (1988) found that *I. pini* also had reduced attraction to its own aggregation pheromone when combined with MPB aggregation pheromones. *Ips pini* and MPB both respond to a number of chemicals from either beetle, which these beetles can use to avoid competition through resource partitioning (Pureswaran et al. 2000). Using this phenomenon of competitive exclusion, Poland and Borden (1998) were able to deter attack of Engelmann spruce by *D. rufipennis* using chemicals emitted by competing insects. Therefore, while these insects do compete for resources, they can use mechanisms to avoid the negative impact of competition though use of allomones and kairomones (i.e., chemicals emitted by organisms that benefit the emitter or benefit the receiver, respectively). However, at the same time insects also use kairomones to find host trees already attacked by bark beetles (Wood 1982, Borden 1989, Allison et al. 2001, 2004). While the interactions I measured between MPB and woodboring beetles in logs most likely occur in lodgepole pine and may occur in jack pine, insects do have mechanisms to avoid competition.

I found that dwarf mistletoe can potentially mediate competition between MPB and other subcortical insect herbivores. Increasing dwarf mistletoe infection severity reduced the

competitive effect of woodboring beetles on MPB success, suggesting that only a full range of plant pathogen infections can capture non-linear responses of intra-guild species interactions (Bertness and Callaway 1994, Barrio et al. 2013). The changes to insect interactions were likely driven by species-specific responses to defense compounds and resource quality of the host jack pine trees. As also hypothesized for other MPB-subcortical insect interactions by Smith et al. (2011), the positive effect of the interaction between tree-infesting organisms on MPB's success may be particularly important when MPB populations are low and it is highly vulnerable to local extinction due to the Allee effect (Liebhold and Tobin 2008, Altieri et al. 2010). The Allee effect is an inverse density dependence and occurs among conspecifics when the positive relationship between population growth rate and population density increases the potential for extinction while at low populations (Courchamp et al. 1999). Allee effects on MPB may be particularly important because of several features of MPB: 1) MPB is subject to a strong Allee effect, where there is a threshold of extinction on an individual tree basis (Goodsman et al. 2016), that could be impacted by several factors such as, tree defenses (Boone et al. 2011), interactions with competing insects or natural predators, and aggregation success (Erbilgin et al. 2014a, Goodsman et al. 2016), and 2) MPB is invading a new host and local extinction could remove it from this new range (Erbilgin et al. 2014a). Therefore, while the negative impact of woodboring beetles on MPB performance has the potential to push MPB populations below an extinction threshold, when both insects are on a host tree with severe dwarf mistletoe infection, the competitive effect of woodboring beetles is reduced and MPB may escape any Allee effect. Consequently, my research has shown that native organisms, such as dwarf mistletoe and the subcortical insect community, could have the potential to impact MPB maintenance in the jack pine forest through tree-mediated interactions.

5.5 Limitations of study system

There are several caveats that should be addressed with respect to the conclusions that can be drawn while using my study system. First, lesion length was assumed to be a proxy for a resistant or susceptible response in jack pine in this thesis. However, *G. clavigera* and jack pine do not have a co-evolved relationship. Therefore, the assumption that a shorter lesion implies a less virulent pathogen and more resistant response should be tested. To better determine whether shorter lesion length indicated a more resistant response, I would need to measure other factors, such as physiological responses, tree mortality, fungal spread within phloem, and xylem penetration by the fungus. However, constitutive monoterpene concentrations in jack pine, which are known to be toxic to *G. clavigera*, had an inverse relationship with lesion length. Furthermore, other related ophiostomatoid fungi are native in jack pine forests, such as *Ophiostoma ips* (Schenk and Benjamin 1969), and jack pine may recognize and respond to this class of fungi using a general class of defense chemicals. Therefore, this negative relationship between monoterpene concentrations and lesion length supports my use of lesion length as a measure of a resistant response.

The use of logs and insects trapped using pheromone baited traps could be a limitation in the extension of my conclusions to the natural population. Bentz (2006) found that MPB caught in pheromone baited traps and those caught from emergence traps were not the same in emergence time, flight periodicity, and lipid content. My use of MPB from pheromone baited traps therefore reflects the populations of beetles that were most attracted to these synthetic baits and beetles used in this research could have been be re-emergent adults along with new brood adults. Also, habitat quality for MPB and woodboring beetles in logs, as used in this research, will be different than in live trees. Changes in logs could include many factors, such as moisture loss, change in volatile defense chemical concentrations, and contamination of tissue with

saprotrophic microorganisms. Furthermore, insects introduced into logs will not experience induced responses that they would have otherwise elicited in a live tree. The use of logs instead of live trees in my research was due to restrictions from using mature trees that were imposed from asking the types of research questions I did. Furthermore, I could not test the combined impact of MPB on live mature trees because experiments with live MPB in jack pine forests were not allowed by the Government of Alberta and both the parasitic plant and MPB do not yet readily co-occur naturally in jack pine, and thus I was restricted to use logs in this research. The similar constraint has been cited in other studies that were focused on different aspects of jack pine-MPB interactions (e.g., Erbilgin et al. 2014a, Lusebrink et al. 2013). However, while logs may not be the most ideal environment to test host-insect interactions, they have been effectively used in the scientific literature (Dyer and Seabrook 1978; Rankin and Borden 1991; Erbilgin et al. 2006, 2014a; Raffa et al. 2013). A similar pattern of facilitation I found in my research was also found by Smith et al. (2011) in live lodgepole pine trees infected by dwarf mistletoe (*A. americanum*) where *Pseudips mexicanus* (Hopkins) presence facilitated MPB performance, though dwarf mistletoe infection severity was not reported. Therefore, although my study was conducted in logs, there is evidence from live host trees that intra-guild interactions may be facilitative in host trees infected with dwarf mistletoe and further research on live trees should be conducted.

As logs and live trees are not the same environment for MPB and its associated fungi, so are there differences in primary and secondary metabolites between seedlings and mature trees (Barton and Korchieva 2010, Erbilgin and Colgan 2012, Lusebrink et al. 2013). Erbilgin and Colgan (2012) showed that jack pine seedlings had greater constitutive monoterpene concentrations than mature live trees. Furthermore, jack pine seedlings showed lower induction of monoterpenes across all induction treatments, including *G. clavigera* inoculation, than mature

live trees (Erbilgin and Colgan 2012). However, there is evidence that defense responses to a number of disturbances (e.g., drought, *G. clavigera* inoculation, phytohormone application) in jack pine or lodgepole pine in greenhouse grown seedlings can correlate with responses in mature trees (Lusebrink et al. 2011, 2013, Erbilgin and Colgan 2012). Therefore, conclusions made using jack pine seedlings, which are not the natural ontogenic stage for MPB nor its associated fungi, should be used to develop further investigations in mature trees.

5.6 Concluding remarks

With MPB continuing to expand in jack pine forests, it will be important to understand factors contributing to the invasion dynamics. My studies contribute both applied and basic science to the field of invasion biology by: (1) Identifying dwarf mistletoe as a factor that influences the relative susceptibility of forest communities, which could be targeted for mitigation to reduce the risk of the invasion; (2) Improving the current understanding of the regulation and coordination of defense signaling, in which an infection by a biotrophic pathogen can make a plant more or less susceptible to an insect or necrotrophic pathogen; and (3) Investigation of how native insects can alter MPB success. This information will be valuable in understanding the invasiveness of MPB in jack pine forests.

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Appendix A

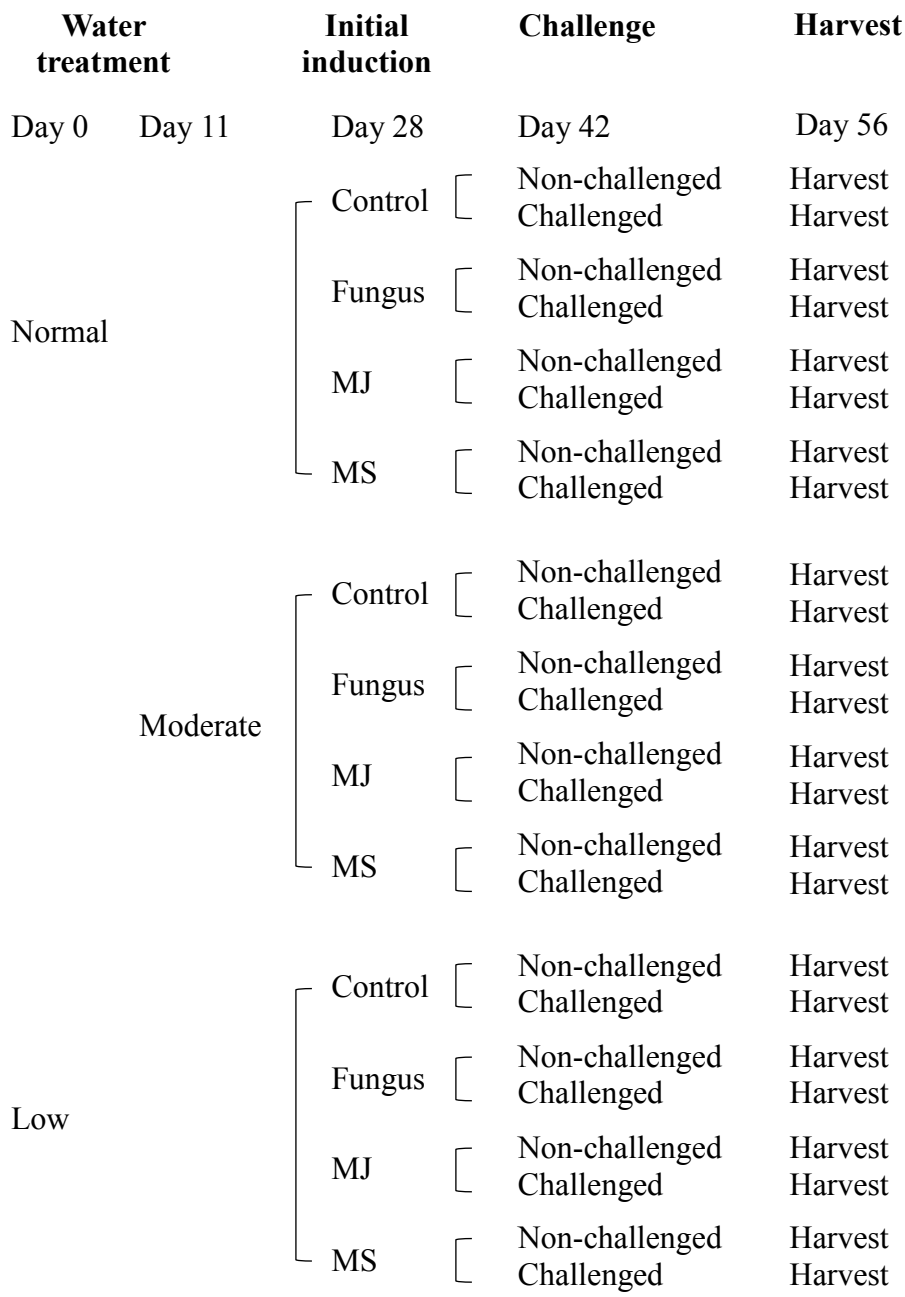

Figure Appendix A.1. Diagram of experimental design and time line for experiment on *Pinus banksiana* response to drought and multiple attackers. The initial induction treatments were applied on lower third of trees as follows: No application of induction agent (Control), inoculation with *Grosmannia clavigera* (Fungus), application of methyl jasmonate (MJ), and

methyl salicylate (MS). Fungal challenge treatment involved the inoculation of *G. clavigera* on middle third of trees.

