University of Alberta

Induced Monoterpene Responses in Jack Pine: Defence against Jack Pine Budworm and a Fungal Associate of the Mountain Pine Beetle

by

Lindsay Jessica Colgan

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Forest Biology and Management

Department of Renewable Resources

Fall 2010
Edmonton, Alberta

Examining Committee

Dr. Nadir Erbilgin, Renewable Resources

Dr. Peter V. Blenis, Renewable Resources

Dr. Maya L. Evenden, Biological Sciences

Dr. John R. Spence, Renewable Resources

Dr. W. Jan A. Volney, Canadian Forest Service, Northern Forestry Center

ABSTRACT

My thesis research investigated monoterpene responses in jack pine (*Pinus banksiana* Lamb.) to different agents to better understand how these responses may influence the spread of the mountain pine beetle (*Dendroctonus ponderosae* Hopkins; MPB) in the boreal forest. The results support that monoterpenes are inducible responses in jack pine. In the first study, methyl jasmonate application elicited the greatest response in juvenile and mature trees suggesting that jasmonic acid plays a role in jack pine defence responses. In the cross-induction study, I found evidence of an increase in resistance to *Grosmannia clavigera* with prior jack pine budworm defoliation (*Choristoneura pinus pinus* Freeman; JPBW). In contrast, needle monoterpenes greatly increased after *G. clavigera* inoculation and continued to increase during JPBW defoliation; however, JPBW increased its feeding rate to compensate for a change in host quality. Overall, monoterpene induction in jack pine depended on the agent(s) involved and their order. The systemic responses that were observed may have implications for MPB spread in the boreal forest.

ACKNOWLEDGEMENTS

First, I would like to thank my supervisor, Dr. Nadir Erbilgin, for his patience and guidance throughout my research and writing, as well as my supervisory committee Drs. Peter Blenis, Maya Evenden, and Jan Volney for their thought-provoking questions. I would also like to thank my professors in my undergraduate and graduate schooling for teaching me much more than course material, especially Drs. Dave Martell and Sean Thomas at the University of Toronto for undergraduate research opportunities which inspired me to pursue graduate studies.

I am grateful for my funding provided by an NSERC CGS M, Walter Johns Fellowship, William McCardell Scholarship, and an NSERC Discovery grant awarded to Nadir Erbilgin.

I am indebted to Taylor Scarr and Dan Rowlinson for providing me with larvae, without which the experiment in my fourth chapter would not have been possible. I also thank the Faculty of Agriculture, Life, and Environmental Science for the greenhouse space and the staff in the Department of Renewable Resources for their support.

Inka Lusebrink had a valuable role in the chemical analyses from teaching me extractions to running my samples on the gas chromatograph. I also appreciated discussions we had both academic and otherwise.

Many people helped with my field research Colin Bergeron, Pei-yu Chen, Tibor Egri, Devin Goodsman, and Inka Lusebrink, and I received support in the greenhouse from Bruce Alexander, Claudia Alaya, and Ahmed Najar. Lisa Lumley and Adrianne Rice showed me techniques and gave me advice for working with my organisms.

I am grateful to the hosts and producers at CBC Radio One for maintaining my sanity through well over a thousand hours of greenhouse and lab work.

Many thanks to my friends and fellow graduate students for providing distraction and sharing the joys and frustrations of graduate school, my friends in Ontario, especially Susan Kidnie, and Neil Cleveland for his continual support, entertainment on weekends, and putting up with endless stories of the wonders of Toronto.

Finally, I never would have reached this point without the love and encouragement of Mom, Dad, Liam, and Babcia who always believed in me when I didn’t believe in myself.

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I. GENERAL INTRODUCTION

Coniferous trees have evolved both constitutive and inducible defence systems that deter or kill insects and inhibit or exclude pathogens both physically and chemically (Johnson and Croteau 1987; Brignolas et al. 1995, 1998; Hudgins et al. 2003, 2004; Salle et al. 2005; Eyles et al. 2007, 2009). Recent fossil evidence suggests that these systems have been operating in the Pinaceae for at least 45 million years (Labandeira et al. 2001; Franceschi et al. 2005). Constitutive defences are the "first tier of defence" that provide immediate resistance to attacking organisms (Franceschi et al. 2005). Some examples of constitutive defences include resin ducts, oleoresin production, stone cells, and a broad spectrum of stored secondary metabolites such as terpenoids, phenolics, and alkaloids. Induced defences are triggered by damage to provide additional protection and act as the "second tier of defence" (Franceschi et al. 2005); however, they are more aptly referred to as induced responses as defence is not always achieved. Some examples of induced responses include anatomical changes, such as the lignification of tissues and the formation of traumatic resin ducts, and changes in the concentration and composition of chemical compounds.

Induced responses can alter host suitability for organisms sharing the same host, which may result in indirect, plant-mediated interactions between attacking organisms. A prior attack can make a host tree more susceptible or more resistant to a subsequent attacker, commonly referred to systemic induced susceptibility (SIS) or systemic induced resistance (SIR), respectively (reviewed by Bonello et al. 2006). Although the study of induced resistance is in its infancy, it holds potential as a component of pest management strategies and research into the mechanisms of induction may yield results for a method for large scale implementation (Bonello 2010).

Climate change may greatly impact insect-plant interactions by affecting insect growth and development (Logan et al. 2003) or tree physiology in ways that alter resistance to herbivores and pathogens (Ayres and Lombardero 2000). Additionally, there is the potential for native species to expand their geographic

range and move into new ecosystems (Logan 2007). In recent decades, range shifts attributed to climate change have been observed in insect species (Parmesan and Yohe 2003; Parmesan 2006). Range expanding species will interact with new communities of organisms and potentially novel hosts in their expanded range (Thuiller et al. 2007). Evolutionary interactions between trees and attacking organisms influence the outcome of host-mediated interactions (Tollrain and Harvell 1999). Thus any changes in resistance or susceptibility of host plants induced by the native organisms could influence the spread of a new species and vice versa. These relationships should be studied under the framework of plant-mediated interactions and can contribute to a richer understanding of ecosystem function and the coordination of plant defences against multiple organisms.

In North America, a pertinent example is the present range expansion of the mountain pine beetle (*Dendroctonus ponderosae* Hopkins [Coleoptera: Curculionidae, Scolytinae]; MPB). MPB is an outbreak bark beetle species and the most destructive insect pest in Canadian pine forests. Its population dynamics are marked by low or endemic populations that build to outbreak or epidemic levels, which decline after several years and return to endemic levels. The range of the MPB extends from British Columbia to northern Mexico (Wood 1982) where it has primarily been hosted by lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.), though it attacks a wide range of *Pinus* species (Safranyik and Carroll 2006). Since the 1970s, forest management, fire suppression, and climate change have increased its suitable habitat by 75% (BCMFR 2006; Carroll et al. 2006). In the last decade, MPB populations have expanded into higher altitudes (Logan 2007), higher latitudes, and eastward into lodgepole *x* jack pine hybrid forests of Alberta, as far east as Slave Lake (ASRD 2009). It is thought that MPB will invade jack pine (*Pinus banksiana* Lamb.)-dominated forests in the boreal (Logan et al. 2003; Logan and Powell 2004; Carroll et al. 2006). If it establishes in jack pine, the MPB has the potential to spread across Canada's boreal forest and into the pine forest of eastern North America (Logan et al. 2003).

Initially, winter temperatures in Alberta's jack pine forests are expected to limit MPB populations to endemic levels (CFS 2008) where beetles will be constrained to attacking trees with lowered defences, such as those weakened by other insects and pathogens (Safranyik and Carroll 2006; Smith et al. 2009). Therefore, any changes in resistance or susceptibility of jack pine induced by native insects or pathogens could influence the spread of MPB. Although a number of research papers have focused on plant-mediated interactions (e.g. Stout et al. 2006; Eyles et al. 2007), few studies focus on if and how these interactions will be affected by climate change and how they may influence the establishment of range expanding species (Van der Putten et al. 2010).

The jack pine budworm (*Choristoneura pinus pinus* Freeman [Lepidoptera: Tortricidae]; JPBW) is sympatrically distributed with jack pine across the boreal and is an important defoliator that may influence the establishment and survival of MPB in the boreal forest. JPBW outbreaks every six to twelve years depending on the region (McCullough 2000) and can result in top-kill, reduced growth, and tree mortality (Kulman et al. 1963). Trees stressed by partial defoliation could have lowered defences and are more likely to be successfully attacked by endemic populations of beetles. The nature of the JPBW – MPB relationship is uncertain as an outbreak defoliator is absent from lodgepole pine forests and an outbreak bark beetle is absent jack pine forests. These two species could affect jack pine suitability for one another by inducing responses in host trees and act synergistically by SIS or antagonistically by SIR. Therefore, studying the indirect interaction between JPBW and MPB is important in forecasting the potential for the establishment and spread of endemic populations of MPB in the boreal forest.

My Master of Science research was conducted to explore the relationship among JPBW, MPB, and jack pine. Originally, this objective was intended to be investigated in a jack pine stand with active MPB and JPBW infestations; however, it was logistically impossible to incorporate these insects in a jack pine stand for the following reasons. Although Alberta has mature jack pine stands and active MPB infestations in lodgepole *x* jack pine hybrid stands, these stands have

low to non-existent levels of JPBW defoliation. Ontario is the only province in Canada that has had active JPBW outbreaks in jack pine forests for the last few years, but MPB infestations have not spread past the Alberta-Saskatchewan border. For understandable regulatory reasons Ontario does not permit any experiments with live MPB or its fungal associates. Thus, my experiments had to be designed around these constraints.

In Chapter II, I provide a framework for the JPBW – MPB – jack pine interaction by placing it in the context of invasion biology. The natural history and population dynamics of JPBW and MPB are reviewed and then after a brief discussion of conifer-mediated interactions, I present a conceptual model of potential scenarios of interactions between different population levels of JPBW and MPB in the boreal forest. Chapter III explores induced responses in juvenile and mature jack pine to different types of damage and plant hormone applications. Juvenile and mature trees were used in this study to characterize differences in their responses. Since it is logistically impossible to use identical experimental protocols in different environments, i.e. juvenile trees in a greenhouse experiment and mature trees in a field experiment, I avoided direct comparisons of their responses but discussed how induced responses compared between juvenile and mature trees to the same or similar treatments. The results of this study were used to determine the best design for the study in Chapter IV. In this chapter, jack pine seedlings received an induction treatment of defoliation by JPBW or inoculation with a fungal associate of MPB, *Grosmannia clavigera*, and then a challenge treatment with the other organism was applied to see if there was a change in jack pine resistance or susceptibility due to prior induction. This experiment is a preliminary exploration of the JPBW – MPB – jack pine relationship, substituting *G. clavigera* inoculations for MPB. Finally, the general discussion in the closing chapter synthesizes my thesis research and discusses its contributions to the understanding of induced responses in jack pine and tree-mediated interactions.

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II. THE ECOLOGICAL INTERACTION OF THE MOUNTAIN PINE BEETLE AND JACK PINE BUDWORM IN THE BOREAL FOREST

2.1 Introduction

Climate change in recent decades has been correlated with shifts in the ranges of insect, bird, and marine species (Parmesan and Yohe 2003; Parmesan 2006). Species that expand their range are of concern because their establishment may impact the integrity of new ecosystems (Thuiller et al. 2007). In North America, one range expansion of great concern is that of the mountain pine beetle (*Dendroctonus ponderosae* Hopkins [Coleoptera: Curculionidae, Scolytinae]; MPB). It is the most destructive insect in Canadian pine forests. Until recently, the MPB was generally limited to lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.)-dominated forests west of the Rocky Mountains but over the last decade populations have moved into higher elevations (Carroll et al. 2006) and eastward into lodgepole *x* jack pine hybrid forests, as far as Slave Lake (ASRD 2009). Beetle populations have also reached epidemic levels in areas where they usually have not (Carroll et al. 2006). Based on the speed of its eastward movement, it is likely that the MPB will invade jack pine (*Pinus banksiana* Lamb.)-dominated forests of the boreal. Prior research has shown that MPB can successfully reproduce (Cerezke 1995) and its fungal associates can grow in jack pine hosts (Rice et al. 2007). If it establishes in jack pine forests, the beetle could cause serious ecological and economic impacts when favourable conditions prevail (Logan et al. 2003; CFS 2008).

Species that have naturally expanded their ranges and those translocated by humans have similar factos that influence their esblishment in new ecosystems and can be studied in the same theoretical framework of invasion ecology (Thuiller et al. 2007). In new ecosystems, invading species encounter a community of organisms with which they have not previously interacted that could influence their spread. Such inter-specific relationships are critical in understanding of ecosystem dynamics and resilience with the uncertainty of

global change. Researchers have made many forecasts of global change impact for forests (e.g. Kurz et al. 2008; Malhi et al. 2010; Rammig et al. 2010) and individual species (e.g. Goldblum and Rigg 2005; Carroll et al. 2006; Bronson et al. 2009), but few have focused on the influences of inter-specific interactions despite their potential importance (Van der Putten et al. 2010).

In jack pine forests, MPB will indirectly interact with a new community of organisms, including jack pine budworm (*Choristoneura pinus pinus* Freeman [Lepidoptera: Tortricidae]; JPBW). JPBW is a major defoliator of jack pine that periodically reaches outbreak or epidemic levels causing large amounts of damage and in some cases mortality. JPBW's range does not extend into the Rocky Mountains and it is uncommon for the MPB to encounter with an outbreak defoliator in lodgepole pine forests of western North America (Duncan 2003). Additionally, although other species of secondary bark beetles, such as *Ips pini* (Say), are present and may kill small numbers of trees, an outbreak bark beetle is absent from jack pine forests. With the potential impacts of two large insect disturbances in the boreal, exploring the JPBW – MPB – jack pine interaction is important to better understand the susceptibility of jack pine forests and the invasive potential of MPB. In this chapter, I investigate this potential interaction by examining the natural history and population dynamics of JPBW and MPB, place their relationship in the context of conifer-mediated interactions, and explore several scenarios of JPBW-MPB interactions at different population levels in the boreal forest.

2.2 Jack Pine Budworm

2.2.1 Natural History

The JPBW is the primary defoliator of jack pine in the Canadian boreal forest (McCullough and Kulman 1991). It is principally hosted by jack pine but also feeds on Scots (*Pinus sylvestris* L.) and red (*P. resinosa* Ait.) pines when they are mixed with jack pine (Kulman and Hodson 1961). Host trees are mature, flowering individuals, generally over 20 to 30 years old.