Appendix B

Details of phenolics analysis

The UPLC has significantly shorter sample run times, superior chromatographic separation, and UV detection compared to the HPLC-MS, and, thus, was preferentially used for determining the peak areas and their UV spectra. Chromatographic separation from UPLC was achieved using a binary solvent system with water and methanol (both acidified to 2% v/v glacial acetic acid) at the following solvent gradient (percentages referring to water solvent): 0-0.75 min hold at 97%; 0.75-9 min 97%-70%; 9-11 min 70%-10%; 11-13 min 10%-0%; 13-14.5 min hold at 0%; 14.5-15 min 0%-97%; 15.5-20.5 min hold at 97%. Waters Empower 3 software was used to determine peaks for quantification at 280 nm using the ApexTrack integration algorithm for selecting peak apexes. The following criteria were used for determining what constituted a peak: peak height = 2000; peak width = 10.0; peak area = 12500; peak threshold (used for determining baselines) = 2.00e+002.

For HPLC-MS analyses, chromatographic separation was achieved using a binary solvent system with water and methanol (both acidified to 0.1% v/v glacial acetic acid) at the following solvent gradient (percentages referring to water): 0-42 min 100%-50%; 42-45 min 50%-15%; 45-53 min 15%-0%; 53-56 min hold at 0%; 56-59 min 0%-100%; 59-65 min hold at 100%. The following MS parameters were used for full scan: electron spray ionization; negative mode scanning 60-800 m/z; -80 capillary volts; ± 5000 needle volts; ± 600 spray shield volts; 50 psi nebulizer gas; 30 psi drying gas; 400 °C drying gas temperature. The same conditions were used for MS^n analysis, with MS^1 fragmentation triggered at 5000 ion counts, subsequent MS^2 fragmentation triggered at 500 ion counts, MS^3 fragmentation triggered at 50 ion counts and MS^4 fragmentation triggered at 10 ion counts. For both full scan and MS^n, other parameters were left

at instrumentation defaults.

Table Appendix B.1. Timeline for sampling of *Pinus banksiana* at a range of dwarf mistletoe infection levels (*Arceuthobium americanum*). Trees were inoculated with *Grosmannia clavigera* (n=24 trees) or left non-inoculated (n=21 trees). Trees rated for dwarf mistletoe infection using Hawksworth Dwarf Mistletoe Rating (DMR) system with a scale of 0 (non-infested) to 6 (more than 50% of branches infested throughout the crown) (Hawksworth and Wiens 1996).

		Week 0		Week 6	
Dwarf mistletoe rating	Height on tree (m)	Inoculated	Non-inoculated	Inoculated	Non-inoculated
0, 2, 3, 5, 6	2.4	--	--	Phloem and wood samples	Phloem and wood samples
	1.4	Inoculate with fungus, sample phloem	--	Lesion, defensive zone, wood samples	Phloem and wood samples
	0	--	--	Phloem and wood samples	Phloem and wood samples

Table Appendix B.2. Percent composition of dichloromethane-extractable compounds in control *Pinus banksiana* and *Grosmannia clavigera*-inoculated trees. *Only present in dwarf mistletoe infected trees or trees inoculated with *Grosmannia clavigera*.

	Control	Pre-inoculation	Defensive Zone	Lesion
α-Pinene	62.47	56.94	59.76	60.92
3-Carene	17.97	19.96	18.66	19.93
β-Pinene	7.00	7.79	8.50	11.50
Limonene	4.28	6.78	4.85	1.45
Myrcene	3.50	3.48	3.58	1.99
Terpinolene	1.84	1.96	1.77	1.79
β-Phellandrene	1.01	1.37	1.38	1.30
Bornyl acetate	0.83	0.52	0.36	0.04
Camphene	0.61	0.60	0.57	0.59
4-Allylanisole	0.13	0.24	0.21	0.13
γ-Terpinene	0.12	0.14	0.15	0.16
Borneol*	0.08	0.03	0.01	0.01
α-Terpineol	0.06	0.08	0.07	0.05
p-Cymene	0.04	0.05	0.05	0.06
α-Terpinene	0.04	0.06	0.07	0.07
cis-Ocimene	0.01	0.01	0.01	0.01
Camphor	0	0	0	0
Sabinene hydrate	0	0	0	0

Table Appendix B.2 (cont.)

	Control	Pre-inoculation	Defensive Zone	Lesion
Pulegone	0	0	0	0

Table Appendix B.3. Characterization of methanol-extractable compounds (phenolic compounds and levopimaric acid) from phloem and wood of *Pinus banksiana* without and with *Grosmannia clavigera* inoculations.

ID	Assigned identity	UPLC Retention time (min)	HPLC-MS [M − H]- ion *m/z*	fragment ion *m/z*	UPLC λ_{max} (nm)
1	RT 1.28	1.28	ND		277
Vah1	Vanillic acid hexoside 1 [1]	1.32	329	167, 152	254 285sh
Vah2	Vanillic acid hexoside 2 [1]	1.49	329	167	254, 287sh
4	RT 1.54	1.54	ND		264
Pah	Phenolic acid hexoside	1.66	373	211, 167	280, 305sh
6	RT 1.75	1.75	373		281, 308sh
Cah	Coumaric acid hexoside [2]	1.96	325	163, 119	295
8	RT 2.34	2.34	ND		285
9	RT 2.54	2.54	ND		290
Ecd	(Epi)catechin derivative [1]	2.67	617	289, 245, 179, 151	281
11	RT 2.76	2.76	327, 147		279
12	RT 2.84	2.84	574, 760		290, 323
Fah1	Ferulic acid hexoside [2]	2.92	355	193, 149	291, 314sh

Table Appendix B.3 (cont.)

ID	Assigned identity	UPLC Retention time (min)	HPLC-MS [M − H]- ion *m/z*	fragment ion *m/z*	UPLC λ_{max} (nm)
Fag	Ferulic acid glucoside [2]	3.16	355	193, 149	292, 315sh
Hh	Hydroxypropiovanillone hexoside [1]	3.82	357	177, 119, 162	275, 305sh
Fah2	Ferulic acid hexoside-like compound [2]	3.88	355, 193		283
17	RT 4.92	4.92	ND		265, 290sh
Un1	Unknown 1 [3]	5.13	315	300, 255, 269, 121	279
Ld	Lignan derivative [4]	5.21	315, 327, 345		281
20	RT 5.24	5.24	ND		288
Lx	Lignan xyloside [2]	5.42	495	363, 165, 315	281
Th	Taxifolin hexoside [2]	5.50	465	303, 285, 241, 213	289
23	RT 5.88	5.88	779		280
24	RT 6.04	6.04	327, 381		278
25	RT 6.14	6.14	329	269, 241	279

Table Appendix B.3 (cont.)

ID	Assigned identity	UPLC Retention time (min)	HPLC-MS [M − H]- ion *m/z*	fragment ion *m/z*	UPLC λ_{max} (nm)
Nh	Naringenin hexoside [5]	6.74	433	271, 151, 107	282
Ld1	Lignan derivative 1 [4]	7.52	315, 327, 345		281
Ld2	Lignan derivative 2 [4]	7.77	315, 327, 345		282
29	RT 8.00	8.00	535, 463		251
30	RT 8.29	8.29	ND		268
31	RT 8.35	8.35	ND		280
32	RT 9.42	9.42	ND		281
33	RT 10.19	10.19	ND		281
34	RT 10.69	10.69	ND		281
35	RT 10.70	10.70	ND		278
36	RT 10.79	10.79	ND		280
37	RT 10.89	10.89	ND		281
38	RT 10.94	10.94	ND		292
39	RT 11.42	11.42	ND		300
40	RT 11.65	11.65	ND		290

Table Appendix B.3 (cont.)

ID	Assigned identity	UPLC Retention time (min)	HPLC-MS [M − H]- ion *m/z*	fragment ion *m/z*	UPLC λ_{max} (nm)
41	RT 11.90	11.90	ND		300
42	RT 12.00	12.00	ND		276
43	RT 12.10	12.10	ND		270
44	RT 12.17	12.17	ND		269, 300
45	RT 12.36	12.36	ND		300
46	RT 12.38	12.38	ND		282, 325
La	Levopimaric acid * [6]	13.05	301	283, 253, 225, 257, 268	273

[no.] UV patterns, full scan and turbo DDS unique fragmentation patterns were used to assign tentative identities to the matched UPLC peaks, based on matches to external standards and the following relevant literature: [1] Karonen et al. 2004, [2] Pan and Lundgren 1996, [3] Villari et al. 2012, [4] Wallis et al. 2011, [5] Weintraub et al. 1995, [6] Kersten et al. 2006. ND = Compounds detected by UPLC but not by HPLC/MS. sh = shoulder.

Table Appendix B.4. Percent composition of methanol-extractable compounds (phenolic compounds and levopimaric acid) from phloem and wood of *Pinus banksiana* without and with *Grosmannia clavigera* inoculations. The amount of phenolics is relative to the total amount of methanol-extractable compounds without levopimaric acid for each tissue type. A blank space indicates non-detection of a compound.

ID	Control phloem	Defensive zone phloem	Lesion phloem	Control wood	Defensive zone wood
1	1.0	0.7			
Vah1	1.0	1.3			
Vah2	1.2	1.7			
4	0.6	0.3			
Pah	0.2	0.1			
6	1.4	1.3			
Cah	10.4	6.2			
8	0.2	0.6			
9	0.9	0.3			
Ecd	1.3	1.9	1.0		
11	3.3	3.1			
12			5.9		
Fah1	33.2	27.3	0.6		
Fag	0.5	0.6			
Hh	19.0	22.1	6.8		

Table Appendix B.4 (cont.)

ID	Control phloem	Defensive zone phloem	Lesion phloem	Control wood	Defensive zone wood
Fah2	0.4	3.5			
17	0.7	0.6			
Un1	0.3	0.3			
Ld	5.7	5.5			
20			8.0		
Lx	0.8	1.1			
Th	2.9	1.9	4.1		
23	0.3	0.4	2.4		
24	3.1	3.9			
25	0.4	1.1			
Nh	0.7	1.3			
Ld1	4.3	6.3	2.8		
Ld2			2.4		
29	0.9	1.0			
30	1.9	1.4			
31	0.4	1.5			
32	0.2	0.3			
33	0.5	0.7			
34	0.3	0.7			

Table Appendix B.4 (cont.)

ID	Control phloem	Defensive zone phloem	Lesion phloem	Control wood	Defensive zone wood
35			6.7		
36	0.1	0.3			
37			12.4		
38			10.8	14.9	9.6
39			9.6	8.5	12.9
40				41.0	39.0
41			8.3	35.5	38.6
42			11.4		
43			3.4		
44			1.8		
45	1.5	0.7			
46			1.5		
La	4.4	5.0	29.2	33.9	30.2

* The percent composition of levopimaric acid is relative to the total of all methanol-extractable compounds.