The JPBW is a univoltine species that overwinters in bark crevasses as second instar larvae in an obligate diapause (McCullough 2000). In the spring, larvae feed for about six weeks, first on pollen cones and later on new foliage, developing through seven instars (Table 2.1) (Nealis 1995). In July, larvae pupate and adults emerge one to two weeks later (McCullough 2000). Females produce pheromones to attract males for mating (Sanders 1971) and after copulation lay eggs on older needles. Eggs hatch in late summer and first instar larvae move to bark crevasses where they moult and form winter hibernacula without feeding. JPBW survival and success appear to be influenced by tree nutrition, site conditions, natural enemies, and defensive host chemicals (Nealis and Lomic 1994; Wallin and Raffa 1999; McCullough 2000). Results from McCullough and Kulman (1991) suggest that changes in needle monoterpenes do not greatly impact larval feeding but negatively affect adult fecundity.

2.2.2 Population Dynamics

The JPBW persists at low populations in open crown jack pine forests (Kulman et al. 1963). The presence of pollen cones on host trees affects population density in the spring and strongly influences the survival of early instar larvae (Nealis and Lomic 1994). Outbreaks last two to four years and occur at six to twelve year intervals, depending on the region and conditions (McCullough 2000). In Wisconsin, JPBW outbreaks at five, six, and ten year intervals (Volney and McCullough 1994) and in Saskatchewan at approximately ten year intervals (Volney 1988). The size of an outbreak is strongly associated with habitat type: shorter cycles are associated with dry and nutrient poor sites (Volney and McCullough 1994). Weather, fire, and water availability likely drive longer ten year outbreak cycles, and weather, site conditions, and other unknown external factors likely drive shorter five and six year cycles (Volney and McCullough 1994). Volney (1988) suggested that the observed increase in outbreak size is due to a greater proportion of susceptible age classes on the landscape as a result of fire suppression. There are currently no significant outbreaks in the Prairie

Provinces but for the last few years there have been areas of defoliation in Ontario (NRC 2009; Taylor Scarr, Ontario Min. Nat. Res., personal comm.).

During population outbreaks, severe defoliation can result in reduced growth, top kill, and tree mortality (Kulman et al. 1963) but such defoliation rarely persists in a stand for more than one year (Gross and Meating 1994; McCullough et al. 1996). Outbreaks collapse quickly due to high parasitism of late larval stages (Nealis 1991) and because of a negative feedback between jack pine pollen cone production and previous defoliation (Nealis and Lomic 1994). Data from Ontario and Michigan reveal 15 % tree mortality in an outbreak and 15 to 20 % of top-kill trees killed in subsequent outbreaks (Gross 1992; Conway et al. 1999).

Jack pine-dominated ecosystems have evolved with JPBW defoliation as a periodic disturbance. Additionally, the accumulation of deadwood and dense jack pine crowns support fires that open the serotinous cones of jack pine and perpetuate even-aged stands (McCullough 2000). Historically, forest fires have left a variety of age classes on the landscape and limited the proportion of stands susceptible to JPBW.

2.3 Mountain Pine Beetle

2.3.1 Natural History

The MPB is an outbreak bark beetle species that attacks pines and is primarily hosted by lodgepole, ponderosa (*P. ponderosa* P. Laws. ex C. Laws), and western white (*P. monticola* Dougl. ex D. Don) pines in its range (Safranyik and Carroll 2006). The beetle is found in forests from the Pacific Coast east to the Black Hills of South Dakota, and from northern British Columbia and western Alberta south to northwestern Mexico (Wood 1982). It attacks mature trees of varying diameter and host quality varies with beetle population density (Elkin and Reid 2010).

Beetles use aggregation pheromones to attack *en masse* to exhaust a tree's defences, access phloem tissue, and kill the host tree. The host tree must die for successful reproduction (see Population Dynamics section below). Pioneer

females use visual and olfactory cues to select host trees (Safranyik and Carroll 2006). Once a host is chosen, females begin to construct galleries and initiate mass attack by converting α-pinene to the aggregation pheromone *trans*-verbenol (Borden et al. 1983). After a few days, anti-aggregation pheromones, such as verbenone (Hunt and Borden 1990), divert flying beetles to other trees, which optimizes attack density and reduces intra-specific competition (Berryman et al. 1985; Safranyik and Carroll 2006). The MPB is associated with a variety of mutualistic fungi that facilitate beetle establishment by weakening tree defences and disrupting transpiration as well as provide a nutritional supplement for larvae and teneral adults (Paine et al. 1997; Bleiker and Six 2007).

Larvae do not have a winter diapause but increase their cold tolerance through the autumn by accumulating antifreeze proteins and polyhydric alcohols (e.g. glycerol, sorbitol) in their haemolymph (Bentz and Mullins 1999) and resume feeding in the spring, or early summer in high latitudes, as soon as temperatures are warm enough. Larvae undergo four instars, pupate in early summer, and new generation adults emerge and fly to new host trees from late June to August depending on the latitude, elevation, and climate (Table 2.1). Once new host trees are colonized, female beetles excavate egg galleries, mate, and lay eggs. After hatch, larvae begin to feed on the phloem.

2.3.2 Population Dynamics

The population dynamics of the MPB can be delineated into four phases: endemic, incipient, epidemic, and post-epidemic (Safranyik and Carroll 2006). At endemic levels, small populations are unable to overcome the defences of vigorous trees and are constrained to attack lower quality and smaller diameter hosts. Host characteristics largely influence the choice of breeding site and beetles frequently target senescent trees or those weakened by insects or diseases. Mass aggregation is not usually achieved and beetles are not successful in depleting tree defences. As a result, many trees are not killed but only partially attacked, commonly referred to as a strip kill. The size of the beetle population remains relatively constant from year to year and affected trees are scattered in a stand. At

this stage, it is thought that natural enemies, such as parasitoids and predators, keep MPB populations under control (Safranyik and Carroll 2006).

In the incipient phase, the transition from endemic to epidemic levels, larger populations allow beetles to overcome the defences of healthy, larger, and more resistant hosts (Raffa and Berryman 1983; Safranyik and Carroll 2006). Attacked trees are clumped in discrete patches in stands as anti-aggregation pheromones divert flying beetles to adjacent uncolonized trees. More hosts are killed each year as MPB populations build.

At epidemic levels, beetles can successfully attack healthy stands and cause widespread mortality across the landscape. Epidemics in western Canada average ten years in duration and persist until hosts are depleted or unsuitably cold temperatures cause high mortality of overwintering larvae (Safranyik and Carroll 2006). During this stage, beetles fly long distances to find new suitable forest stands. Long distance dispersals of beetles from British Columbia have resulted in the invasion of lodgepole pine forests in western Alberta. In the post-epidemic phase, beetles continue to attack healthy hosts but declining populations result in many partially attacked trees and reduced generation sizes (Safranyik and Carroll 2006).

Generational success of MPB is greatly influenced by seasonal conditions. Weather can influence beetle dispersal and water deficits can influence tree resistance (Taylor et al. 2006). Cold snaps in the late fall and winter temperatures below $-40^{o}C$ may result in high rates of larval mortality (Bentz and Mullins 1999).

Deadwood in MPB-killed stands promotes high intensity fires that result in even-aged lodgepole pine regeneration. Prior to forest management, fires limited the proportion of stands suitable for MPB, constraining population growth. This pattern perpetuated a mosaic of age classes on the landscape (Taylor and Carroll 2004).

2.4 Conifer-Mediated Interactions

Conifers are subject to attack by a variety of organisms that use them for food, habitat, and oviposition sites. As a result, trees have evolved an array of defences to minimize damage by attackers. Constitutive defences are the "first tier of defence" (Franceschi et al. 2005) that are always maintained by a tree and are present when a tree is attacked (Karban and Baldwin 1997). If the organism is not deterred, the "second tier of defence" or a series of induced responses are triggered to further protect the plant (Franceschi et al. 2005). These physical and chemical changes occur over temporal scales of a few hours to the next season (Eyles et al. 2009). More specialized insects often have adapted to exploit conifer defences. For example, pines have specific induced responses to bark beetle attack (reviewed by Franceschi et al. 2005); however, bark beetles mass attack to overwhelm a tree's defence system. As a result, bark beetles remain a major disturbance in coniferous forests (Berryman 1972; Paine et al. 1997; Raffa et al. 2008).

Induced tree responses lead to multipartite interactions in a community through indirect interactions as one organism may change a host's suitability for others and hosts become less or more suitable for the subsequent attackers. If induced responses increase a tree's defences for a period of time it is referred to as systemic induced resistance (SIR), whereas when tree resistance declines following attack it is referred to as systemic induced susceptibility (SIS) (Bonello et al. 2006). Bonello et al. (2006) suggested a hypothetical threshold for tree resistance in which an induction event increases defence above the constitutive level, which can remain constant as SIR or decline as SIS. When new herbivores invade an ecosystem, indirect interactions may influence the species' invasive potential because of SIS or SIR from native organism attack or they may affect native organisms. Thus, examining tree-mediated interactions may provide insight into the potential interaction of JPBW, MPB, and jack pine.

2.4.1 Systemic Induced Resistance

There are many examples of how an initial induction treatment can affect resistance to a subsequent challenge treatment in conifers. For example, pre-treatment of Norway spruce (*Picea abies* L. Karst.) with the blue-stain fungal associate of the Eurasian spruce bark beetle (*Ips typographus* L.), *Ceratocystis polonica* (Siem.) C. Moreau, induces resistance to simulated bark beetle attack. Inoculation with medium and high fungal densities (50 and 100 inoculations/m^2) reduced colonization success of a mass inoculation with the same fungus by 76 % and 97 %, respectively (Krokene et al. 1999). Likewise, Scots pine was less susceptible to mass inoculation with a blue-stain fungus *Leptographium wingfieldii*, a fungal associate of the pine shoot beetle (*Tomicus piniperda* L.), when pre-treated with *L. wingfieldii* (Krokene et al. 2000). Additionally, pre-treatment of Japanese black pine (*Pinus thunbergii*) and Japanese red pine (*P. densiflora*) with a non-virulent form of the pine wood nematode (*Bursaphelenchus xylophilus* [Steiner & Buhrer] Nickle) resulted in systemic resistance to a virulent form, though high concentrations of pre-treatments were necessary for effective induction (Kosaka et al. 2001).

The defence responses in the studies above could have been organism-specific as the same species were used for the induction and challenge treatments; however, similar results have been observed among multiple species of organisms. In lodgepole pine, defence induction of feeding by the pine beauty moth (*Panolis flammea* D&S) and the European sawfly (*Neodiprion sertifer* Geoff.) were tested on the other species' fitness (Trewhella et al. 1997). Feeding by both insects led to significant changes in needle chemistry, which reduced the fitness of *P. flammea* but not that of *N. sertifer*, suggesting that plant defences have different impacts on herbivores. In Finland, Scots pine defoliated by the sawfly *Diprion pini* (L.) had increased resistance to the pine shoot beetle (Annila et al. 1999).

2.4.2 Systemic Induced Susceptibility

There is also research that supports that an initial attack can increase host susceptibility to subsequent attack. At low populations, bark beetles target trees with reduced defences, like those weakened by herbivores and pathogens (Safranyik and Carroll 2006). Heavy defoliation of Douglas fir (*Pseudotsuga menziesii* [Beissn.] Franco) by the Douglas-fir tussock moth (*Orgyia psuedotsugata* McDunnough) reduced the resistance to colonization by the fir engraver beetle (*Scolytus ventralis* LeConte) and the Douglas-fir bark beetle (*Dendroctonus pseudotsugae* Hopkins), allowing lower densities of beetles to successfully attack trees (Wright et al. 1984). Wallin and Raffa (2001) examined the effect of JPBW defoliation on colonization by the five-spined engraver beetle (*Ips grandicollis* Eichhoff) and the pine sawyer beetle (*Monochamus carolinensis* Olivier) in jack pine trees in Wisconsin and found that heavily defoliated trees had reduced resin flow. Further, beetle-colonized trees coincided with JPBW defoliation, and mortality in colonized trees was positively related to defoliation with 30, 82, and 100 % mortality in the moderate, heavy, and severe defoliation classes, respectively. Similarly, artificially defoliated red pines were more suitable hosts for the pales weevil (*Hylobius pales* Herbst) the same year, and the pine engraver two years after defoliation compared to the control (Raffa et al. 1998).

There are also examples in the literature demonstrating the complex interactions between species based on the amount initial damage they caused. For example, defoliation by the pine looper (*Bupalus piniaria* L.) resulted in a strong decline in the resistance of Scots pine to the blue-stain fungus *L. wingfieldii* (Långström et al. 2001). Trees in the lowest defoliation classes were less susceptible to *L. wingfieldii* than those in higher defoliation classes.

While numerous studies address induced resistance or susceptibility when multiple organisms act on a tree, it is difficult to compare different systems because tree-mediated relationships depend on the interacting organisms, intensity of damage, time since induction, and tree responses to multiple organisms simultaneously. The studies presented above suggest that initial treatment with a pathogen or light defoliation increases tree resistance to fungi and bark beetles,

while severe defoliation increases tree susceptibility. If jack pine forests follow this trend, timing of JPBW and MPB interactions will be important for determining whether host resistance or susceptibility is induced by the first attack.

2.5 The JPBW – MPB – Jack Pine Interaction

Predicting the outcome of the JPBW– MPB – jack pine interaction could be made by examining how MPB and *Choristoneura* spp. interact with insects and diseases in their respective forest ecosystems. Attacks by endemic populations of MPB are consistently associated with lodgepole pine infected by *Armillaria mellea* (Vahl. ex. Fr.) Kummer (Tkacz and Schmitz 1986) or comandra blister rust (*Cronartium comandrae* Pk.) (Rasmussen 1987). Similarly, lodgepole pine colonized by Monterey pine ips (*Pseudips mexicanus* Hopkins) were more attractive and a better resource for endemic populations of MPB than those attacked solely by MPB (Smith 2008). Ponderosa pines heavily affected by south western dwarf mistletoe (*Arceuthobium vaginatum* [Willd.] Presl subsp. *crytopodum* [Engelm.] Hawksw. & Wiens) were at greatest risk for bark beetle colonization in Arizona (Kenaley et al. 2006, 2008). In Newfoundland, the success of the four-eyed spruce bark beetle (*Polygraphus rufipennis* Kirby) colonizing black spruce (*Picea mariana* Mill) was positively related to damage by the spruce budworm (*C. fumiferana* Clem.) and bark beetle attacks increased rapidly following the collapse of budworm populations (Bowers et al. 1996). Colonization of jack pine by the five-spined engraver beetle and pine sawyer beetle increase exponentially in relation to JPBW defoliation (Wallin and Raffa 2001).