Table Appendix B.5. Equations for regression models identifying relationship between control and inoculated *Pinus banksiana* with *Grosmannia clavigera* at different sampling heights across the gradient of *Arceuthobium americanum* infection severity (DMR) for total monoterpene (natural log transformed) and non-structural carbohydrate concentrations. Bolded parameter estimates (standard errors) are significant at α=0.05. (Parameters displayed in Figs. 3.1 and 3.3)

Height		Inter-								P-
(m)	No.	cept		Tissue types		DMR	DMR^2	R^2	*F*	value
			Control	Defensive	Lesion					
nl(Total monoterpenes (ng mg^{-1}))										
0	45	**7.58**	-0.27	0	**--**	**0.31**	**-0.06**	0.189	3.18	0.034
		(0.16)	(0.17)			**(0.13)**	**(0.02)**			
1.4	69	**10.20**	**-3.51**	**-2.77**	**0**	-0.01	ns	--	--	--
		(0.13)	**(0.15)**	**(0.15)**		(0.03)				
2.4	45	7.20	-0.27	0	--	-0.03	ns	0.055	1.21	0.307
		(0.18)	(0.20)			(0.04)				
Percent sugar concentration (w/w)										
0	45	15.93	-0.16	0	--	0.01	ns	0.001	0.03	0.972
		(0.65)	(0.73)			(0.16)				
1.4	67	**4.83**	**11.58**	**15.88**	**0**	-0.02	ns	--	--	--
		(0.74)	**(0.91)**	**(0.87)**		(0.16)				
2.4	43	18.20	0.10	0	--	0.04	ns	0.002	0.04	0.962
		(0.63)	(0.72)			(0.16)				

Table Appendix B.5 (cont.)

Height (m)	No.	Inter-cept	Tissue types			DMR	DMR^2	R^2	*F*	P-value
			Control	Defensive	Lesion					
Percent starch concentration (w/w)										
0	45	3.07	-0.34	0	--	-0.08	ns	0.061	1.36	0.269
		(0.28)	(0.32)			(0.07)				
1.4	67	**0.50**	**1.69**	**1.70**	**0**	-0.06	ns	--	--	--
		(0.15)	**(0.19)**	**(0.18)**		(0.03)				
2.4	43	1.87	0.27	0	--	-0.04	ns	0.051	1.07	0.352
		(1.87)	(0.22)			(0.04)				
nl(Total phenolic concentration (AU)) – Phloem										
1.4	29	**13.63**	**0.87**	**0.60**	**0**	**0.04**	ns	--	--	--
		(0.09)	**(0.10)**	**(0.11)**		**(0.02)**				
nl(Total phenolic concentration (AU)) – Wood										
1.4	21	13.19	-0.27	0	--	**0.23**	ns	0.222	2.56	0.105
		(0.48)	(0.46)			**(0.10)**				
nl(Levopimaric acid concentration (AU)) – Phloem										
1.4	29	**13.10**	**-1.54**	**-1.58**	**0**	**-0.06**	ns	--	--	--
		(0.21)	**(0.22)**	**(0.24)**		**(0.04)**				
nl(Levopimaric acid concentration (AU)) – Wood										
1.4	21	13.46	0.16	0	--	0.02	ns	0.066	0.64	0.540
		(0.18)	(0.18)			(0.04)				

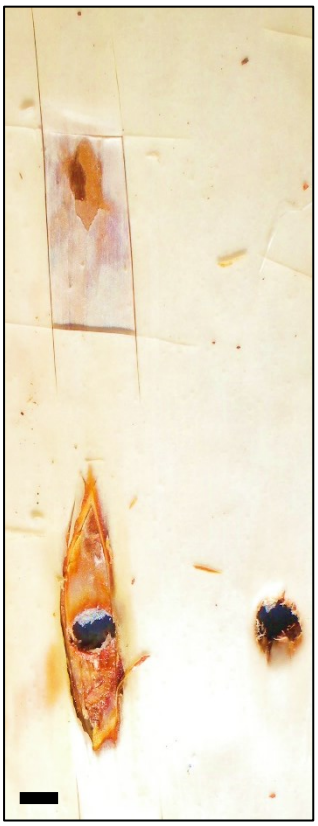

Figure Appendix B.1. Picture of lesion (lower left hole and discolored tissue that has been removed) from inoculation with *Grosmannia clavigera*, defensive zone (removed phloem in the upper left), and wound from sampling sapwood (lower right hole) in *Pinus banksiana*. Bar is 1 cm.

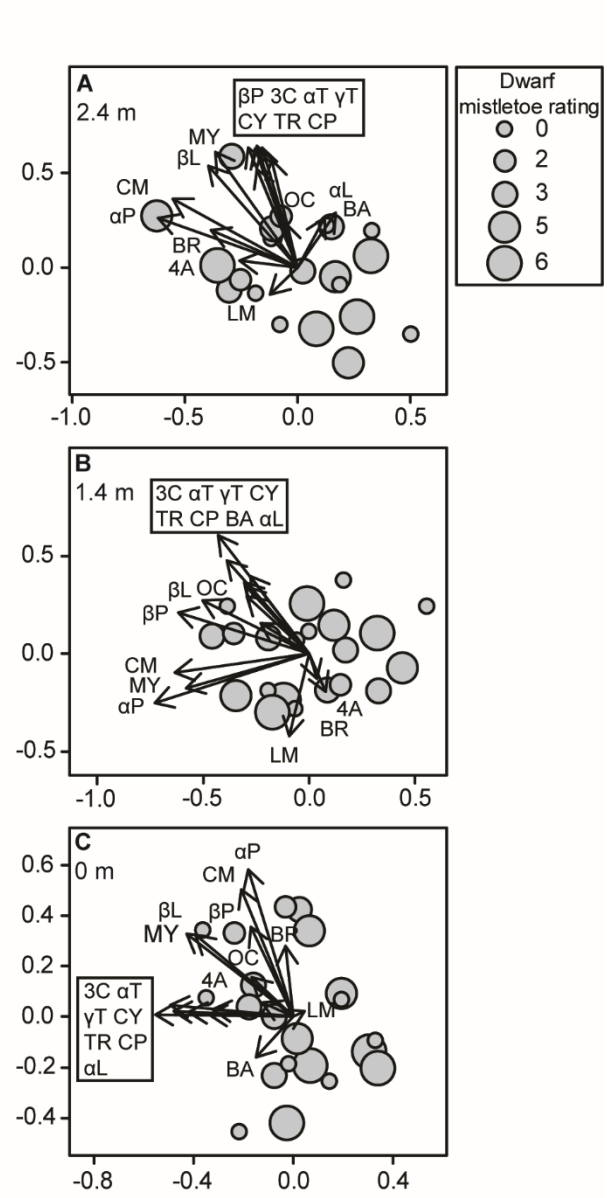

Figure Appendix B.2. Relationship between *Arceuthobium americanum* infection severity (dwarf mistletoe rating) and concentration of monoterpenes in *Pinus banksiana* phloem at three sample heights: (A) 2.4 m, (B) 1.4 m, and (C) 0 m. Analysis done by non-metric multidimensional scaling with Bray-Curtis distance ordination. Monoterpene compounds are represented by overlaid vectors and indicate the correlation with dwarf mistletoe rating. Longer monoterpene vectors show stronger relationships with the ordination configuration. For sample heights of 2.4,

1.4, and 0 m the minimum stress was 0.15, 0.13, and 0.10, respectively. Acronyms for monoterpenes: αP = α-pinene, CM = camphene, βP = β-pinene, 3C = 3-carene, MY = myrcene, αT = α-terpinene, LM = limonene, βL = β-phellandrene, OC = ocimene, γT = γ-terpinene, CY = p-cymene, TR = terpinolene, CP = camphor, BA = bornyl acetate, 4A = 4-allylanisole, αL = α-terpineol, BR = borneol.

Appendix C

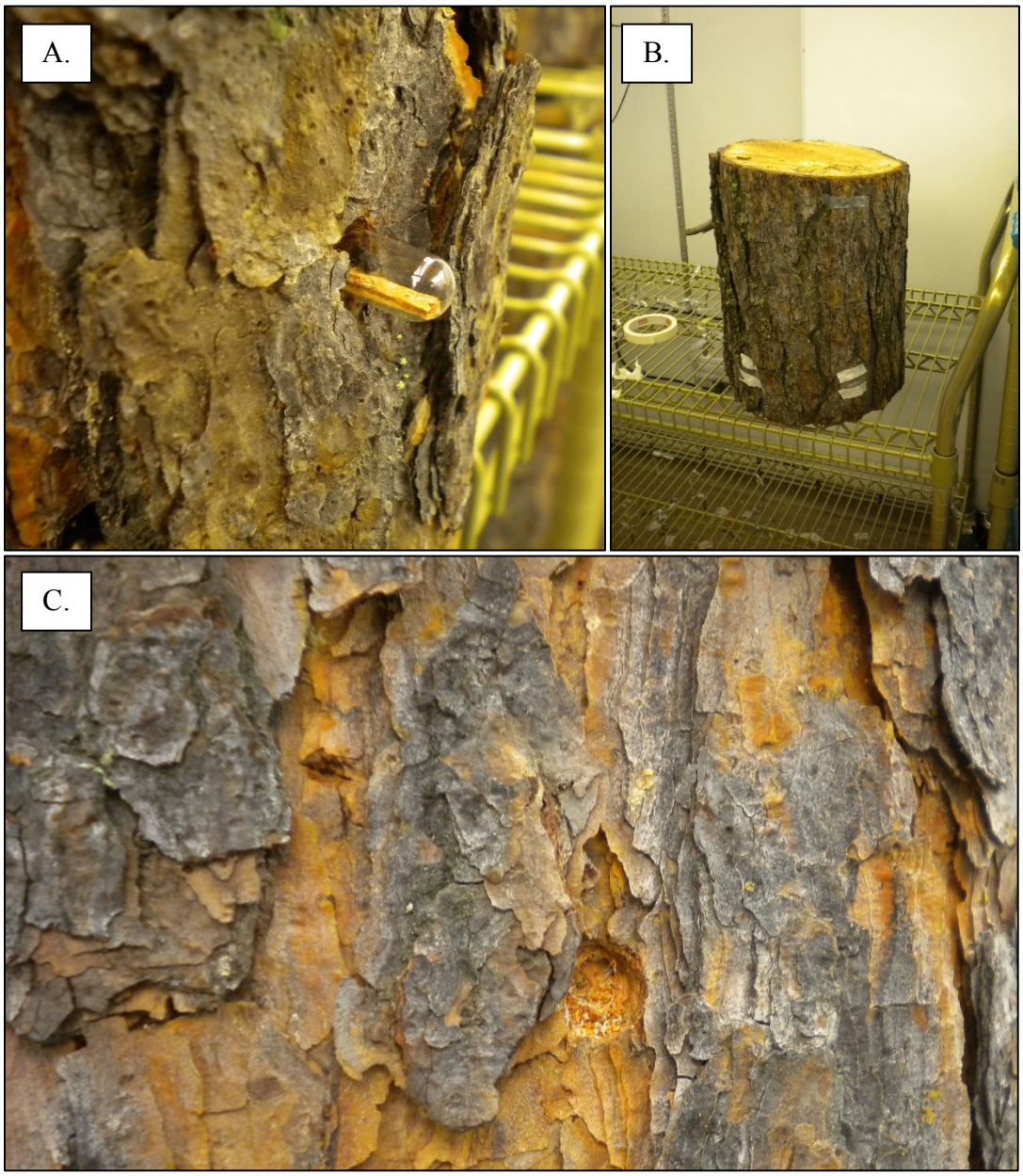

Figure Appendix C.1. Pictures of the process of introducing *Dendroctonus ponderosae* pairs into *Pinus banksiana* logs. A. Adult *D. ponderosa* within capsule placed in a hole drilled through the bark. B. Covered introduction holes. C. Successful introduction of *D. ponderosa* below an egg niche made by a woodboring beetle (Coleoptera: Cerambycidae).

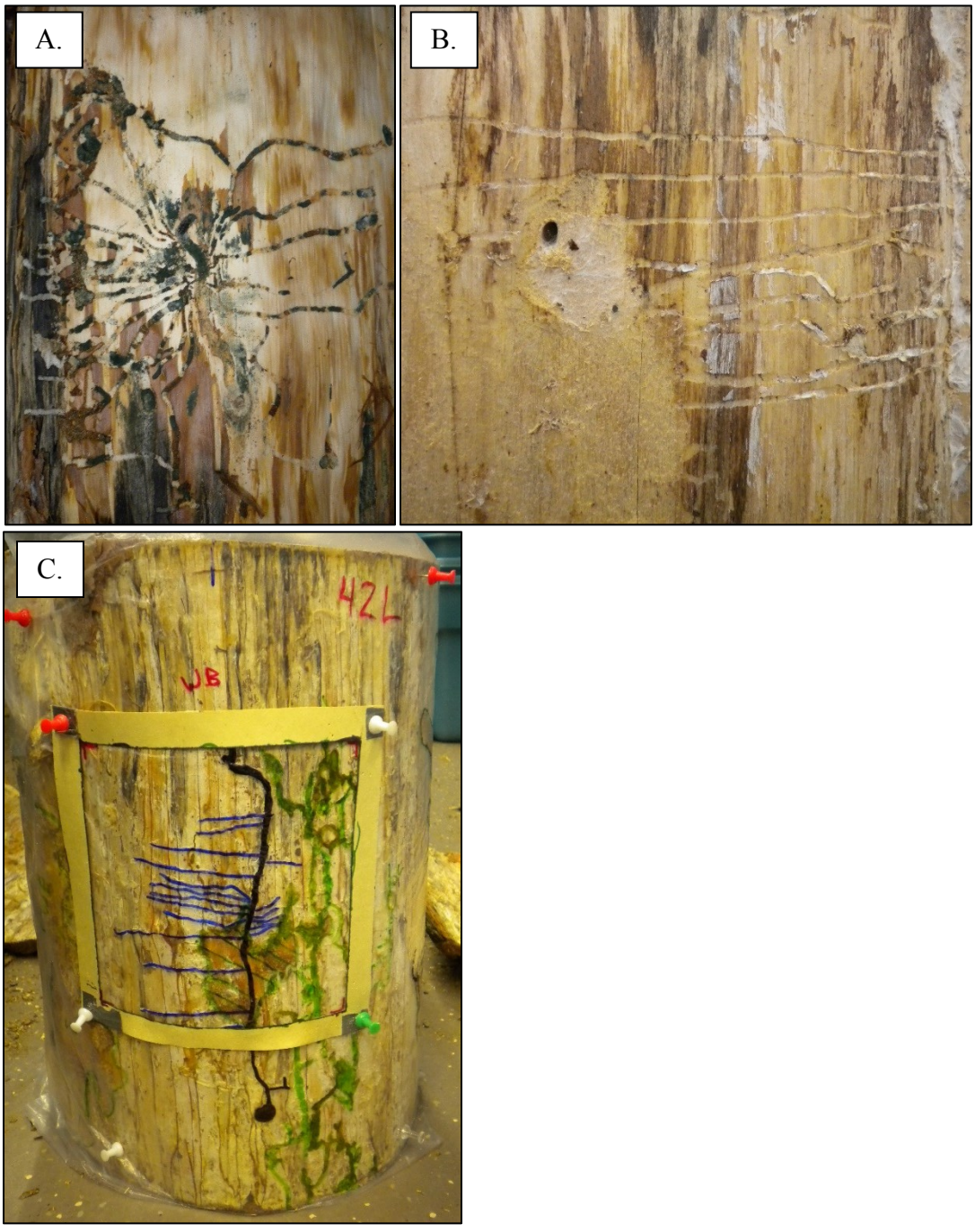

Figure Appendix C.2. Pictures of (A, B, C) *Dendroctonus ponderosae* galleries and (B, C) woodboring beetle feeding under the bark of *Pinus banksiana* logs.

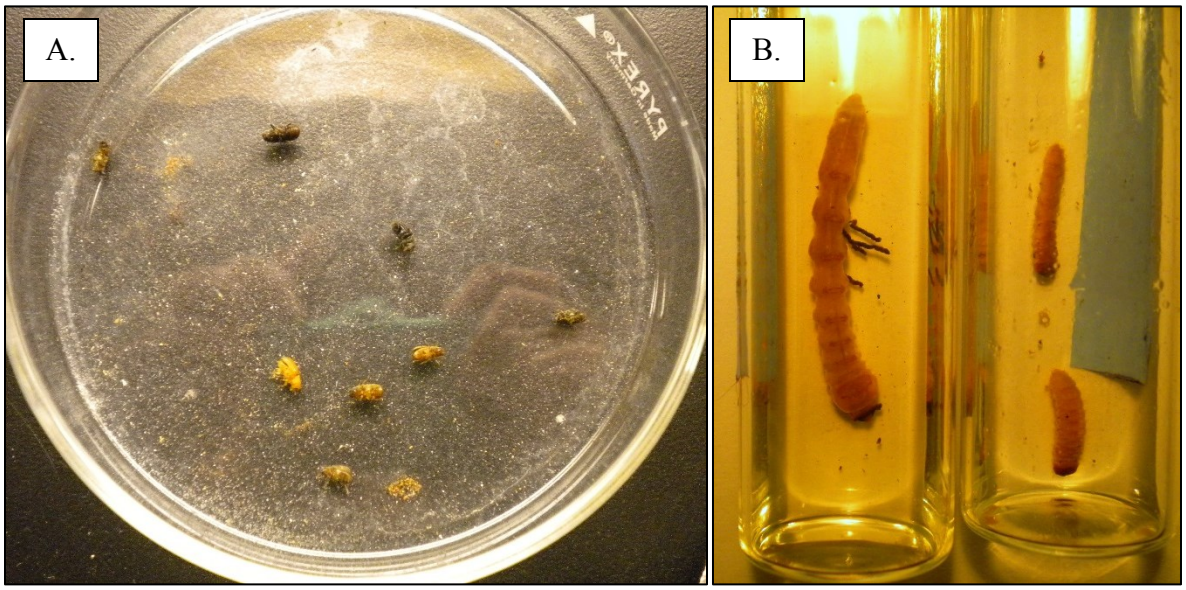

Figure Appendix C.3. Adult and teneral adults of (A) *Dendroctonus ponderosae* and larvae of (B) woodboring beetles (Coleoptera: Cerambycidae) in *Pinus banksiana* logs.

Figure Appendix C.4. Layout for *Pinus banksiana* logs in a forest with an active *Dendroctonus ponderosae* population. Each station baited with commercially available *D. ponderosae* lure.

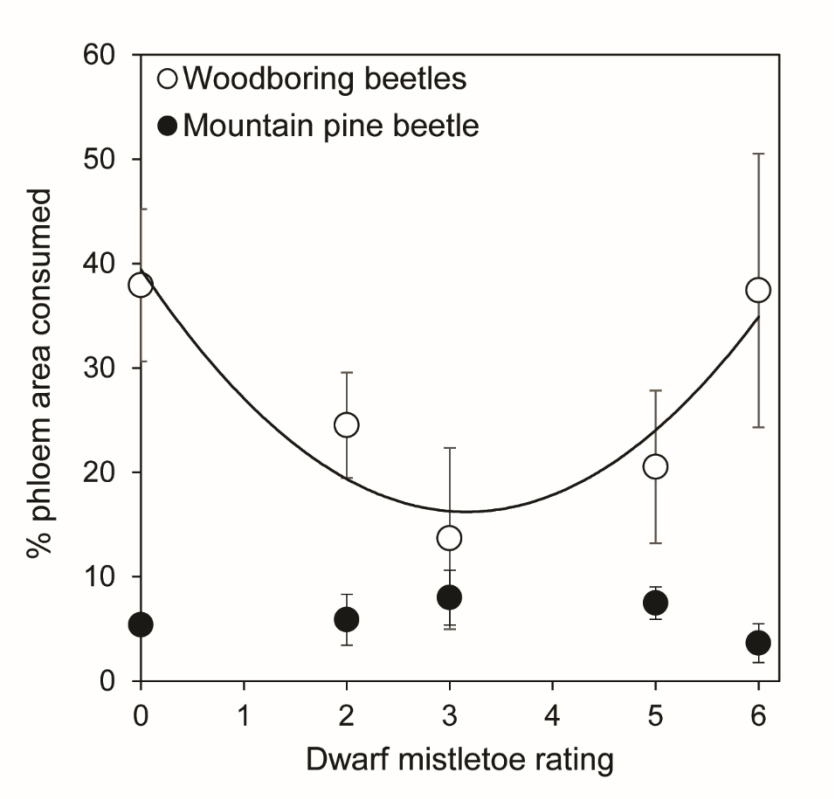

Figure Appendix C.5. Relationship between phloem area consumed by woodboring beetles and the mountain pine beetle (*Dendroctonus ponderosae*) and dwarf mistletoe (*Arceuthobium americanum*) rating in jack pine (*Pinus banksiana*). Dwarf mistletoe infection intensity quantified by dwarf mistletoe rating (DMR). Mean presented with standard error bars. Average of phloem area consumed by *D. ponderosae* (27.4 cm^2) was used for woodboring beetle feeding regression equation: woodboring beetles = 237.87 - (45.64*DMR) + (6.59*DMR^2) - (2.75* *D. ponderosae*), $R^2 = 0.45$, $F_{3,26} = 7.08$, $P = 0.001$, $P < 0.05$ for $\beta_{intercept}$ and $\beta_{D.\ ponderosae}$, $P < 0.1$ for β_{DMR} and $\beta_{DMR\text{^}2}$.

Table Appendix C.1. Methods timeline for introducing *Dendroctonus ponderosae* (MPB) and woodboring beetles into jack pine (*Pinus banksiana*) at different intensities of dwarf mistletoe (*Arceuthobium americanum*) infection, Smoky Lake, Alberta.