Although MPB can successfully reproduce in jack pine (Cerezke 1995), initially its populations in Alberta will likely be constrained to endemic levels by winter temperatures (Régnière and Bentz 2007); therefore, established populations will be limited to attacking weakened or stressed trees. Their spread and survival will be affected by indirect interactions with other organisms in the boreal, which could include those root diseases, such as *Armillaria* and *Tomentosus* root rots, dwarf mistletoe (*Arceuthobium americanum* Nutt. ex Englem.), competitor

beetles, such as pine engraver beetles, JPBW, and other defoliators (Mallet and Volney 1990; Mallet 1992; Brandt et al. 1998).

Since JPBW periodically reaches outbreak levels, changes in its population size could influence stand susceptibility and thus the spread of MPB. If MPB establishes in jack pine forests as predicted, JPBW and MPB could indirectly interact through defence responses in jack pine, which could result in competition for host trees. Since both are agents of large scale disturbance in their ecosystem, their interaction is important in understanding the behaviour of MPB populations in jack pine ecosystems and cascading effects for other species.

In the same forest, JPBW and MPB will temporally overlap on host trees (Table 2.1). Density-dependent processes affect the generational survival of JPBW (Nealis and Lomic 1994) and MPB (Trzcinski and Reid 2009). JPBW attacks flowering jack pine trees that are over 20 to 30 years of age and there is a strongly positive relationship between pollen cone production and larval survival (Nealis and Lomic 1994). Therefore overlap in host selection is likely to be important when both populations are at low levels. JPBW and MPB may act synergistically by lowering tree defences (thus increasing tree susceptibility), or they may act antagonistically by raising tree defences (thus increasing tree resistance). The outcome of the JPBW – MPB – jack pine interaction will depend on the population size of each species, host availability, weather conditions, and host suitability, which is influenced by weather, site conditions, and any defence response that has been induced. Below are four possible scenarios to generalize interactions between these species (Fig. 2.1).

I) If both JPBW and MPB populations were at endemic levels, there would be little effect on forest stand dynamics. Light JPBW defoliation would minimally impact tree growth (Volney 1988) while MPB would target senescent and weakened trees along with other competitor beetles, such as pine engravers. Low populations would result in a high proportion of unsuccessful attacks (Safranyik and Carroll 2006) and these partial attacks would impact pollen cone production and foliar quality for JPBW larvae in the spring. Similarly, if JPBW eggs were on

a tree attacked by MPB in the late summer, the tree's suitability in the following spring would be altered for JPBW larvae. Pollen cones are critical to the survival of early instar JPBW, so MPB attacks would have an immediate negative effect on budworm survival. Changes in foliar chemistry may also affect adult fecundity (McCullough and Kulman 1991) and thus population levels in the following years. On the other hand, partially attacked jack pines could produce a stress crop of pollen cones; however, this food source would be unlikely to affect JPBW populations as these trees would be scattered in a stand and it would take several years for JPBW populations to build to destructive levels (Volney 1988). As a result, in this scenario there could be occasional JPBW – MPB interactions but overall the populations of both species would remain low with minimal impact on the forest.

II) If JPBW populations were at epidemic levels and MPB populations were at endemic levels, jack pines stressed by heavy defoliation would be preferentially attacked by MPB. The damage to a tree would depend on the induction of jack pine defence, time since defoliation, severity and longevity of defoliation, and the resulting tree condition of resistance or susceptibility to MPB. If heavy or multiple years of defoliation lower tree defences then lower densities of beetles could successfully colonize and kill a tree (Wright et al. 1984). This would result in higher rates of jack pine mortality than in a JPBW outbreak without MPB. MPB populations could build in subsequent years with more successful colonization. On the other hand if jack pine defences increase, a much greater proportion of MPB attacks would be unsuccessful and many trees would be only partially attacked. Higher densities of beetles would be necessary to overcome increased defences and successfully colonize a tree, which would negatively impact MPB population growth. As a result, the damage and mortality in a stand would be similar to a normal JPBW outbreak as MPB would not add to tree mortality.

III) If JPBW populations were at endemic levels and MPB populations were at epidemic levels, the impact on a stand would be similar to a normal MPB outbreak as MPB would mass attack and kill healthy, mature hosts (Safranyik and Carroll 2006). JPBW oviposits in the summer before MPB colonizes trees and trees with JPBW eggs subsequently colonized by MPB would be dead in the spring. As a result, no pollen cones would be produced and JPBW larvae would die from starvation. JPBW larvae would disperse to find suitable host but the rate of survival would be low. JPBW populations would remain small and may even decrease because of larval mortality and a reduction in the number of host trees. The MPB would continue at epidemic levels with high rates of tree mortality.

IV) It is possible that JPBW and MPB might reach epidemic populations simultaneously. This would have devastating impacts on the forest in a short period of time. Because MPB mass attack can overcome even well defended trees, MPB would not be affected by defences induced by prior JPBW defoliation. However, JPBW populations would be sensitive to the timing of MPB outbreak. If MPB populations reached epidemic levels prior to JPBW outbreak, beetle-caused tree mortality and partial attacks could result in JPBW starvation. Small diameter trees and those less desirable to MPB, would be vulnerable to JPBW defoliation. On the other hand, if JPBW populations were large in the spring prior to MPB flight in the summer, heavy defoliation would kill some trees and those remaining would be attacked by MPB. Outbreaks of both species could rapidly deplete hosts and may lead to the collapse of both populations in the years following.

The scenarios summarized above are a few possibilities on a continuum of plausible JPBW – MPB interactions at the tree level. Competitor beetles and other organisms may also play a role in host selection and colonization of endemic populations of MPB. The outcome of JPBW – MPB interactions are more dependent on MPB as at high populations it will function independently of jack pine defence induction. Modelling population dynamics of each species along

with research of jack pine-mediated interactions between species would yield a more accurate relationship between these organisms. There are no biological impediments for MPB in the boreal (CFS 2008) and as mentioned MPB can reproduce (Cerezke 1995) and fungal associates can grow (Rice et al. 2007) in jack pine. However, the role of host defences have not been investigated but likely is the cornerstone of this relationship. The temporal scales of jack pine induction and the duration of systemic induced resistance or susceptibility remain largely unexplored. Results from Karban (1990) and Raffa et al. (1998) reveal important time delays in induced resistance and susceptibility, which could further complicate the relationship between JPBW and MPB.

Focusing on several basic questions would significantly deepen our knowledge of this interaction. Does defoliation change phloem chemistry in ways that inhibit or promote colonization of jack pine by MPB? Does MPB partial attack change needle chemistry and affect JPBW larval development and survival through changes in needle chemistry and pollen cone production? How does tree susceptibility change with the intensity and frequency of defoliation and the level of beetle attack: will trees that are moderately to heavily defoliated and then partially attacked by MPB die? Answering these questions will allow us to predict if JPBW and MPB will act synergistically or antagonistically in the boreal forest. This knowledge will provide a platform to examine further aspects of their relationship, explore MPB's relationship with other organisms, and help forest entomologists make the best recommendations for multi-species management of boreal forest pests in jack pine.

Management of invasive non-native species usually focuses on reducing the rate of spread as often species are not detected until after they have established (Liebhold and Tobin 2008). Range expansion of pest species, like the MPB, provides the unique opportunity for the management of invasive species as their behaviour in the new system can be investigated prior to their arrival. A thorough understanding of how system reacts to the new organism can help forest managers develop effective pest management strategies. For example, if heavily defoliated jack pine trees are susceptible to MPB attack, these stands can be targeted for

sanitation harvesting or other mitigation activities intended to reduce MPB risk. As a result, management of range-expanding species could focus on proactive actions to minimize population growth and slow the rate of spread rather than suppressing outbreaks.

Tree-mediated interactions are potentially important determinants of the population dynamics of herbivores and pathogens in forests and could become increasingly important under climate change. The field of chemical ecology is a critical component of tree-mediated interactions and focuses on the mechanistic understanding of plant chemical responses to insects and diseases and their direct and indirect interactions. Studying these relationships in boreal forest can provide valuable information of how jack pine defensive chemistry is coordinated against multiple organisms, including MPB.

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Table 2.1. The life cycles of the jack pine budworm (JPBW) and mountain pine beetle (MPB).

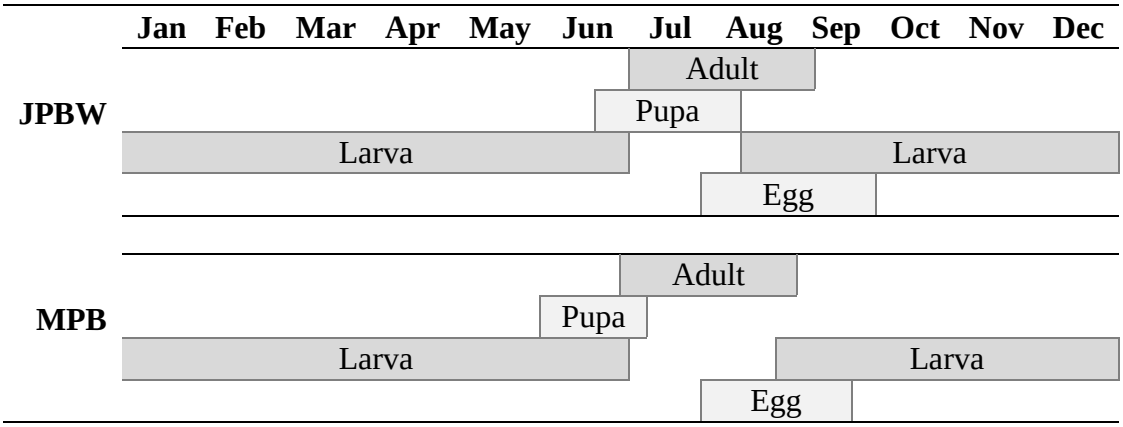
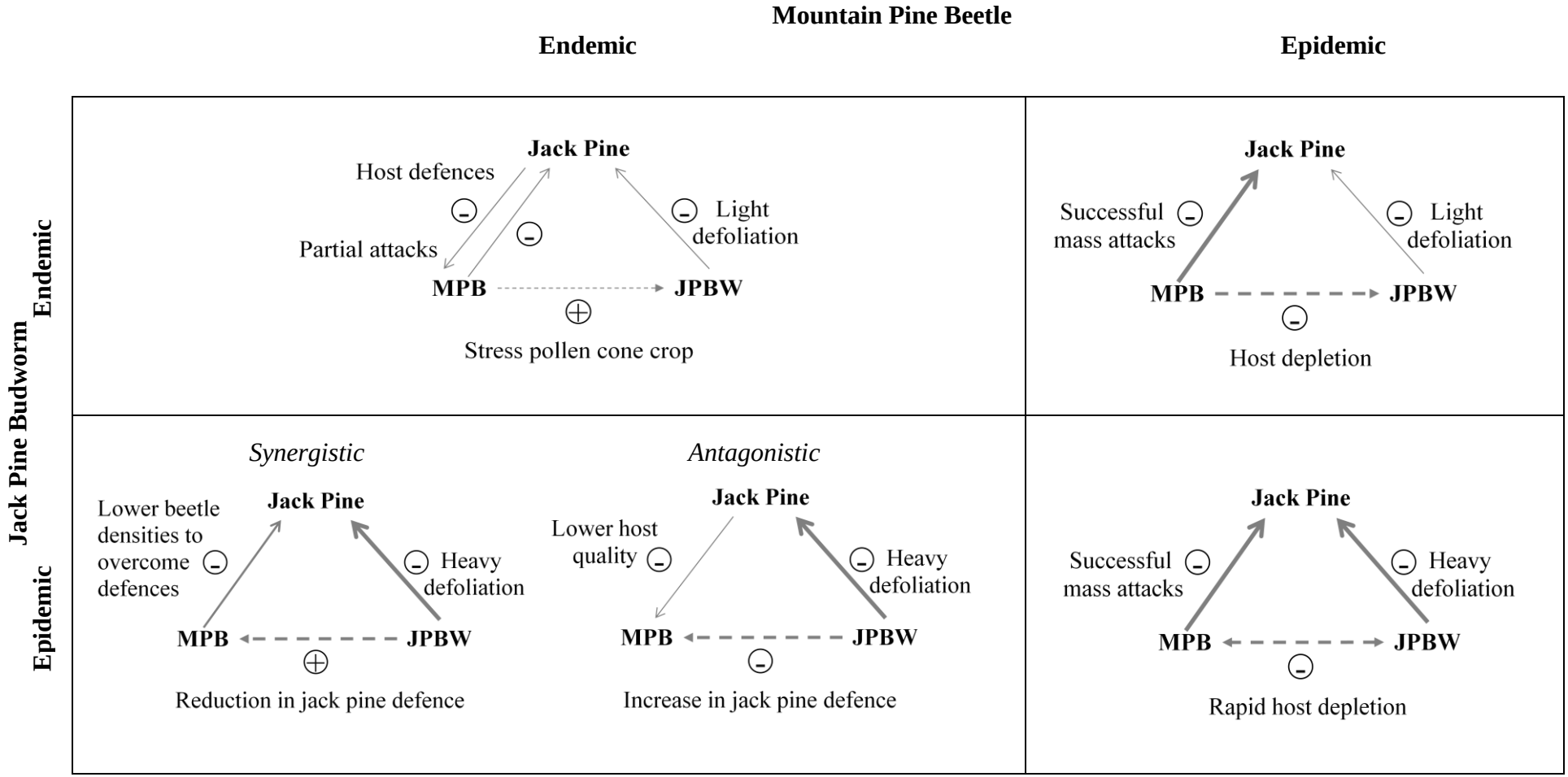


Figure 2.1 Diagram of potential interactions of different population levels of jack pine budworm (JPBW) and mountain pine beetle (MPB) in jack pine forests. Thicker arrows indicate a larger effect.