Week	MPB only	Woodboring beetle and MPB
1	Cut trees with DMR 0, 2, 3, 5, 6 and removed one log per tree. Logs transported to growth chamber.	Cut trees with DMR 0, 2, 3, 5, 6 and removed one log per tree. Logs transported to stand with active MPB population and baited (See Figure Appendix C.4).
2	Introduced pairs of MPB into logs (See Figure Appendix C.1A,B).	Moved logs to growth chamber and two days later introduce pairs of MPB into logs (See Figure Appendix C.1).
3		
4		
5		
6	Moved logs to cold room.	Moved logs to cold room.
7		
8		
9		
10	Moved logs to growth chamber and monitored for emergence.	Moved logs to growth chamber and monitored for emergence.

Table Appendix C.1 (cont.)

Week	MPB only	Woodboring beetle and MPB
11		
12		
13		
14		
15		
16		
17		
18	Peeled back bark and measured MPB gallery characteristics (See Figure Appendix C.2A, C.3A).	Peeled back bark and measured MPB gallery characteristics and woodboring beetle feeding (See Figure Appendix C.2, C.3).

Table Appendix C.2. Chemical profiles presented as percent of total monoterpene concentration from phloem of jack pine (*Pinus banksiana*) at different intensities of dwarf mistletoe (*Arceuthobium americanum*) infection, Smoky Lake, Alberta. Means and standard errors are presented.

	Dwarf mistletoe ratings				
	0	2	3	5	6
α-Pinene	52.8 (4.7)	52.8 (2.1)	72.8 (9.1)	53.9 (8.4)	66.7 (12.6)
3-Carene	22.1 (4.4)	28.1 (4.1)	0.4 (0.2)	14.7 (8.4)	19.3 (11.1)
Limonene	9.0 (3.6)	2.2 (1.7)	12.3 (8.7)	13.3 (8.2)	2.7 (1.3)
β-Pinene	6.3 (1.2)	8.6 (3.8)	8.7 (2.4)	6.3 (1.1)	5.1 (3.1)
Myrcene	3.9 (0.8)	3.5 (0.7)	2.1 (0.4)	4.6 (1.2)	3.7 (2.1)
Terpinolene	2.8 (1.1)	2.0 (0.3)	0.1 (0.1)	2.0 (1.1)	1.2 (0.9)
β-Phellandrene	0.9 (0.2)	0.9 (0.2)	1.1 (0.3)	3.8 (3.3)	0.8 (0.1)
Bornyl acetate	0.7 (0.6)	0.8 (0.4)	1.2 (1.2)	0.2 (0.1)	0.0 (0.0)
Camphene	0.6 (0.1)	0.6 (0.2)	1.0 (0.1)	0.5 (0.1)	0.4 (0.1)
4-Allylanisole	0.4 (0.1)	0.2 (0.2)	0.3 (0.2)	0.2 (0.1)	0.0 (0.0)
γ-Terpinene	0.2 (0.1)	0.1 (0.1)	0.0 (0.0)	0.1 (0.1)	0.1 (0.1)
α-Terpinene	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)
α-Terpineol	0.1 (0.1)	0.0 (0.0)	0.0 (0.0)	0.2 (0.1)	0.0 (0.0)
Borneol	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)
cis-Ocimene	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
p-Cymene	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)	0.1 (0.1)	0.0 (0.0)

Appendix D

Impact of dwarf mistletoe on chemical and anatomical defenses, growth, and physical characteristics in jack pine

The purpose of the following section is to report data for a project that investigated the impact of dwarf mistletoe (*Arceuthobium americanum* Nutt. ex Engelm.) on jack pine (*Pinus banksiana* Lamb.) chemical and anatomical defenses, radial growth, and phloem physical characteristics at multiple sites in Alberta. The objectives were to: 1) identify the pattern of change for a number of different measures of chemical and anatomical defenses, radial increment growth, and phloem characteristics over a gradient of dwarf mistletoe infection severities, and 2) determine the effect of site on these patterns.

Methods and Materials

Site Description and Sampling

We selected three sites with jack pine-dominated forests near the towns of Bruderheim, Smoky Lake, and Lac La Biche, Alberta, Canada (Table Appendix D.1). In July 2012, dwarf mistletoe infection severity on jack pine with diameter at breast height > 18 cm (DBH) at each site was rated using the Hawksworth Dwarf Mistletoe Rating (DMR) system with a scale of 0 (non-infested) to 6 (more than 50% of branches infested throughout the crown) (Hawksworth and Wiens 1996). Trees were chosen based on their DMR (0, 2, 3, 5, or 6) and the following

measures were taken on trees at Bruderheim (n=39), Smoky Lake (n=53), Lac La Biche (n=50): DBH, height, and height to bottom of crown. On the north and south facing side of each tree at 1.3 m, a 5 x 5 cm section of phloem was taken for monoterpene analysis (methods described below). Phloem thickness was measured from another 5 x 5 cm section taken directly above the 1.3 m sampling height on the north and south facing sides. All phloem samples were wrapped in aluminum foil, sealed in air-tight bags, and transported in dry ice. North and south facing samples reserved for monoterpene analysis were stored at -40°C and then ground to a fine powder in liquid nitrogen. The wet weight of the remaining phloem samples (previously used in the field for phloem thickness) was measured and then samples were dried at 70°C for 4 days and dry weight was measured. Percent phloem moisture was calculated as: (wet-dry) / dry * 100.

Radial increment growth and resin duct production measures were calculated from increment core samples taken on the north side of trees next to the 1.3 m phloem sampling height. We used increment core borers (5.15 mm in width) to take cores that went about 5 - 10 cm into the wood. We prepared cores using standard techniques, such as drying, mounting and sanding with incrementally fine sand paper. Cores were scanned at 1200 d.p.i. and ring boundaries were assigned and widths measured using WinDendro (Regent Instruments 2009). Ring width series were then visually cross-dated to assign a calendar year to each ring (Grissino-Mayer 2001). Measures of resin ducts were analyzed in ImageJ (ver. 1.50i, Schneider et al. 2012). A sampling area (width of 3 mm) was drawn on each core photo and the number and area of resin ducts per ring were measured. The following parameters were calculated from increment and resin duct data averaged for the last 5 and 10 years: ring width (mm), number of resin ducts yr^{-1}, resin duct

density (number cm^{-2}), resin duct area (mm^2), and percent resin duct area (percent ring increment area composed of resin ducts).

Analysis of Monoterpenes

Concentrations of monoterpene and other dichloromethane-extractable compounds were measured using established methods (Klutsch et al. 2016). Briefly, ground samples from north and south facing sides of each trees were combined and 100 mg was extracted twice with 0.5 ml of dichloromethane and 0.004% tridecane (internal standard). Each extraction was vortexed for 30 s, sonicated for 10 min, and centrifuged at 16,100 rcf at 2°C for 15 min, before the two extracts were pooled. Sample extract (1 µl) was injected in splitless mode into a Gas Chromatograph/Mass Spectrometer (GC/MS, Agilent 7890A/5062C, Agilent Tech., Santa Clara, CA, USA) equipped with a HPInnowax column (I.D. 0.25 mm, length 30 m) (Agilent Tech.) with helium carrier gas flow at 1 ml min^{-1}, and a temperature of 55°C for 0.5 min, increased to 60°C by 2°C min^{-1}, held for 1 min, then increased to 120°C by 10°C min^{-1}, held for 1 min, and finally increased to 250°C by 30°C min^{-1}. To quantify individual and total compounds (mainly monoterpenes) (ng mg^{-1} of fresh tissue, hereafter concentration), the following standards were used: borneol, pulegone, α-terpinene, γ-terpinene, α-terpineol, camphor (Sigma-Aldrich, St. Louis, MO, USA), 3-carene, terpinolene, α-pinene, β-pinene, limonene, myrcene, camphene, *p*-cymene, 4-allylanisole (Fluka, Sigma-Aldrich, Buchs, Switzerland), bornyl acetate, *cis*-ocimene (SAFC Supply Solutions, St. Louis, MO, USA), and β-phellandrene (Glidco Inc., Jacksonville, FL, USA).

Data analysis

To identify the impact of dwarf mistletoe infection severity and site on phloem physical characteristics and measures of chemical defenses, resin duct and increment growth, an ANOVA was constructed (PROC GLM in SAS, ver. 9.3). An interaction term between dwarf mistletoe infection and site was tested to determine whether patterns of change with dwarf mistletoe severity differed between sites, but was not significant for any model and was removed. Differences between sites were tested using a Tukey's adjusted P-value at an α=0.05. Model assumptions of normality and homogeneity of variance were visually assessed using Q-Q normality and residual plots. To meet these assumptions, resin duct and increment growth measures were square root transformed, and dichloromethane-extractable compounds (mostly monoterpenes) were natural log transformed. Quadratic relationships between dwarf mistletoe rating and monoterpene concentrations (total and individual) were assessed by comparing the Akaike's information criterion between linear models with and without a squared dwarf mistletoe rating variable and selecting the model with the lowest criterion.

Results

Physical characteristics

Phloem thickness and moisture both decreased with increasing dwarf mistletoe severity (Fig. Appendix D.1, Table Appendix D.2). There was also a significant effect of site on both phloem measures, but there was no interaction between site and dwarf mistletoe rating. Phloem thickness was 15% less in Bruderheim than in Lac La Biche and Smoky Lake.

Monoterpene concentrations

Across all sites, total monoterpenes along with α-pinene, camphene, myrcene, β-phellandrene, and terpinolene increased with increasing infection severity in trees with low dwarf mistletoe ratings, but concentrations decreased in trees with higher severity infections (Fig. Appendix D.3). The percent concentration of β-pinene also varied non-linearly with dwarf mistletoe rating, but the lowest composition of β-pinene was in trees with moderate infection severity (R^2=0.13, $F_{(4,141)}$=4.90, P=0.001). Furthermore, the percent composition of camphene decreased with increasing severity of dwarf mistletoe infection across all sites (R^2=0.08, $F_{(4,141)}$=3.90, P=0.010). There was an effect of site, though there was no interaction between site and dwarf mistletoe rating for any monoterpene concentrations or percent compositions (Tables Appendix D.2, C.3, C.4). Trees in Bruderheim had 54% greater total monoterpene concentration than Smoky Lake. Most individual monoterpenes also followed this pattern.

Resin duct measures

Mean annual resin duct production and area decreased with increasing dwarf mistletoe infection severity across all sites (Figs. Appendix D.4 and C.5, Table Appendix D.2). This pattern of decreased resin duct production and area with dwarf mistletoe severity was found in tree rings from the last five years and last 10 years. There was an effect of site, though there was no interaction between site and dwarf mistletoe severity. Trees in Bruderheim had twice the annual production of resin ducts than trees in the other two sites for growth in the last five and 10 years (Fig. Appendix D.4). Furthermore, trees in Bruderheim had 77% and 65% greater resin duct area than trees in the other two sites for growth in the last five and 10 years, respectively (Fig. Appendix D.5). Resin duct density and percent resin duct area, which are measures of relative investment in defense compared to growth, did not vary with dwarf mistletoe rating (Fig.

Appendix D.6). However, trees in Bruderheim had 80% greater percent resin duct area than the other two sites, showing that a greater percentage of the area in each annual increment was made up of resin ducts in trees in Bruderheim (Fig. Appendix D.7).

Increment growth

In all sites, mean annual increment growth of trees decreased with increasing dwarf mistletoe severity (Fig. Appendix D.8, Table Appendix D.2). As with other tree measures, there was a significant effect of site but no interaction between site and dwarf mistletoe severity. Trees in Bruderheim had 60% and 81% greater increment growth than trees in the other sites for the last five and 10 years, respectively.

Table Appendix D.1. Characteristics of sites and jack pine (*Pinus banksiana*). Means (SE) are reported for tree measures. Diameter at breast height (1.3 m) is abbreviated as DBH.

Site	Latitude	Longitude	N trees	DBH (cm)	Height (m)	Height to bottom of live crown (m)
Bruderheim	53°51.8' N	112°55.7' W	39	28.2 (1.3)	14.9 (0.5)	3.3 (0.4)
Lac La Biche	54°59.9' N	112°00.0' W	50	24.0 (0.4)	16.2 (0.2)	2.9 (0.3)
Smoky Lake	54°05.8' N	112°15.5' W	53	22.4 (0.3)	16.3 (0.3)	5.9 (0.4)

Table Appendix D.2. Relationship among measures of physical characteristics, anatomical defenses, and increment growth with site and *Arceuthobium americanum* infection severity (DMR) in *Pinus banksiana*. for total monoterpene (natural log transformed) and non-structural carbohydrate concentrations. Bolded parameter estimates (standard errors) are significant at α=0.05. *Variable square root transformed. (Parameters used to build equations for Figs. Appendix-1, 2, 4-8)

		Site						
	Intercept	Bruderheim	Lac La Biche	Smoky Lake	DMR	R^2	*F*	P-value
Phloem thickness (mm)	**1.60**	**-0.18**	**0.10**	**0**	**-0.4**	0.17	8.47	<0.0001
	(0.07)	**(0.07)**	**(0.07)**		**(0.01)**			
Percent moisture (dry weight)	**207.46**	**-2.27**	**-14.49**	**0**	**-3.68**	0.16	8.73	<0.0001
	(4.50)	**(5.23)**	**(4.91)**		**(0.95)**			
Last 5 years								
Annual resin duct production*	**1.04**	**0.37**	**0.02**	**0**	**-0.08**	0.24	14.16	<0.0001
	(0.08)	**(0.10)**	**(0.09)**		**(0.02)**			
Resin duct area (mm^2)*	**0.13**	**0.04**	**0.01**	**0**	**-0.01**	0.25	14.61	<0.0001
	(0.01)	**(0.01)**	**(0.01)**		**(0.01)**			

Table Appendix D.2 (cont.)

		Site						
	Intercept	Bruderheim	Lac La Biche	Smoky Lake	DMR	R^2	*F*	P-value
Resin duct density (no. cm^{-2})*	1.89	0.33	-0.14	0	-0.04	0.05	2.16	0.0952
	(0.18)	(0.21)	(0.20)		(0.04)			
Percent resin duct area*	**0.93**	**0.26**	**-0.04**	**0**	-0.03	0.09	4.54	0.0046
	(0.08)	**(0.10)**	**(0.09)**		(0.02)			
Annual increment growth	**1.03**	**0.24**	**0.04**	**0**	**-0.07**	0.28	17.63	<0.0001
(mm)*	**(0.05)**	**(0.06)**	**(0.06)**		**(0.01)**			
Last 10 years								
Annual resin duct production*	**1.08**	**0.37**	**0.02**	**0**	**-0.06**	0.28	17.29	<0.0001
	(0.07)	**(0.08)**	**(0.07)**		**(0.01)**			
Resin duct area (mm^2)*	**0.13**	**0.03**	**0.01**	**0**	**-0.01**	0.25	14.7	<0.0001
	(0.01)	**(0.01)**	**(0.01)**		**(0.00)**			

Table Appendix D.2 (cont.)

		Site						
	Intercept	Bruderheim	Lac La Biche	Smoky Lake	DMR	R^2	*F*	P-value
Resin duct density (no. cm^{-2})*	1.97	0.08	-0.21	0	-0.00	0.03	1.3	0.2774
	(0.13)	(0.16)	(0.14)		(0.03)			
Percent resin duct area*	**0.98**	**0.13**	**-0.08**	**0**	-0.00	0.06	3.1	0.0288
	(0.06)	**(0.07)**	**(0.07)**		(0.01)			
Annual increment growth	**1.03**	**0.35**	**0.07**	**0**	**-0.05**	0.33	21.88	<0.0001
(mm)*	**(0.05)**	**(0.06)**	**(0.06)**		**(0.01)**			

Table Appendix D.3. Monoterpene concentrations (ng mg^{-1}) and standard errors (ln back-transformed) from jack pine (*Pinus banksiana*) infected with dwarf mistletoe (*Arceuthobium americanum*) from Bruderheim, AB.

	Dwarf mistletoe rating									
	0		2		3		5		6	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
α-pinene	777.5	(202.0,273.0)	1052.8	(195.4,239.9)	365.7	(106.1,149.4)	642.2	(151.4,198.1)	331.7	(99.2,141.5)
β-pinene	126.1	(32.3,43.4)	117.1	(29.0,38.6)	49.8	(14.9,21.3)	83.3	(22.0,29.9)	61.0	(19.8,29.4)
myrcene	70.4	(21.6,31.2)	74.8	(11.6,13.7)	32.9	(9.9,14.1)	53.1	(14.1,19.3)	29.3	(5.6,6.9)
limonene	62.3	(28.7,53.4)	55.2	(23.2,40)	46.1	(25.9,59.1)	9.2	(7.7,48.2)	5.5	(4.5,24.6)
β-phellandrene	11.8	(3.6,5.2)	8.6	(1.6,1.9)	5.0	(1.5,2.1)	5.4	(1.1,1.4)	5.5	(1.0,1.3)
camphene	6.2	(1.4,1.9)	8.0	(1.9,2.6)	2.7	(0.8,1.0)	4.7	(1.1,1.5)	2.6	(0.8,1.1)
3-carene	3.5	(2.8,14.5)	4.4	(3.3,13.3)	194.2	(69.7,108.6)	4.6	(3.8,22)	50.0	(38.1,160)
p-cymene	1.1	(0.8,3.0)	0.6	(0.5,1.8)	18.8	(6.8,10.7)	0.6	(0.5,2.0)	5.0	(3.3,9.5)
bornyl acetate	0.5	(0.3,1.0)	1.9	(1.3,3.6)	0	(0,0.1)	0.1	(0,0.1)	0.1	(0.1,0.2)
γ-terpinene	0.1	(0.1,0.1)	0	(0,0)	0.1	(0,0.1)	0	(0,0)	0.1	(0,0.1)
4-allylanisole	0.1	(0,0.1)	0.1	(0.1,0.2)	0	(0,0.1)	0.1	(0.1,0.2)	0	(0,0)
ocimene	0.1	(0,0.1)	0	(0,0)	0.1	(0.1,0.2)	0	(0,0)	0	(0,0)
α-terpineol	0	(0,0)	0	(0,0)	0.1	(0,0.1)	0	(0,0)	0	(0,0)

Table Appendix D.3 (cont.)

	Dwarf mistletoe rating									
	0		2		3		5		6	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
pulegone	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)
borneol	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)
terpinolene	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)
α-terpinene	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)

Table Appendix D.4. Monoterpene concentrations (ng mg^{-1}) and standard errors (ln back-transformed) from jack pine (*Pinus banksiana*) infected with dwarf mistletoe (*Arceuthobium americanum*) from Lac La Biche, AB.

	Dwarf mistletoe rating									
	0		2		3		5		6	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
α-pinene	359.1	(69.5,86.2)	710	(188.4,256.4)	540.9	(118.8,152.2)	562.9	(122.1,156.0)	315.6	(70.6,91.0)
β-pinene	35.6	(9.0,12.0)	82.2	(22.3,30.6)	37.2	(7.8,9.9)	42.8	(12.3,17.2)	44.7	(13.6,19.5)
myrcene	21.5	(6.7,9.6)	56.5	(14.3,19.2)	38.6	(10.2,13.8)	38.5	(8.8,11.4)	9.8	(5.5,12.3)
limonene	15.8	(11.5,42.4)	22.5	(14.0,36.8)	31.2	(20.5,59.7)	83.7	(31.4,50.3)	7	(4.8,14.9)
camphene	1.8	(0.8,1.5)	3.3	(1.7,3.4)	2.5	(1.2,2.3)	4	(0.9,1.1)	0.4	(0.2,0.6)
β-phellandrene	0.7	(0.4,1.1)	3	(1.9,5.0)	1.5	(0.9,2.2)	4.9	(2.6,5.8)	1.4	(1.0,2.9)
bornyl acetate	0.2	(0.1,0.3)	0.3	(0.2,0.6)	0.2	(0.2,0.4)	0.2	(0.1,0.4)	0.1	(0.1,0.1)
3-carene	0.2	(0.1,0.6)	29.6	(22.1,86.6)	4	(3.3,17.0)	10.2	(8.0,36.4)	0.4	(0.3,1.5)
terpinolene	0.1	(0.1,0.2)	3.6	(2.7,10.0)	0.5	(0.4,1.4)	2	(1.4,4.6)	0.1	(0.1,0.2)
4-allylanisole	0.1	(0,0.1)	0.1	(0,0.1)	0	(0,0.1)	0	(0,0)	0	(0,0)
ocimene	0	(0,0.1)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)
γ-terpinene	0	(0,0)	0.1	(0,0.1)	0.1	(0,0.1)	0	(0,0)	0	(0,0)
p-cymene	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)

Table Appendix D.4 (cont.)

	Dwarf mistletoe rating									
	0		2		3		5		6	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
pulegone	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)
borneol	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)
α-terpineol	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)
α-terpinene	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)

Table Appendix D.5. Monoterpene concentrations (ng mg^{-1}) and standard errors (ln back-transformed) from jack pine (*Pinus banksiana*) infected with dwarf mistletoe (*Arceuthobium americanum*) from Smoky Lake, AB.

	Dwarf mistletoe rating									
	0		2		3		5		6	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
α pinene	483.3	(79.6,95.2)	471.6	(99.1,125.4)	591.5	(150.1,201.1)	420.3	(21.7,22.8)	302.3	(47.1,55.7)
β-pinene	36.8	(6.5,7.8)	31.8	(7.1,9.1)	56.8	(16.1,22.5)	47.1	(4.8,5.4)	25.0	(5.7,7.5)
myrcene	27.8	(6.1,7.9)	31.1	(9.5,13.6)	41.8	(11.9,16.7)	29.0	(4.5,5.4)	13.3	(2.7,3.4)
limonene	26.6	(15.6,37.5)	30.9	(14.3,26.7)	46.7	(19.9,34.8)	27.4	(11.2,19.0)	19.9	(12.6,34.3)
camphene	3.7	(0.6,0.7)	3.9	(0.8,1.1)	4.8	(1.2,1.6)	2.9	(0.1,0.2)	2.1	(0.4,0.4)
3-carene	2.9	(2.2,9.6)	2.6	(2,9.2.0)	31.9	(22.6,77.9)	6.1	(4.6,19.4)	2.7	(2.2,10.1)
β-phellandrene	1.4	(0.7,1.4)	3.4	(0.7,0.9)	8.5	(2.2,3.1)	3.5	(0.5,0.6)	0.9	(0.5,1.1)
terpinolene	0.7	(0.4,1.3)	1.0	(0.6,1.7)	6.7	(4.3,11.9)	0.9	(0.7,2.3)	0.8	(0.5,1.8)
bornyl acetate	0	(0,0.1)	0.1	(0.1,0.1)	0.1	(0.1,0.2)	0	(0,0)	0	(0,0)
4-allylanisole	0	(0,0)	0	(0,0)	0.1	(0.1,0.1)	0	(0,0.1)	0	(0,0)
γ-terpinene	0	(0,0)	0	(0,0)	0.1	(0.1,0.2)	0	(0,0)	0	(0,0)
α-terpinene	0	(0,0)	0	(0,0)	0.1	(0,0.1)	0	(0,0)	0	(0,0)
p-cymene	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)

Table Appendix D.5 (cont.)