III. INDUCED RESPONSES OF JUVENILE AND MATURE JACK PINE TO VARIOUS INDUCTION AGENTS

3.1 Introduction

Conifers are attacked by a wide variety of organisms and have evolved physical and chemical defences to protect their tissues from herbivory and infection. Constitutive defences are always present in a tree to discourage attackers, whereas induced responses are triggered by damage and aimed to limit damage from attacking organisms (Franceschi et al. 2005). Constitutive defences include resinosis, calcium oxalate crystals, and stored secondary metabolites, such as terpenoids, phenolics, and alkaloids, and induced responses include polyphenolic parenchyma cell and traumatic resin duct formation and the synthesis of additional defence compounds (Krekling et al. 2000; Franceschi et al. 2005). Plant induced responses are important in ecosystem dynamics as they can alter a host's suitability for subsequent colonization by the same or different organisms, and as a result, host plants mediate the interactions between organisms (Stout et al. 2006).

Herbivore attack and oviposition can induce changes in terpenoid composition of host trees (Mumm and Hilker 2006). Induced terpenoid responses have been observed in many conifer species (Erbilgin et al. 2006; Mumm and Hilker 2006; Moreira et al. 2009). Terpenoids are a family of carbon-based, defensive compounds that include monoterpenes, diterpenes, and sesquiterpenes (reviewed by Keeling and Bohlmann 2006 and Mumm and Hilker 2006). Terpenoids can be toxic to insects or negatively impact their development and survival and are correlated with insect resistance in conifers (Keeling and Bohlmann 2006). For example, monoterpenes can influence herbivore host selection and colonization (Latta et al. 2000; Raffa et al. 2008).

In this research study, I investigated constitutive and induced defences of jack pine (*Pinus banksiana* Lamb.) against a number of agents including a fungal associate of mountain pine beetle (*Dendroctonus ponderosae* Hopkins [Coleoptera: Curculionidae, Scolytinae]; MPB). The MPB has expanded its range

from its historical habitat in lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm)-dominated forests west of the Rocky Mountains to lodgepole *x* jack pine forests of western Alberta (Logan et al. 2003; Carroll et al. 2006) and continues to spread eastward. Jack pine is a dominant tree species in the Canadian boreal forest, extending from northern Alberta to Nova Scotia, and is likely to become a host for MPB in the boreal (Logan et al. 2003; Carroll et al. 2006). Since MPB use host tree terpenoids to locate their host trees and synthesize their pheromones (Borden et al. 1983; Conn et al. 1983; Conn et al. 1984), my investigation of jack pine responses to different agents will contribute to understanding the invasion success of the mountain pine beetle in boreal forest.

The present study focused on monoterpene induction in mature and juvenile jack pine to different levels and types of induction agents. In a field experiment, I examined the phloem response of mature trees to fungal inoculations and methyl jasmonate (MeJa) application. In a greenhouse experiment, I examined needle and phloem responses in seedlings to mechanical defoliation, fungal inoculations, MeJa and methyl salicylate (MeSa) applications.

MeJa and MeSa are methyl esters of the phytohormones jasmonic acid (JA) and salicylic acid (SA), which among many roles, are important in signalling defence responses in plants to insect and pathogen attack (Creelman and Mullet 1997; Felton and Korth 2000; Walling 2000; de Bruxelles and Roberts 2001; Stout et al. 2006). In conifers, the exogenous application of MeJa can induce terpenoid accumulation and other defence responses, including traumatic resin duct formation and polyphenolic parenchyma cell formation in the secondary phloem (Franceschi et al. 2000, 2005; Martin et al. 2002; Hudgins et al. 2003; Erbilgin et al. 2006; Zeneli et al. 2006; Krokene et al. 2008). In contrast, there are few studies that explore the role of SA in conifers, but studies demonstrated that SA accumulated after pathogen attack in Norway spruce (*Picea abies* Karst.) tissues (Kozlowski et al. 1999), and *Pinus* species expressed chitinase homologs after SA was applied as a soil drench (Davis et al. 2002). Additionally, MeJa application induced SA accumulation in Norway spruce, suggesting a possible relationship, or *cross-talk*, between these two chemicals (Kozlowoski et al. 1999).

MeJa and MeSa were applied in this study to stimulate the biochemical pathways of defence responses and to compare jack pine responses to these compounds and those to real damage.

The first type of real damage was inoculation with *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer and Wingfield (Northern Forestry Culture Collection, NOF 2896), formerly known as *Ophiostoma clavigerum* (Robinson-Jeffrey and Davidson) Harrington. This blue-stain fungus is an associate of the MPB that is likely adapted to survive the cold winters of the boreal forest (Rice et al. 2007). Inoculations with fungi are commonly used in experiments to induce tree responses and observe changes in tree resistance to subsequent treatments (Bonello and Blodgett 2003; Christiansen et al. 1999; Krokene et al. 1999, 2000). Wound inoculations were included to ensure that *G. clavigera* inoculation induced responses beyond the physical damage cause by inoculation. Wound inoculations were not included as an induction type in the analysis but were compared to *G. clavigera* inoculations in a separate analysis.

In the greenhouse experiment, the second type of real damage was mechanical defoliation. Needles were clipped to mimic jack pine budworm (*Choristoneura pinus pinus* Freeman [Lepidoptera: Tortricidae]; JPBW) defoliation. JPBW is a common defoliator of jack pine trees in the boreal forest, which outbreaks every six to twelve years depending on the region (McCullough 2000). JPBW defoliation can induce monoterpene responses (Wallin and Raffa 1999) and can result in decreased growth, top-kill, and tree mortality (Kulman et al. 1963). Since larvae were not available at the time of experiment, mechanical foliage removal was used to simulate JPBW defoliation by clipping needles near the base; mechanical defoliation induced monoterpene cyclases in several conifer species (Litvak and Monson 1998).

The objectives of this study were to: a) compare total monoterpene concentration and composition among induction types in each plant tissue (mature phloem, juvenile phloem, and juvenile needle); b) evaluate response to MeJa and MeSa application compared to real damage by *G. clavigera* inoculation and mechanical defoliation; c) compare response of needles and phloem tissue in

juvenile trees; and d) qualitatively explore the relationship between responses in mature trees from the field experiments and juvenile trees from the greenhouse experiments.

3.2 Methods

3.2.1 Field Experiment

In July 2008, a healthy stand of jack pine trees without any visible insect and disease symptoms was selected at a site about 200 km north of Edmonton, AB (N55^{o}02.32', W114^{o}02.97'). Seventy mature trees (diameter at breast height of 20.03 ± 0.04 cm) were chosen and randomly assigned to a treatment. The experiment had two induction types, each with two levels, two densities of wound inoculations, and a control group for a total of seven treatments. The treatments were: 25 and 100 mM MeJa application; 12 and 36 *G. clavigera* inoculations; 12 and 36 wound inoculations; and an untreated control. There were ten trees in each treatment.

For MeJa applications, outer bark (cork layer) was scraped off the bole between 80 and 155 cm and a 25 or 100 mM MeJa solution (similar to Martin et al. 2002) with 0.1% (v/v) Tween 20 was applied to the area using a spray bottle (Erbilgin et al. 2006). Since the cork is not living, removing the outer bark does not harm the tree. Tween 20 is a non-biologically active surfactant and was used to emulsify MeJa (Martin et al. 2002; Hudgins et al. 2003, 2004). In many species of Pinaceae, application of 100 mM MeJa induced responses similar to those to wounding (Hudgins et al. 2003) and was chosen as the highest concentration in this experiment following other studies (Martin et al. 2002; Krokene et al. 2008).

For *G. clavigera* inoculations, 12 evenly spaced 5 mm holes were drilled to the sapwood in a ring(s) around the bole (Krokene et al. 1999). For trees with 12 inoculations, holes were drilled in a ring 130 cm (breast height) above the ground. For trees with 36 inoculations, 12 holes were drilled around the stem at heights of 105, 130, and 155 cm above the ground. A 4 mm plug of *G. clavigera* colonized malt extract agar was placed in each hole. Each inoculation ring was covered in masking tape and the section of the stem was wrapped with cling wrap

and sealed with duct tape to minimize possible contamination. We chose 36 inoculations/tree as the upper inoculation density because Raffa and Berryman (1983) reported that MPB minimizes intra-specific competition by the optimization of mass attack density around 60 attacks/m^2 or 34 inoculations/tree. Wound inoculations were prepared by the same method expect drill holes were left empty. Control trees were not treated.

After treatment, trees were left for 8 ½ weeks until they were sampled. One 3 x 3 cm phloem sample was taken from each tree between 5 and 20 cm above the MeJa treated area or the top inoculation ring. The mean height (and standard error) of phloem sample location was 10.7 ± 1.44 cm. The lengths of four evenly spaced lesions were measured from every inoculation/wound ring, i.e. four measurements from 12 inoculation/wound trees and twelve measurements from 36 inoculation/wound trees. A lesion is an area of necrotic tissue that forms around a site of pathogen entry and is commonly used to quantify induced response to pathogens, where smaller lesions indicate better tree defences (Bonello and Blodgett 2003; Krokene et al. 2008). For inoculated or wounded trees, one phloem sample from inside inoculated or wounded lesions was flame sterilized and put on malt extract agar to reculture *G. clavigera* and any organisms present. Although the common air borne *Penicillium* spp. and endophytic bacteria were present all samples, *G. clavigera* was present in most *G. clavigera* inoculation (65 %) and few of the wound inoculation trees (<15 %).

3.2.2 Greenhouse Experiment

Two-year-old jack pine seedlings were obtained from Boreal Horticultural Services Ltd. (Bonnyville, AB). Seedlings were watered daily and fertilized weekly in greenhouses at the University of Alberta (Edmonton, AB). After treatments were applied to seedlings, fertilizer was not added to exclude any nutrient-defence interactions (Zhao et al. 2007). The healthiest seedlings of a similar height (44.2 ± 0.04 cm) and diameter at the base (8.3 ± 0.01 mm) were chosen and randomly assigned to treatments. The treatments were: 10, 25, and 50 mM MeJa; 10, 25, and 50 mM MeSa; 10, 25, and 40 % mechanical defoliation; 1,

3, and 6 *G. clavigera* inoculations; 1, 3, and 6 wound inoculations; and untreated control. There were ten seedlings in each treatment, except in wound inoculation treatments and controls, which each had five seedlings. Smaller samples sizes were chosen for the wound inoculations and control because without any induction treatment, variation among seedling chemistry is expected to be small.

Treatments were applied on July 23rd, 2008. For MeJa and MeSa application, 10, 25, or 50 mM solutions with 0.1 % (v/v) Tween 20 were applied to the bottom third of the stem using a paintbrush. Needles and branches were not painted. Seedlings were not watered for 24 hrs to ensure that the solutions were absorbed. For mechanical defoliation treatments, scissors were used to clip needles near the base to mimic JPBW feeding. Foliage was removed daily over the first 10 days of the experiment and was visually quantified to a final defoliation of 10, 25, or 40%. For 1, 3, or 6 *G. clavigera* inoculations, the outer bark and phloem of the seedlings was removed using a 3 mm cork borer and placing a plug of agar with mycelia in the hole. The stem section was wrapped in Parafilm to secure the inoculum and minimize possible contamination. Inoculation points were spread over the stem below the crown and adjacent holes were placed at 180^{o} from one another. Wound inoculations were again included to test that fungus had an effect beyond inoculation damage, but were not analyzed as a type of induction. Wound inoculations were prepared by the same method but the holes were left empty and wrapped in Parafilm. Controls were not treated during the experiment.

After 3 weeks, seedlings were harvested and needles and stem sections were stored at – 40^{o}C until tissue was sampled for monoterpene analysis. Needles were sampled from new growth in the crown and undamaged needles were sampled in mechanically defoliated seedlings. Phloem samples were taken close to the location of induction as follows: above the MeJa/MeSa treated portion of the stem; above *G. clavigera*/wound inoculations; and below the branches of the crown on the stem in mechanically defoliated and untreated control seedlings.

3.2.3 Chemical Analysis

All tissue was ground in liquid nitrogen using a mortar and pestle, and monoterpenes were extracted using a method modified from Wallis et al. (2008). Five hundred microlitres of dichloromethane (CH_2Cl_2) with 0.1 % tridecane internal standard was added to 100 mg of ground tissue in a 1.5 mL microcentrifuge tube. The samples were vortexed for 30 sec, placed in an ultrasonic bath for 10 min, and then centrifuged at 0^{o}C and 13.2 rpm for 15 min. The extract was pipetted into a vial and stored at – 40^{o}C. Then the sample was extracted for a second time using the same method. The second extract was added into the vial with the first and stored at – 40^{o}C until gas chromatography analysis.

Samples (1 μL) were injected in an Agilent 7890A Gas Chromatograph (Palo Alto, CA, USA) with an HP Innowax column (I.D. 0.32 mm, length 30 m), helium carrier gas flow at 1.8 mL/min and the temperature 50°C for 2 min, increased to 160°C by 5°C/min, and then to 250°C by 20°C/min. Peaks were identified using the following standards: borneol, pulegone, α-terpinene, *γ*-terpinene, α-terpineol (Sigma-Aldrich, St. Louis, MO, USA), camphor, 3-carene, α-humulene, terpinolene, α-thujone, β-thujone, (–)-α-pinene, (–)-β-pinene, (S)-(–)-limonene, sabinene hydrate, myrcene, (–)-camphene, p-cymene (Fluka, Sigma-Aldrich, Buchs, Switzerland), α-phellandrene, bornyl acetate, ocimene (SAFC Supply Solutions, St. Louis, MO, USA), β-phellandrene (Glidco Inc., Jacksonville, FL, USA), and by comparison of the retention indices (Table 3.1). For all samples the peaks were integrated and peak area was compared for qualitative and quantitative differences between samples of different treated seedlings at different time points.

3.2.4 Statistical Analyses

For both experiments, fungal growth was quantified by comparing the lesion lengths of *G. clavigera* and wound inoculated jack pines. The length of *G. clavigera* lesions in mature trees and seedlings could not be transformed to meet the assumptions of parametric statistics and instead were analyzed using non-parametric Kruskal-Wallis and Wilcoxon two-sample tests. The effect of

inoculation density was tested by comparing lesion lengths among the levels of inoculation in an experiment. For mature trees that received 36 inoculations/ wounds, I also compared lesion length of inoculations at 105, 130, and 155 cm to see if there was an effect of inoculation height.

Total monoterpene concentrations in inductions were tested using Proc GLM in SAS (v9.1 SAS Institute, NC, USA). The same analyses were performed for total monoterpene concentrations in each of mature phloem, seedling needle, and seedling phloem tissues. In mature trees, phloem monoterpenes were transformed (ln) for ANOVA. In seedlings, total needle monoterpenes met ANOVA assumptions and total phloem monoterpenes were transformed (ln).