	Dwarf mistletoe rating									
	0		2		3		5		6	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
pulegone	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)
borneol	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)
ocimene	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)
α-terpineol	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)	0	(0,0)

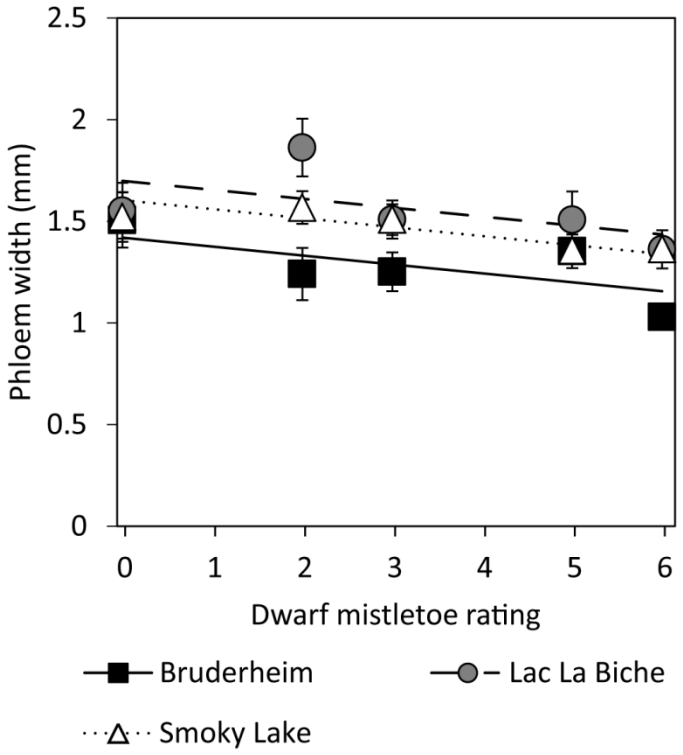

Figure Appendix D.1. Mean phloem thickness in *Pinus banksiana* with varying levels of *Arceuthobium americanum* (dwarf mistletoe) at three sites in Alberta, Canada.

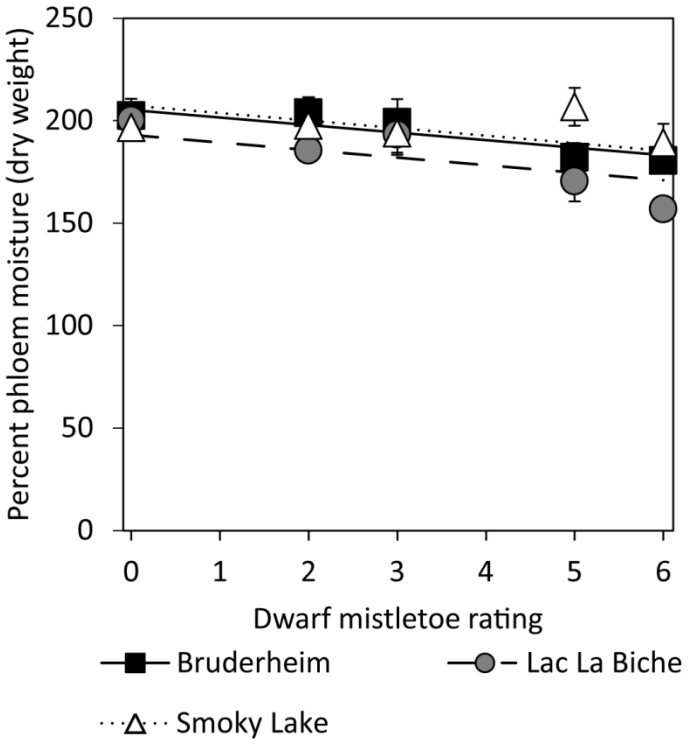

Figure Appendix D.2. Mean percent phloem moisture in *Pinus banksiana* with varying levels of *Arceuthobium americanum* (dwarf mistletoe) at three sites in Alberta, Canada.

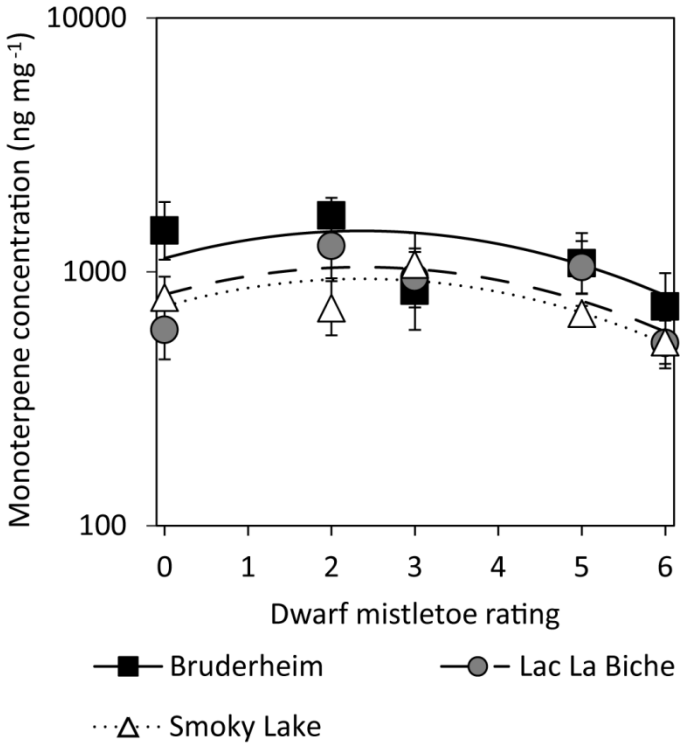

Figure Appendix D.3. Mean concentration of monoterpenes (ln back-transformed, ng mg^{-1}) in *Pinus banksiana* with varying levels of *Arceuthobium americanum* (dwarf mistletoe) at three sites in Alberta, Canada.

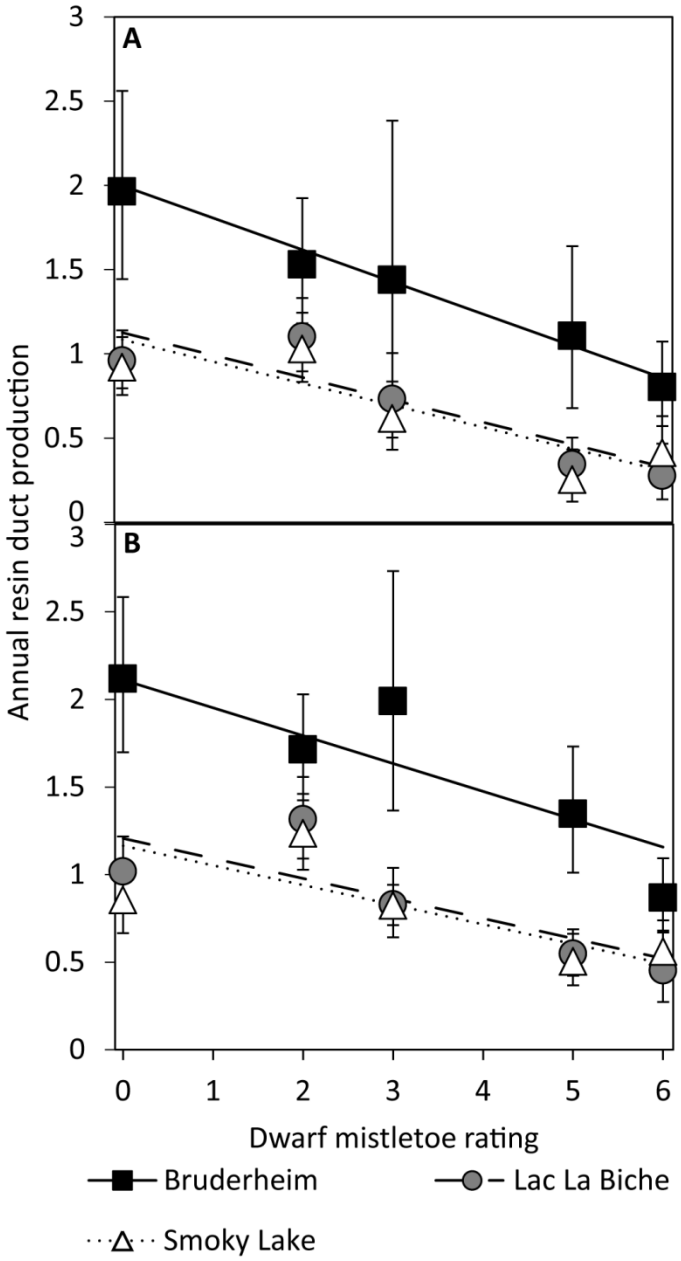

Figure Appendix D.4. Mean annual resin duct production (square root back-transformed, no. yr^{-1}) in *Pinus banksiana* with varying levels of *Arceuthobium americanum* (dwarf mistletoe) at three sites in Alberta, Canada. A. Mean of last 5 yrs. B. Mean of last 10 yrs.