First, an ANOVA of total monoterpene concentration was conducted with induction type, level, and their interaction to see if the interaction between induction and level was significant. When the interaction was not significant, an ANOVA of total monoterpene concentration and induction type was conducted to examine the differences among the inductions and the control. An lsmeans statement was included to compare each induction type to the control using Dunnett's test. Also, specific contrast statements were used to observe differences between chemical applications and real damage: MeJa and *G. clavigera* in mature trees; and MeJa and mechanical defoliation, MeJa and *G. clavigera* inoculation, MeSa and mechanical defoliation, and MeSa and *G. clavigera* inoculation in seedlings. A second GLM procedure was used to conduct to examine the total monoterpene concentrations among levels within each induction type. Finally, t-tests were used to compare total monoterpene concentrations of *G. clavigera* and wound inoculations by level and across all levels.

Monoterpene composition in all tissues was analyzed by examining individual monoterpenes as a proportion of total concentration. For mature trees, α-pinene, β-pinene, 3-carene, limonene, and myrcene were examined as they composed over 90% of the total phloem monoterpene concentration. For juveniles, α-pinene, β-pinene, 3-carene, limonene, myrcene, and bornyl acetate were tested as they composed over 90 % of the total monoterpene concentration in needles and phloem. The same statistical analyses were performed without

transformations for each individual monoterpene proportion for mature phloem, juvenile needle, and juvenile phloem tissue.

For each monoterpene proportion, an ANOVA of induction type, level, and their interaction was conducted. When the interaction was not significant an ANOVA of monoterpene proportion and induction type was conducted. Again, an lsmeans statement was included to compare the proportion in each induction to that of the control using Dunnett's test. Specific contrast statements were also included to test differences between hormone application and real damage for the pairs listed for total monoterpene comparisons.

Total monoterpene concentration in juvenile needles and phloem were transformed (ln) to achieve normality and induction types were compared using t-tests to observe any differences in the tissues. The Folded F test was used to test equality of variance and the appropriate pooled variance or Satterthwaite t-test was used. First, total monoterpene concentrations were compared between needles and phloem irrespective of treatment and then by induction type. The analysis continued by examining the proportional change in monoterpene concentration from the untreated control in each induction (Δ= [Induced – Untreated]/ Untreated). Wilcoxon two-sample tests were used to examine differences between needles and phloem by induction type. Individual monoterpenes α-pinene, β-pinene, 3-carene, limonene, myrcene, and bornyl acetate were assessed in terms of a change in proportion from the untreated control, as described above, and then by induction type using Wilcoxon two-sample tests.

3.3 Results

3.3.1 Field Experiment

Trees with *G. clavigera* inoculation had significantly longer lesions than the wound inoculations (Fig. 3.1). Inoculation density (12 or 36) did not affect lesions length (55.1 ± 6.43 mm for 12, 59.6 ± 3.22 mm for 36) (Z=-0.90, p=0.368). There was no apparent effect of ring height on lesion length for the 36 *G. clavigera* (χ^2=3.95, df=2, p=0.139) or wound (χ^2=1.45, DF=2, p=0.485) inoculation trees.

In an ANOVA of total phloem monoterpene concentration with induction type (MeJa application, *G. clavigera* inoculation, untreated control), level (e.g. 25 and 100 mM), and the interaction of induction type and level, the interaction term was not significant (F=0.07, df=1, p=0.787), so the data were tested by induction type, regardless of level. When the ANOVA was run with induction type, there were differences among induction types (F=4.34, df=2, p=0.019; Table 3.2). Dunnett's test revealed that total monoterpene concentrations in MeJa treated trees were significantly higher (p=0.0009) and *G. clavigera* inoculated trees were marginally higher (p=0.106) than the untreated control. Comparisons between hormone and real treatments indicated that total monoterperne concentrations did not differ between MeJa treated and *G. clavigera* inoculated trees (F=1.76, df=1, p=0.191). Similarly, monoterpene concentrations did not differ between *G. clavigera* and wound inoculated trees (Table 3.3). There were no differences in the levels within *G. clavigera* inoculation (F=1.01, df=1, p=0.327) nor MeJa application (F=1.06, df=1, p=0.317).

The proportions of α-pinene, β-pinene, 3-carene, limonene, and myrcene were examined for mature phloem and monoterpene compositions for each treatment are listed in Appendix Table A.1. For each monoterpene proportion, an ANOVA of induction type, level, and their interaction was conducted and none of the interactions was significant. As a result, each monoterpene proportion was tested among MeJa application, *G. clavierga* inoculation, and the untreated control in an ANOVA. Myrcene (F=4.59, df=2, p=0.015) was significantly different and β-pinene was marginally different among inductions and the control (F=2.74, df=2, p=0.075). Dunnett's test revealed that the proportion of myrcene was significantly higher (p=0.016) in MeJa treated trees than the untreated control. In the phytohormone-real damage contrasts, trees with MeJa application had higher concentrations of myrcene (F=5.32, df=1, p=0.026) and β-pinene (F=4.82, df=1, p=0.033) than trees with *G. clavigera* inoculation.

3.3.2 Greenhouse Experiment

Grosmannia clavigera inoculated seedlings had significantly longer lesions than those only wound inoculated. Lesion length varied among the fungal inoculation densities (χ^2_2=9.91, p=0.007) with one inoculation having significantly longer lesions than three or six inoculations (Fig. 3.3).

In needle tissue, an ANOVA of total monoterpene concentration with induction type (mechanical defoliation, *G. clavigera* inoculation, MeJa, MeSa, control), level, and the interaction of induction and level revealed that the interaction term was not significant (F=1.56, df=6, p=0.166). When an ANOVA was conducted with induction type, total monoterpene concentration were significantly different (F=4.53, df=4, p=0.002) (Table 3.4). A Bonferroni corrected *post-hoc* test indicated that MeJa treated seedlings had significantly higher monoterpene concentrations than both mechanically defoliated and *G. clavigera* inoculated seedlings (Fig. 3.4). However, none of the induction types was different from the untreated control in Dunnett's test. Levels within each induction type only differed in mechanically defoliated seedlings (F=3.37, df=2, p=0.049). Total monoterpene concentration needles did not differ between *G. clavigera* and wound inoculated trees (Table 3.5). In planned contrasts of phytohormone application and real damage, total monoterpene concentrations differed significantly between mechanical defoliation and MeJa (F=16.85, df=1, p<0.0001), *G. clavigera* inoculation and MeJa (F= 8.57, df=1, p=0.004), and marginally different between mechanical defoliation and MeSa (F=3.58, df=1, p=0.061).

The proportions of α-pinene, β-pinene, 3-carene, limonene, myrcene, and bornyl acetate were analyzed for seedling needles and monoterpene composition for each treatment is listed in Appendix Table A.2. The interaction of induction and level was not significant for any of the monoterpene proportions and the ANOVAs were rerun with induction type. Individual monoterpene proportions did not differ among induction types (all models p>0.05, results not shown) nor between each induction type and the control (p>0.05, results not shown). In planned contrasts of hormone application and real damage, the proportion of

myrcene was marginally higher in mechanically defoliated than MeSa treated seedlings (F=3.54, df=1, p=0.062) and 3-carene was marginally higher in *G. clavigera* inoculated than MeSa treated seedlings (F=3.22, df=1, p=0.075).

In phloem tissue, an ANOVA of total monoterpene concentration with induction type (mechanical defoliation, *G. clavigera* inoculation, MeJa, MeSa, control), level, and their interaction did not reveal a significant interaction between induction type and level (F=0.26, df=6, p=0.951). The ANOVA of induction type with total monoterpene concentration was not significant (Table 3.6), nor were comparisons to the untreated control using Dunnett's test. Also, planned contrasts of phytohormone application and real damage were not significant (p>0.05, results not shown). The ANOVAs of levels within each induction were not significant for any of the induction types. Total monoterpene concentrations in *G. clavigera* and wound inoculated seedlings were different (Table 3.7).

The proportions of α-pinene, β-pinene, 3-carene, limonene, myrcene, and bornyl acetate in seedling phloem were analyzed and the monoterpene composition for each treatment is listed in Appendix Table A.3. In ANOVAs of monoterpene proportion with induction type, level, and their interaction, the interaction between level and induction was not significant (p>0.05) for any of the compounds tested so the proportions were analyzed in ANOVAs with only induction type. In these ANOVAs, the proportion of myrcene significantly differed (F=2.64, df=4, p=0.037) and α-pinene marginally differed (F=1.97, df=4, p=0.104) among induction types. Myrcene was significantly higher in seedlings with MeJa application than the control (p=0.012) and was marginally lower in mechanically defoliated seedlings that the control (p=0.073). Dunnett's test did not show differences between the induction types and the control. In pair-wise comparisons of monoterpene proportions between hormone application and real damage: myrcene was higher in MeJa treated than *G. clavigera* inoculated seedlings (F=3.95, df=1, p=0.049); bornyl acetate was higher in MeJa treated than mechanically defoliated seedlings (F=4.79, df=1, p=0.031); and 3-carene was marginally higher in mechanically defoliated than MeSa treated seedlings

(F=3.14, df=1, p=0.079) as well as in *G. clavigera* inoculated than MeSa treated seedlings (F=3.39, df=1, p=0.068).

3.3.3 Phloem vs. Needle Monoterpene Concentrations in Seedlings

Phloem had a significantly higher concentration (3964.8 ± 150.3 μg/g) of total monoterpenes than needles (2379.9 ± 86.3 μg/g) across the inductions and control (t= –10.15, p<0.0001), as well as when compared by induction type (mechanical defoliation, *G. clavigera* inoculation, MeJa, and MeSa) (Table 3.8). When total monoterpene concentration in each induction was examined as a proportional change from the concentration of the control, the magnitude of induction, phloem had a larger increase in monoterpenes than needles overall and across induction types (Fig. 3.6a; Z= –7.59, p<0.0001). In addition, individual monoterpenes, α-pinene, β-pinene, 3-carene, limonene, and myrcene, were also examined as a change from the proportion in the control (Fig. 6b-f). The proportional increase of β-pinene differed between phloem and needles (Fig. 3.6c; Z= –3.57, p=0.0004). The proportion of myrcene increased more in phloem than needles (Fig. 3.6f; Z= –4.61, p<0.0001) while the proportion of Δ-3-carene increased more in needles than phloem (Fig. 3.6d; Z=4.89, p<0.0001).

3.3.4 Difference between Phloem Monoterpene Concentrations in Mature and Juvenile Trees

Total monoterpene concentrations averaged across MeJa treated, *G. clavigera* inoculated, wound inoculated, and control was higher in juvenile (3964.8 ± 150.25 μg/g) than mature (1123.3 ± 149.76 μg/g) trees, as well as in each induction type (values not shown). When examining the magnitude of induction, the change in monoterpene concentration from the control, mature trees had a larger magnitude of induction compared to juveniles (Fig. 3.7a). Examining proportions of α-pinene, β-pinene, myrcene, and limonene show that with the exception of limonene, these compounds have a larger magnitude of induction in mature than juvenile trees (Fig. 3.7b-e).

3.4 Discussion

The results of this study demonstrate that changes in monoterpene concentration and composition are inducible responses in mature and juvenile jack pine. MeJa application resulted in the greatest monoterpene increase in both mature and juvenile trees. Additionally, induction treatments affected proportions of myrcene and β-pinene in mature phloem and myrcene and α-pinene in juvenile phloem. Overall, monoterpene concentrations were higher in phloem than needle tissue of juveniles. Although the monoterpene concentrations were higher in juvenile than mature phloem, it appears that mature phloem has a greater magnitude of induction.

Jack pine showed the greatest monterpene response to MeJa application in both field and greenhouse experiments. Total monoterpene concentrations increased significantly by 283.6 ± 70.34 % in mature and 63.1 ± 15.52 % in juvenile phloem compared to the untreated control. In juvenile needles, total concentrations were significantly higher in MeJa treated seedlings than *G. clavigera* inoculated and mechanically defoliated seedlings, although these concentrations were not different than the control. It is well established that exogenous application of MeJa can induce production of terpenoid defences in conifers (Martin et al. 2002; Hudgins et al. 2003, 2004; Erbilgin et al. 2006; Zeneli et al. 2006; Krokene et al. 2008). Since MeJa influenced monoterpene composition of both juvenile and mature trees, this suggests that MeJa application can be used to induce responses in future experiments that focus on jack pine defences.

In contrast, exogenous application of MeSa did not affect monoterpene concentrations in juvenile jack pine. This result contradicts that the role of SA reported in studies of plant resistance and defence signalling in conifers (Durrant and Dong 2004). For example, SA application induced multiple chinitnase homologs in slash pine (*P. elliottii* Engelm. var *elliotti*) after inoculation with *Fusarium subglutinans* (Wollenweb. & Reinking) P.E. Nelson, T.A. Toussen & Marasas f. sp. *pini* (Davis et al. 2002). Further, Norway spruce accumulated free and bound SA in cotyledons, hypocotyls, and roots when treated with volatile

MeJa (Kozolowski et al. 1999). MeSa may not have had an effect because either it does not induce monoterpenes but perhaps other secondary metabolites, or 21 days may have been too short to observe an induced monoterpene response in jack pine. The use of MeSa in inducing jack pine defence responses requires further research.

Mechanical defoliation of juvenile jack pine was used to simulate JPBW herbivory but total monoterpene concentrations in neither needles nor phloem changed compared to the control. Simulated herbivory induced monoterpene cyclases in needles of ponderosa pine (*Pinus ponderosa* Lawson), lodgepole pine, and white fir (*Abies concolor* Lindl. and Gordon) (Litvak and Monson 1998), though very few studies make direct comparisons of real and mechanical defoliation. Real damage by herbivores usually causes stronger changes in trees than mechanical damage alone. For example, ponderosa pine by *Halisdota ingens* Hy. Edwards (Lepidoptera: Arctiidae) resulted in a 2.5 fold larger monoterpene response compared to mechanical damage alone (Litvak and Monson 1998). Likewise, Hartley and Lawton (1987) observed that total phenolics were higher after caterpillar grazing than artificial damage or leaf mining. Mechanical defoliation was spread over 10 days and the damage period may have been too short or there may have been too little damage to induce a significant change in jack pine monoterpenes. Alternatively, there may have been changes at the anatomical level that were not measured in the current study. Careful analyses are required to conclude the role of mechanical defoliation in inducing defences in jack pine.