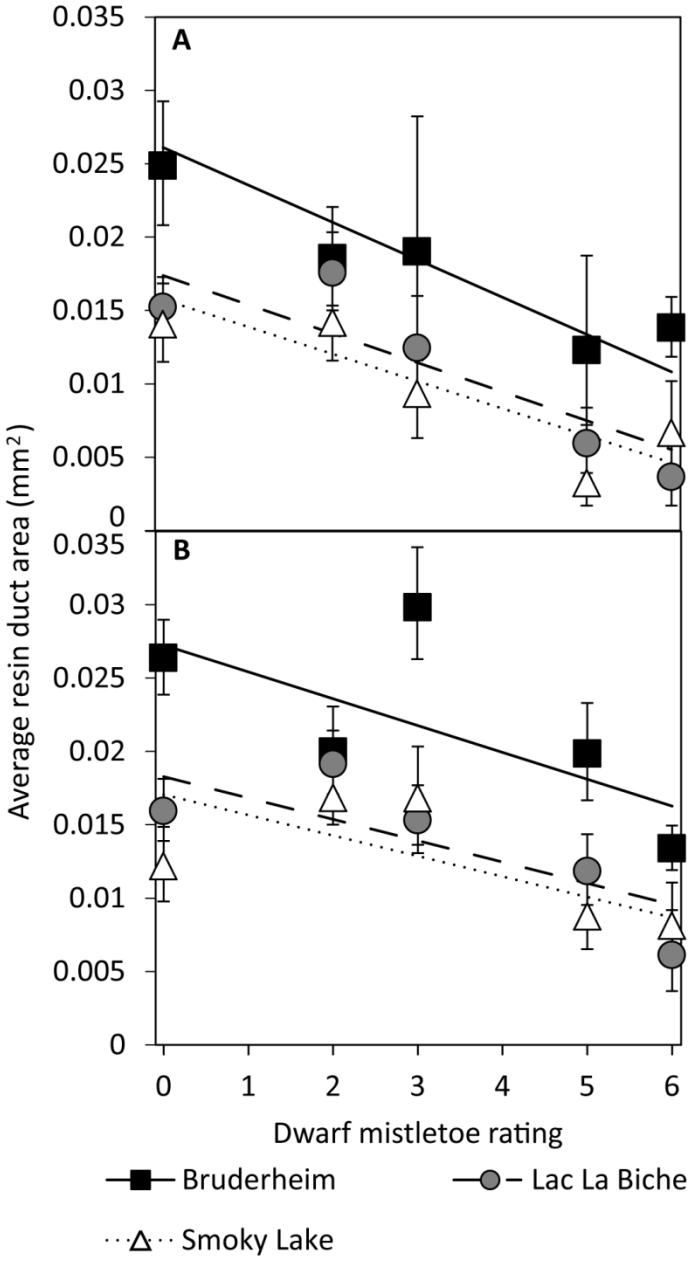

Figure Appendix D.5. Mean resin duct area (square root back-transformed, mm^2) in *Pinus banksiana* with varying levels of *Arceuthobium americanum* (dwarf mistletoe) at three sites in Alberta, Canada. A. Mean of last 5 yrs. B. Mean of last 10 yrs.

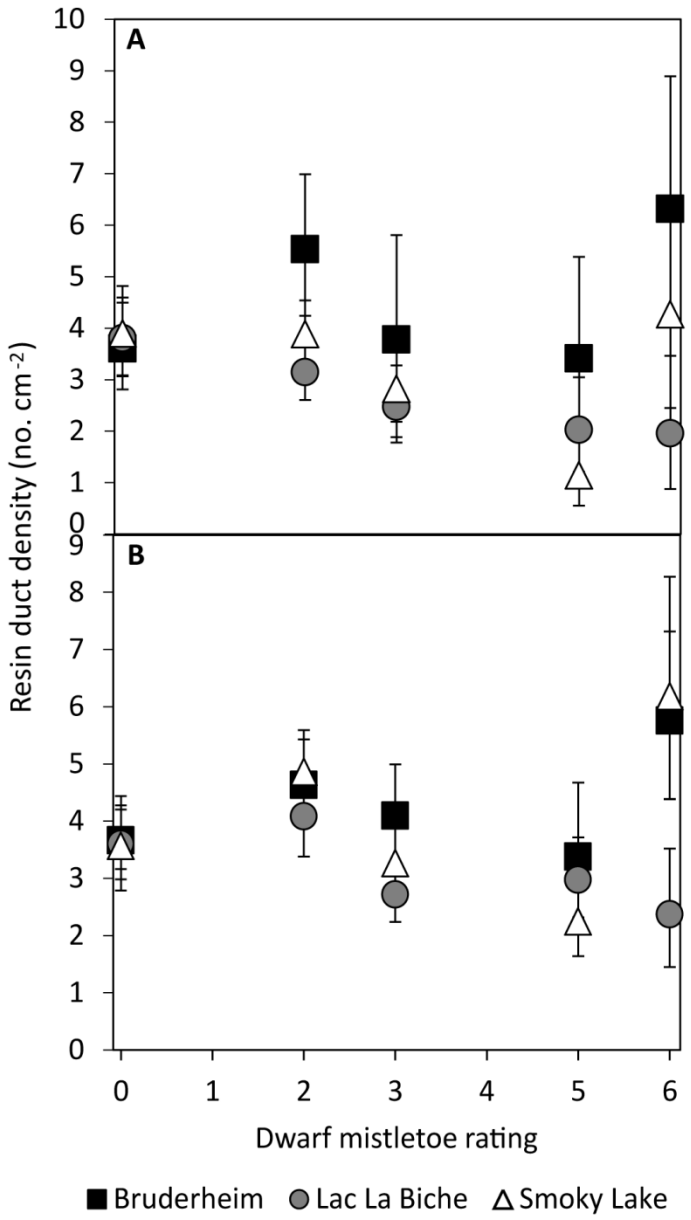

Figure Appendix D.6. Mean annual resin duct density (square root back-transformed, no. cm^{-2}) in *Pinus banksiana* with varying levels of *Arceuthobium americanum* (dwarf mistletoe) at three sites in Alberta, Canada. A. Mean of last 5 yrs. B. Mean of last 10 yrs.

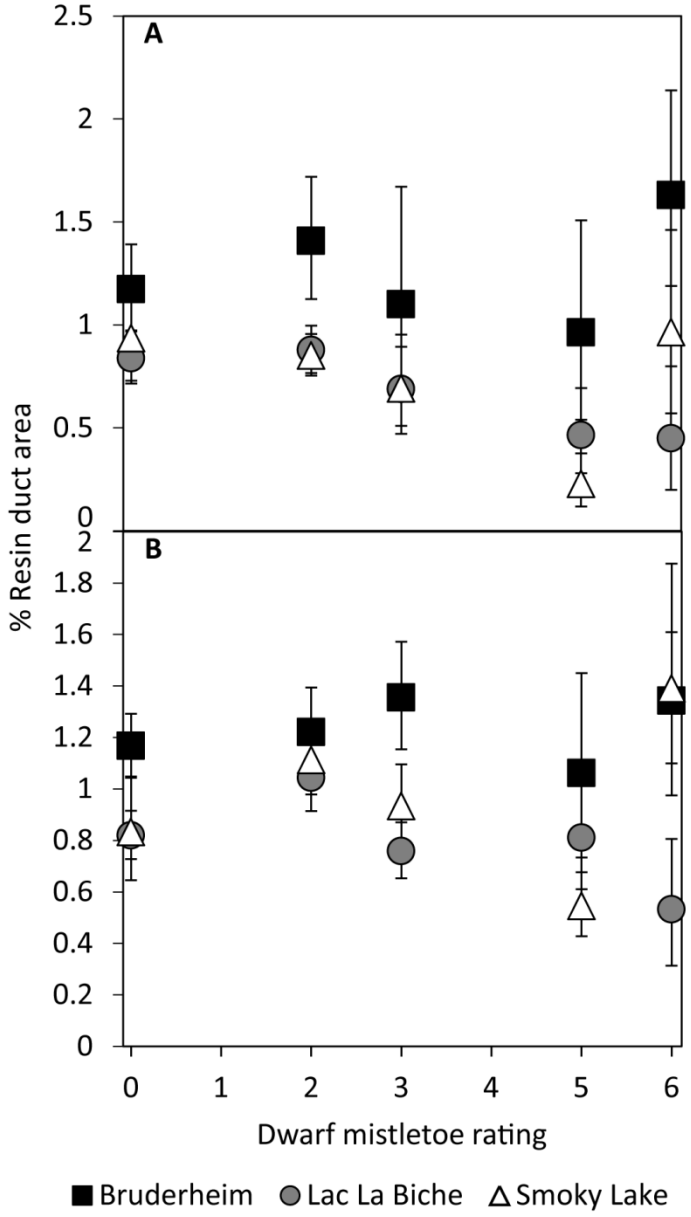

Figure Appendix D.7. Mean percent resin duct area (square root back-transformed, %) in *Pinus banksiana* with varying levels of *Arceuthobium americanum* (dwarf mistletoe) at three sites in Alberta, Canada. A. Mean of last 5 yrs. B. Mean of last 10 yrs.

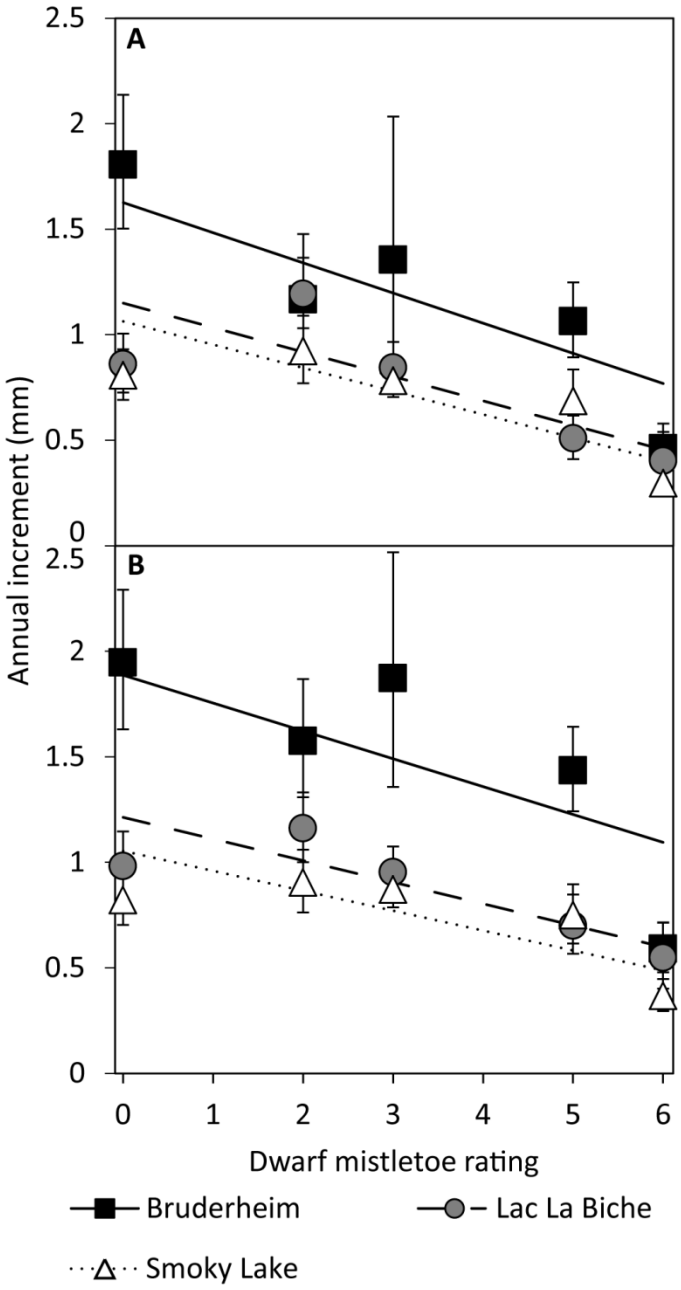

Figure Appendix D.8. Mean annual increment growth (square root back-transformed, mm) in *Pinus banksiana* with varying levels of *Arceuthobium americanum* (dwarf mistletoe) at three sites in Alberta, Canada. A. Mean of last 5 yrs. B. Mean of last 10 yrs.