Finally, although *G. clavigera* inoculations resulted in longer lesions than the wound inoculations, monoterpene concentrations did not differ from the untreated control in mature nor juvenile phloem. Longer lesions in *G. clavigera* inoculated trees indicate that fungus has a greater effect than wounding alone. However, a single *G. clavigera* inoculation in juveniles surprisingly resulted in a longer lesion compared to 3 or 6 inoculations, although monoterpene responses did not vary between different levels of inoculations. Currently I do not have any explanation for this difference; however, literature available suggests that tree

responses to pathogens or fungal inoculations are non-linear and can only be explained by characterizing the full defence response though changes in chemistry and anatomy (see review by Franceschi et al. 2005; Wallis et al. 2008; Eyles et al. 2009).

In juvenile jack pines, total monoterpene concentrations were consistently higher in phloem than needles, within and across induction types. Further, phloem also had a higher magnitude of induction than needles, calculated as the proportional change from the control. Defences are allocated within a plant depending on the value of the organ and the probability of attack, which may explain the differences observed between needle and phloem tissues**.** Zangerl and Bazzaz (1992) reported that since phloem is vital connective tissue between the roots and the needles and the stem is often more valuable to plants than leaves, roots, and even seeds and fruit, it is evolutionarily reasonable to expect them to have a higher concentration and inducible monoterpenes that plants invest more in stem tissues than other plant tissues.

Total monoterpene concentrations in phloem were consistently higher in juvenile than mature jack pine trees. The concentrations of monoterpenes may be lower in mature trees as they have a multitude of defences (see Franceschi et al. 2005) and secondary metabolites may play a smaller role in defence. In contrast, juvenile trees usually have poor physical defences and may require higher chemical defences to deter herbivores (Barton and Koricheva 2010). In a recent meta-analysis of the defence of woody plants against herbivores, seedlings showed greater chemical defences, whereas physical defences increased during the vegetative juvenile stage (Barton and Koricheva 2010).

However, the discrepancy between mature and juvenile monoterpene concentrations in the current study was not reflected in the magnitude of induction: the magnitude of induction was much larger in mature than juvenile trees. Mature trees appear to have a larger potential for monoterpene induction than seedlings, suggesting that ontogeny plays a role in jack pine defences. Juveniles of many species rely on chemical defences but trees are less affected by tissue losses to herbivory as they age (Barton and Koricheva 2010). For example,

the damage to willows (*Salix serica, S. eriocephala,* and their hybrid) by the slug *Arion subfuscus* Draparnaud [Mollusca: Arionidae] decreased over time (Fritz et al. 2001). Since they are deployed after attack has been detected, induced responses may be less costly than maintaining constitutive defences (Keeling and Bohlmann 2006). Further, induced responses may better accommodate the unpredictability of herbivory and allow trees to respond to a multitude of attackers (Lerdau and Gershenzon 1997). Mature and juvenile trees appear to have different defence strategies and research is needed to examine how resource allocation affects their defences since mature trees have more stores and resources available for the synthesis of defences than juvenile trees.

Examining monoterpene composition is important as individual compounds can be more important in defence than the total concentration (Berenbaum 1995). In this study, jack pine altered the proportion of some monoterpenes after induction treatments. Myrcene was most affected by induction in both juvenile and mature trees and its concentrations were much higher after MeJa application compared to the controls. The concentration of β-pinene increased in mature trees with MeJa application and *G. clavigera* inoculation. Wallin and Raffa (1998) found lower concentrations of myrcene in tissues preferred by JPBW. Further studies showed that JPBW defoliation significantly affected the relative proportions of α-pinene and β-pinene in mature jack pine (Wallin and Raffa 1999). In the boreal ecosystem, changes in individual compounds could affect the patterns of herbivory and infection as myrcene is also an important synergist with bark beetle pheromones for mass attack (Borden et al. 1983; Conn et al. 1983).

Finally, the results of this study reveal differences in jack pine responses to phytohormone application and real damage. In mature phloem, total monoterpene concentrations did not differ between MeJa application and *G. clavigera* inoculation but their proportions of myrcene and β-pinene differed. In juvenile needles, total monoterpene concentration differed between mechanical defoliation and MeJa application, mechanical defoilation and MeSa application, and *G. clavigera* inoculation and MeJa appliation. In juvenile phloem, total

monoterpene concentration did not differ among phytohormone applications and real damages, but there were differences in some comparisons in the proportions of 3-carene, myrcene, and bornyl acetate. As mentioned, terpenoid accumulation in conifers can be induced by MeJa application (Martin et al. 2002), fungal inoculation (Zeneli et al. 2006), and herbivore attack (Mumm and Hilker 2006), but the relationship between the amount of real damage and the concentration of phytohormones in monoterpene response has not been explored, despite the frequent use of MeJa to induce response. Further, Heijari et al. (2005) observed that monoterpenes in Scots pine did not change after MeJa treatment but seedlings were more resistant to the weevil *Hylobius abietis* (L.), suggesting that other types of defences are induced by MeJa. This study does not provide clear evidence for the relationship between responses induced by phytohormone application and real damage and more research in this area is required.

Overall, my results support other research showing that monoterpenes are an inducible response that depends on the type of damage (Raffa and Smalley 1995; Wallin and Raffa 1999; Eyles et al. 2009). Monoterpene concentration in jack pine phloem can rapidly increase after inoculation and level off after an initial peak (Raffa and Smalley 1995) but an increase in monoterpene concentration may not correspond with an increase defence (e.g. Heijari et al. 2005). Although I focused on monoterpenes, herbivores and pathogens can also affected by other defensive compounds, such as phenolics and alkaloids, and physical defences (Franceschi et al. 2005; Mumm and Hilker 2006). Unlike primary metabolites which are present in virtually all cells, the abundance of secondary metabolites depends on the environment and development regulation (Berenbaum 1995). For example, the production of terpenoids and phenolics, both carbon-based compounds, have an inverse relationship (Wallis et al. 2008). Terpenoids are only aspect of defence and as suggested by my results, may not fully represent the inducible response of trees.

3.5 References

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Table 3.1. Retention times for gas chromatograph.

Compound	Retention Time (min)
α-pinene	4.339
Camphene	4.996
β-pinene	5.724
3-carene	6.616
α-phellandrene	6.902
Myrcene	6.926
α-terpinene	7.214
Limonene	7.662
β-phellandrene	7.846
Ocimene	8.511
γ-terpinene	8.741
p-cymene	9.341
Terpinolene	9.631
Tridecane	10.046
α-thujone	12.967
β-thujone	13.416
Sabinene hydrate	14.008
Camphor	14.967
Linalool	16.049
Bornyl acetate	16.735
Pulgeone	18.203
α-humulene	18.785
α-terpineol	19.389
Borneol	19.454

Table 3.2. An ANOVA table of total phloem monoterpene concentration and induction type for mature trees in the field experiment.

Source	df	Type III SS	MS	F	*p*
Induction type	2	7.821	3.911	4.34	*0.019*
Error	46	41.449	0.901		
Corrected Total	48	49.270			

Table 3.3. Total phloem monoterpene concentrations of *G. clavigera* and wound inoculated mature trees. Data were transformed (ln) to meet the assumption of normality.

Number of Inoculations	Monoterpene Concentration (μg/g)		t	df	*p*
	G. clavigera	Wound			
12	874.0 ± 188.21	970.3 ± 253.78	0.58	18	*0.572*
36	1726.5 ± 767.22	920.5 ± 206.39	- 0.73	18	*0.478*
All	1300.2 ± 396.69	945.4 ± 159.29	-0.04	31.93	*0.969*

Table 3.4. An ANOVA table of total needle monoterpene concentration and induction type for juvenile trees in the greenhouse experiment.

Source	df	Type III SS	MS	F	*p*
Induction type	4	17397202.1	4349300.5	4.53	*0.002*
Error	120	115137759.3	959481.3		
Corrected Total	124	132534961.4			

Table 3.5. Total needle monoterpene concentrations of *G. clavigera* and wound inoculated juvenile trees.

Number of Inoculations	Monoterpene Concentration (μg/g)		t	df	*p*
	G. clavigera	Wound			
1	1817.3 ± 272.68	2494.8 ± 622.49	1.17	13	*0.262*
3	2484.4 ± 434.37	2024.5 ± 76.15	- 1.04	9.54	*0.323*
6	2365.1 ± 292.46	2447.5 ± 435.39	0.16	13	*0.875*
All	2222.3 ± 197.40	2322.3 ± 242.29	0.30	43	*0.762*

Table 3.6. An ANOVA table of total phloem monoterpene concentration and induction type for juvenile trees in the greenhouse experiment. Data were transformed (ln) to meet the assumptions of ANOVA.

Source	df	Type III SS	MS	F	*p*
Induction type	4	0.808	0.202	1.14	*0.341*
Error	120	21.254	0.177		
Corrected Total	124	22.062			

Table 3.7. Total phloem monoterpene concentrations of *G. clavigera* and wound inoculated juvenile trees. Data were transformed (ln) to meet the assumption of normality.

Number of Inoculations	Monoterpene Concentration (μg/g)		t	df	*p*
	G. clavigera	Wound			
1	3887.3 ± 264.49	3600.3 ± 708.47	- 0.71	13	*0.488*
3	4256.7 ± 747.23	2527.9 ± 317.03	- 1.51	13	*0.154*
6	3432.3 ± 291.44	3294.3 ± 499.29	- 0.35	13	*0.730*
All	3858.8 ± 278.76	3140.8 ± 309.29	-1.66	43	*0.104*

Table 3.8. Comparison of total monoterpene concentrations of needle and phloem tissue of juveniles by induction type. Data were transformed (ln) to meet the assumption of normality.

Induction Type	t	df	*p*
Mechanical Defoliation	- 6.31	58	*<0.0001*
G. clavigera Inoculation	- 5.38	58	*<0.0001*
Methyl Jasmonate	- 3.55	58	*0.001*
Methyl Salicylate	- 5.39	58	*<0.0001*

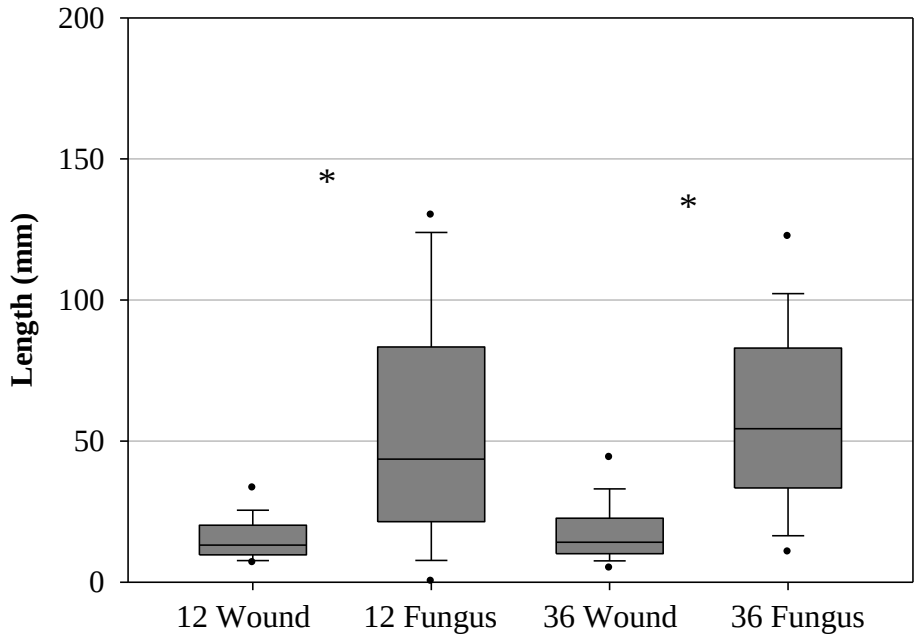

Figure 3.1. Box plot of lesion lengths of *Grosmannia clavigera* and wound inoculated trees from the field experiment. Box indicates the 25th and 75th percentile, the line through the box is the median, the capped lines indicate the 10th and 90th percentile, and the dots are the 5th and 95th percentile. * indicates that there is a significant difference (p<0.05) between lesion length of the *G. clavigera* and wound inoculation using a two-tailed Wilcoxon two-sample test.

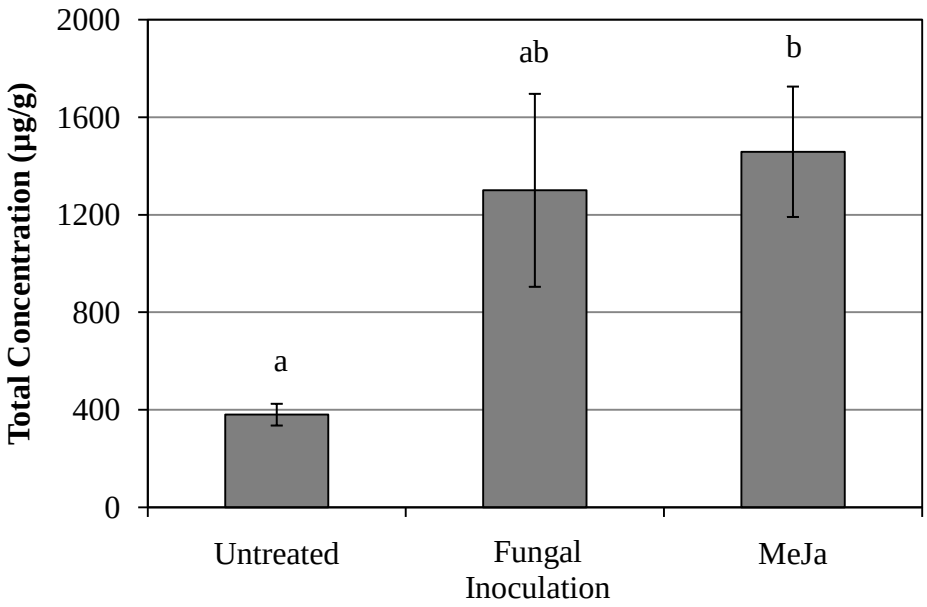

Figure 3.2. Total phloem monoterpene concentrations of mature trees from the field experiment (mean ± standard error) ($F_{2,45}$=4.34 p=0.019). Data were transformed (ln) for analysis. Letters indicate significant differences in a Bonferroni corrected *post-hoc* test.

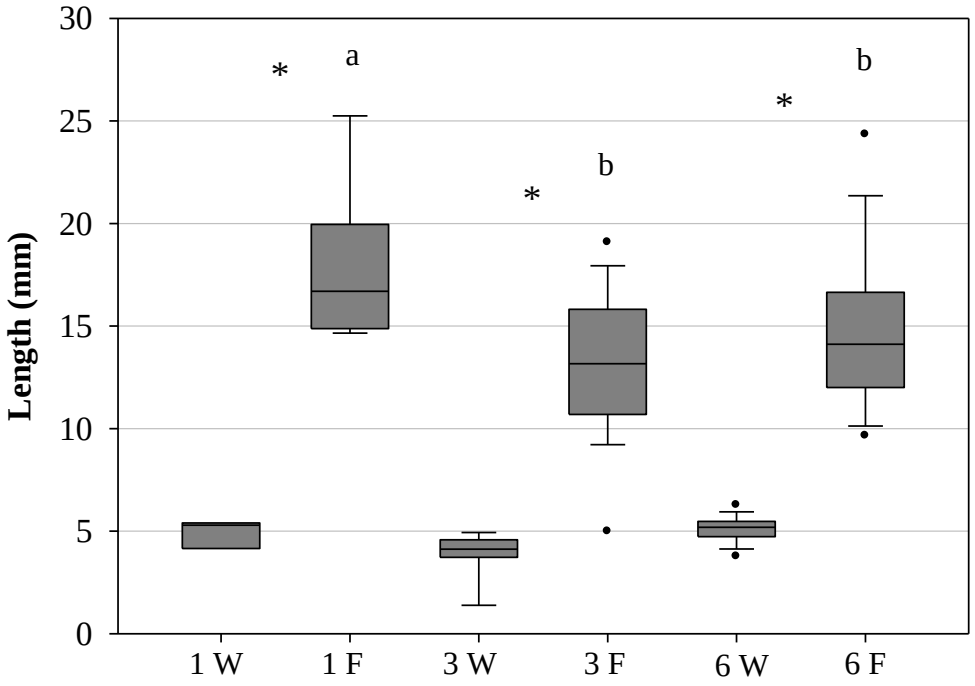

Figure 3.3. Box plot of lesion lengths of *Grosmannia clavigera* inoculated and wounded control juveniles from greenhouse experiment. Box indicates the 25th and 75th percentile, the line through the box is the median, the capped lines indicate the 10th and 90th percentile, and the dots are the 5th and 95th percentile. Letters indicate significant differences between *G. clavigera* inoculated juveniles in a Bonferroni corrected *post-hoc* test. F = *G. clavigera* inoculation; W = Wounded inoculation. * indicates that there is a significant difference (p<0.05) between lesion length of the wound and *G.clavigera* inoculation using a two-tailed Wilcoxon two-sample test.

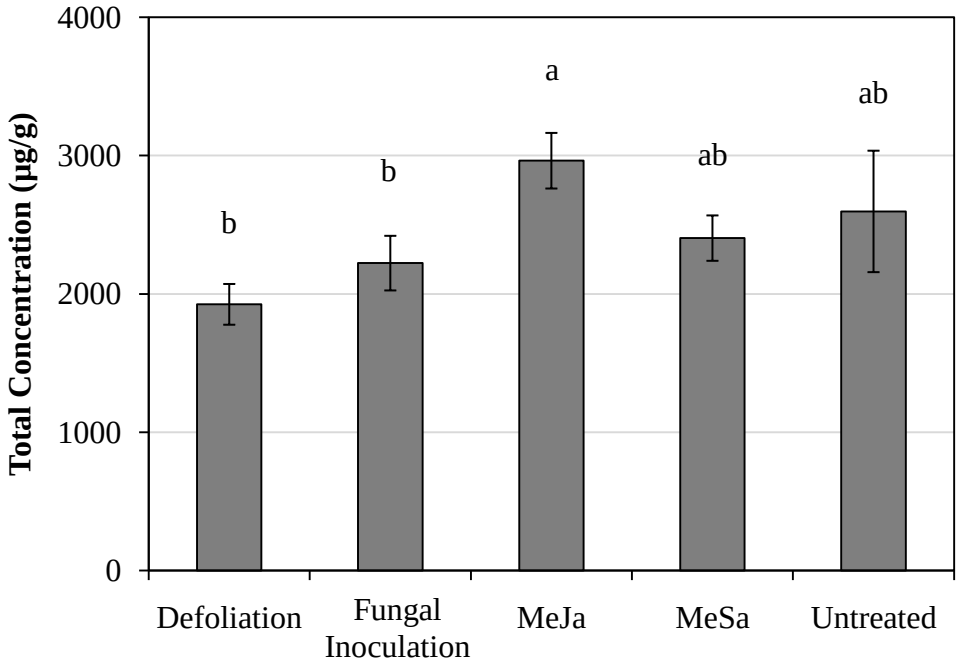

Figure 3.4. Total needle monoterpene concentrations of juveniles from the greenhouse experiment (mean ± standard error) ($F_{4,199}$= 4.53 and p=0.002). Letters indicate significant differences among induction types in a Bonferroni corrected *post-hoc* test. Defoliation = Mechanical defoliation; Fungal Inoculation = *Grosmannia clavigera* inoculation; MeJa = Methyl jasmonate; MeSa = Methyl salicylate; Untreated = Untreated control.

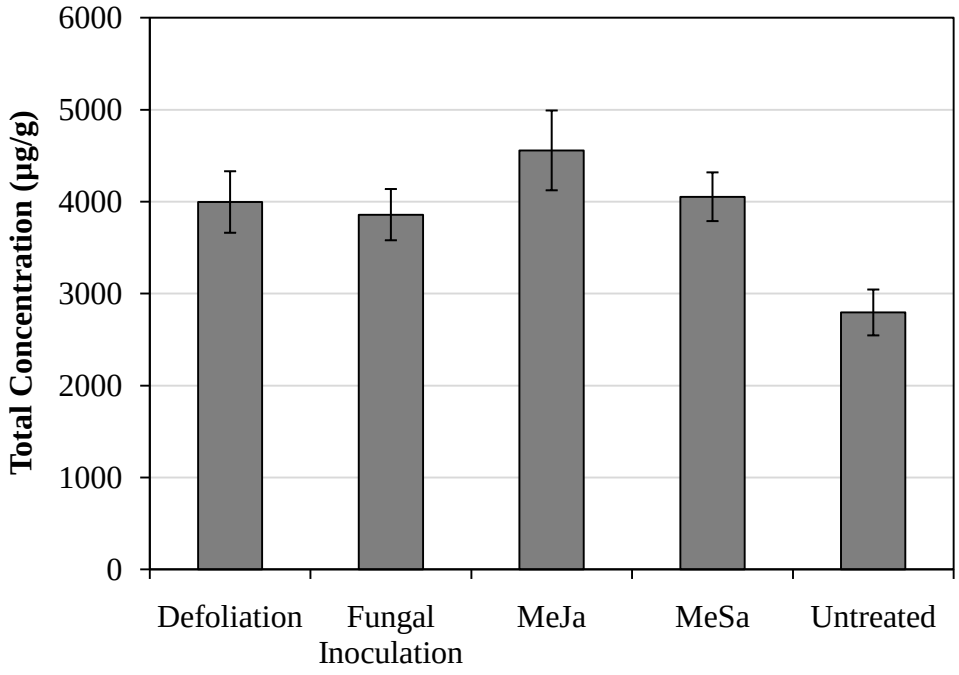

Figure 3.5. Total phloem monoterpene concentrations of juveniles from the greenhouse experiment (mean ± standard error) ($F_{4,119}$=1.16, p=0.333). Data were transformed (ln) for the analysis. Defoliation = Mechanical defoliation; Fungal Inoculation = *Grosmannia clavigera* inoculation; MeJa = Methyl jasmonate; MeSa = Methyl salicylate; Untreated = Untreated control.

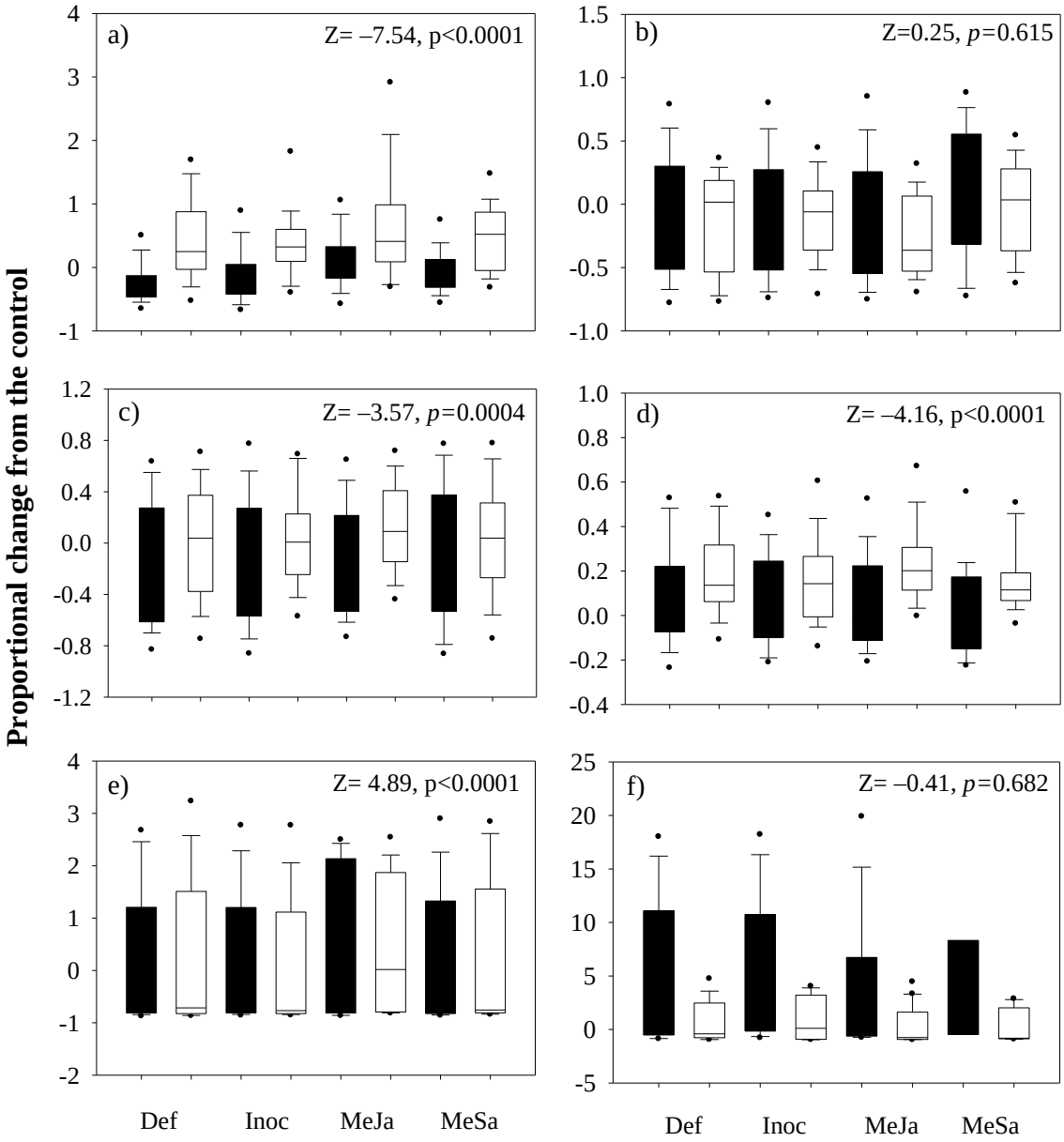

Figure 3.6. Box plots of change in individual monoterpenes relative to the concentration of the untreated control in needles (white boxes) and phloem (black boxes) of juveniles. a) Total monoterpenes, b) α-pinene, c) β-pinene, d) Myrcene, e) Limonene, f) 3-carene. Z and p value are results of Wilcoxon two-sample test with all inductions grouped together for juvenile vs. needle phloem. Def = mechanical defoliation; Inoc = *Grosmannia clavigera* inoculation; MeJa = Methyl jasmonate; MeSa = Methyl salicylate.

* indicates a significant difference in a Wilcoxon two-sample test between needles and phloem in the same induction type.

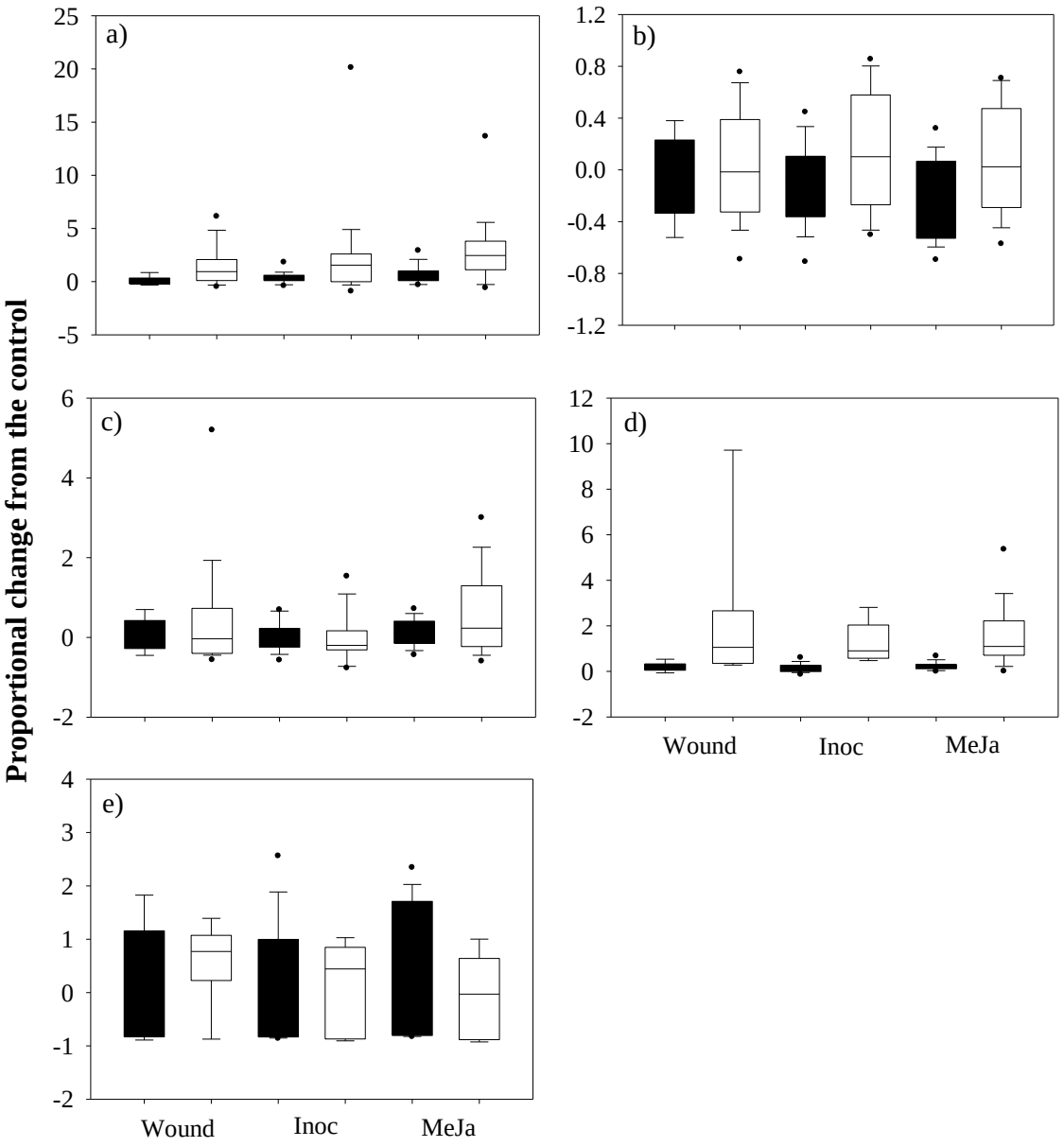

Figure 3.7. Box plots of the change in individual phloem monoterpenes relative to the concentration of the untreated control for juveniles (black boxes) and mature (white boxes) trees. a) Total monoterpenes, b) α-pinene, c) β-pinene, d) Myrcene, e) Limonene. Inoc = *Grosmannia clavigera* inoculation; MeJa = Methyl jasmonate; Wound = Wounded control.

IV. THE JACK PINE-MEDIATED INTERACTION BETWEEN THE JACK PINE BUDWORM AND A MOUNTAIN PINE BEETLE FUNGAL ASSOCIATE

4.1 Introduction

Coniferous trees have evolved both constitutive and inducible defence systems that deter or kill insects and inhibit or exclude pathogens physically and chemically (Johnson and Croteau 1987; Brignolas et al. 1995, 1998; Hudgins et al. 2003; Franceschi et al. 2005; Salle et al. 2005; Eyles et al. 2007, 2009). Recent fossil evidence suggests that these systems have been operating in the Pinaceae for at least 45 million years (Labandeira et al. 2001; Franceschi et al. 2005). Constitutive defences are general defences constantly maintained by a tree that are present at the time of attack to provide immediate resistance to an invasion (Franceschi et al. 2005). Examples of these include resin ducts, stone cells, and oleoresin, and a broad spectrum of secondary plant metabolites, such as terpenoids, alkaloids, and phenolics. Induced responses are triggered by the threat of imminent tissue damage and are deployed to increase tree defence (Franceschi et al. 2005). Upon damage, conifers initiate numerous anatomical changes, such as the formation of traumatic resin ducts and the activation of polyphenolic parenchyma cells, as well as quantitative and qualitative changes of defence compounds.

Induced responses may alter a host's suitability and increase its resistance or susceptibility to subsequent attacking organisms, respectively termed systemic induced resistance (SIR) and systemic induced susceptibility (SIS). As a result, interactions among attacking organisms are mediated by the host tree. There are many examples of changes in conifer resistance (Wright et al. 1984; Raffa et al. 1998; Wallin and Raffa 2001) and susceptibility (Annila et al. 1999; Christiansen and Krokene 1999; Krokene et al. 1999, 2000; Kosaka et al. 2001) after damage. Studies have documented that response induction depend on the organisms attacking and plant tissues involved (Karban 1990; Blodgett et al. 2007; Bonello 2010), as well as resource availability (Herms and Mattson 1992; Bonello 2010).

Climate change is allowing insect species to move beyond their historical ranges (Parmesan and Yohe 2003; Parmesan 2006). Range-expanding insect species interact with a new suite of organisms, including new host plants and their assemblages, which could influence their invasion success. The evolutionary history between trees and attacking organisms will influence the outcome of host-mediated interactions (Tollrain and Harvell 1999). Thus their interactions with the native organisms in a new ecosystem should be studied under the framework of plant-mediated interactions. In this chapter, I focus on invasion of the Canadian boreal forest by a native pest species, the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae, Scolytinae; MPB), and its potential relationship with a native herbivore, the jack pine budworm, *Choristoneura pinus pinus* Freeman (Lepidoptera: Tortricidae; JPBW), mediated by the host tree jack pine, *Pinus banksiana* Lamb., which is a new host for MPB. Both the MPB and JPBW are outbreak species that have periods of high or epidemic population levels followed by periods of low or endemic population levels.

The MPB is the most destructive pest of pine forests in western North America. Females lay eggs in the phloem tissue of host trees in late summer and after hatching larvae feed on the phloem. Larvae overwinter in the phloem and resume feeding in the spring as soon as temperatures warm enough. Larvae undergo four instars, pupate in early summer, and adults emerge and fly to new host trees. Once new host trees are colonized, they excavate galleries, mate, and lay eggs. Beetles use aggregation pheromones to attack *en masse* to exhaust a tree's defences and access phloem tissue. The host tree must die for successful reproduction. The MPB carries a variety of pathogenic fungi, including *Grosmannia clavigera*, *Ophiostoma montinum* and *Leptographium longiclavatum* that facilitate beetle establishment by weakening tree defences and disrupting transpiration, and provide a nutritional supplement for larvae and teneral adults (Paine et al. 1997; Six and Klepzig 2004; Bleiker and Six 2007).

Since the 1970s, fire suppression, forest management, and climate change have increased the habitat suitable for MPB in Canada by 75% (BCMFR 2006;

Carroll et al. 2006). Over the last decade, the MPB has greatly expanded its range into higher elevations and moved eastward from lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.) forests in British Columbia over the Rocky Mountains to invade lodgepole *x* jack pine hybrid forests in northwestern Alberta. It is expected to invade jack pine (*Pinus banksiana* Lamb.) forests in the boreal (Logan et al. 2003) where it has the potential to be invasive. In jack pine forests, MPB will interact with new organisms associated with the jack pine ecosystem that it has never before encountered.

Winter temperatures in Alberta may initially limit MPB populations to endemic levels (Régnière and Bentz 2007; CFS 2008). This could influence its spread because endemic beetle populations are constrained to attack trees with lowered defences, such as those weakened by other insects and pathogens (Safranyik and Carroll 2006). The movement of the MPB is of great concern as once it establishes in the boreal zone of northern Alberta it has the potential to expand across Canada and into more susceptible eastern pine forests of North America (Logan and Powell 2004).

A recent Canadian Forest Service (2008) publication identified knowledge gaps in MPB ecology and areas for future research. The role of jack pine defence and MPB's interactions with other species were listed as areas of uncertainty that require further study. The JPBW is an important defoliator in the boreal forest and outbreaks every six to twelve years depending on the region (McCullough 2000). Severe defoliation can result in growth reduction, top-kill, and tree mortality (Kulman et al. 1963). After overwintering as second instar larva in diapause, JPBW larvae feed on pollen before moving to new foliage in later instars.

The lifecycles of JPBW and MPB overlap with larvae of both species feeding on host trees in the spring and MPB colonization and JPBW oviposition occurring in mid to late summer. As a result, host trees colonized by JPBW or MPB may be colonized by the other species. These two insects could affect jack pine suitability for one another by the induction of responses in host trees: effects could be synergistic (SIS) or antagonistic (SIR). For example, jack pine trees stressed by partial JPBW defoliation could be vulnerable to colonization by

endemic populations of MPB. Outcomes of this potential interaction are uncertain because an outbreak defoliator is absent from lodgepole pine ecosystems and an outbreak bark beetle is absent from jack pine ecosystems. Since tree-mediated interactions depend on the organisms involved (Bonello 2010), studying the indirect interaction between JPBW and MPB could help to forecast the establishment and spread of endemic populations of MPB in jack pine forests.

I conducted a greenhouse experiment to test this relationship as extremely low populations of JPBW in Alberta made *in situ* studies impossible (discussed in Ch. I). Jack pine seedlings were either defoliated by JPBW (*induction*) and subsequently inoculated with *G. clavigera* (*challenge*), or inoculated with *G. clavigera* (*induction*) and subsequently defoliated by JPBW (*challenge*). Monoterpenes, carbon-based secondary compounds, were used to quantify jack pine responses to JPBW and *G. clavigera*. Monoterpenes were chosen as they are well studied in bark beetle-conifer systems (Raffa et al. 2005), are part of the inducible defence response of jack pines to defoliators (Wallin and Raffa 1999) and bark beetles in general (Franceschi et al. 2005), are important in herbivore host selection (Latta et al. 2000), and play a major role in inter- and intra-specific communication in conifer-bark beetle systems (Paine et al. 1997; Raffa et al. 2005, 2008). Budworm larvae were collected in northeastern Ontario by Dan Rowlinson and Taylor Scarr (Ontario Ministry of Natural Resources, ON, Canada) and couriered to the University of Alberta. Following previous studies (Krokene et al. 1999, 2000; Lieutier et al. 2009), *G. clavigera* inoculations were used to induce jack pine defences. This fungus was chosen for my experiments as it is well adapted to cold temperatures and is likely to become more prevalent in the fungal community of MPB in the boreal forest (Rice et al. 2007).

My objectives were to: a) examine if the growth of JPBW or *G. clavigera* is affected by prior induction with the other organism; b) compare monoterpene responses to the induction, challenge, and control treatments; c) explore the relationships among jack pine monoterpenes, JPBW growth, and *G. clavigera* lesions; and d) investigate if changes in jack pine monoterpenes can explain changes in seedling resistance or susceptibility to JPBW or *G. clavigera*.

4.2 Methods

4.2.1 Experiment Design and Data Collection

In March 2009, two-year-old jack pine seedlings were obtained from Boreal Horticultural Services Ltd. (Bonnyville, AB). Seedlings were planted in one gallon pots and grown in a greenhouse at the University of Alberta (Edmonton, AB). After one month, individuals with the healthiest appearance and of similar diameter (0.8 ± 0.001 cm) and height (43.8 ± 0.05 cm) were selected and randomly assigned to a treatment. Seedlings were watered three times a week and pots were rotated twice a week to exclude any variation in greenhouse conditions. Fertilization was stopped two weeks before the experiment began to exclude confounding effects of nutrient-defence interactions (Herms 2002; Zhao et al. 2007).

The experiment had two stages: an induction stage of defoliation by JPBW or inoculation with *G. clavigera*, followed by a challenge stage with the other organism. There were two levels of each induction followed by one level of challenge, the corresponding induction controls, i.e. defoliation controls for defoliation-inoculation seedlings and inoculation controls for inoculation-defoliation seedlings. Wounded controls and an untreated control were also included for a total of eleven treatments (Table 4.1). The inoculation and defoliation stages were staggered so that JPBW would feed simultaneously in all treatments. The experiment was originally designed with equal time periods for the fungal and defoliation stages; however, inclement weather delayed larval collection until after seedlings had received their inoculation induction. As a result, the fungal stages of the experiment were lengthened from four to nine weeks.

The defoliated-inoculated seedlings received either two or four fifth instar larvae in the induction stage that fed until pupation (~ three weeks). One week after JPBW pupation, four weeks from the start of defoliation, seedlings were inoculated with three plugs of *G. clavigera* and harvested nine weeks later. Induction controls for defoliation had two or four larvae feed but were not inoculated in the challenge stage. The inoculated-defoliated seedlings were

inoculated with three or six plugs of *G. clavigera* in the induction stage. After nine weeks, two fifth instar larvae were placed on seedlings and allowed to feed until pupation. These seedlings were harvested four weeks after defoliation began. Induction controls for inoculation were inoculated with three or six plugs of *G. clavigera* but did not received JPBW larvae. To test the effect of fungus compared to the physical damage of inoculation, a wounded control with three or six mechanical wounds 'inoculated' with sterile agar was included. These seedlings did not have JPBW larvae feed during the challenge stage. An untreated control was also included in the experiment.

Second and third instar larvae were reared on a modified McMorran diet (CFS Insect Production Services, Sault Ste. Marie, ON). When enough fifth instar larvae were available they were placed on the seedlings in screen bags that were secured around the crown. Seedlings that did not have larvae feeding were also bagged to control for confounding effects. I observed that larvae ceased feeding in direct sunlight and tried to escape from the bags. Consequently, during the defoliation stage all seedlings were kept under 40 % shade cloth. Pupae were collected every other day, weighed to the nearest 0.001 g, and left individually in containers at room temperature (~ 23^{o}C) to emerge. Adults that emerged were weighed within 18 hrs. After they were killed in a freezer, forewings were removed, scanned, and area was measured using ImageJ 1.43 (Rasband 2009). JPBW feeding was quantified by counting the number of needle bundles damaged in the crown.

A 3 mm cork borer was used to remove the outer bark and phloem for inoculations and an agar plug with *G. clavigera* mycelia was placed in the hole. For wounded controls, sterile agar was placed in the hole. The inoculum was secured with parafilm, which also minimized the possibility of contamination. Inoculation points were spread evenly over the stem below the crown and adjacent holes were placed 180^{o} from one another. Tree response to *G. clavigera* and wounding was quantified by measuring lesion length, which is commonly used as an indicator of induced response to pathogens (Bonello and Blodgett 2003; Krokene et al. 2008). Better defended trees generally have smaller lesions.

The bark was removed around each inoculation point using a scalpel and the length of the lesion was measured using callipers. A xylem sample from each seedling was taken from inside a lesion to reculture fungus.

Needles were sampled for monoterpene analysis before the induction stage (T_0), after the induction but before the challenge stage (T_1), and after the challenge stage (T_2) for all seedlings, including untreated controls. Sample collection was staggered according to when the induction treatment was applied. Since monoterpenes can be highly variable (Latta et al. 2000), undamaged, current year growth was taken from different branches in the crown to obtain a bulk sample. When seedlings were harvested, stem sections were stored and phloem was later sampled 1 cm above the *G. clavigera*/wound lesions and on the stem below the crown in seedlings that were not inoculated. All samples were stored on dry ice after collection until they were transferred to a – 40^{o}C freezer and stored until chemical analysis.

4.2.2 Chemical Analysis

Monoterpenes were extracted from both needle and phloem tissue following the same method. Samples were ground in liquid nitrogen using a mortar and pestle and monoterpenes were extracted twice using a method modified from Wallis et al. (2008). In a 1.5 mL microcentrifuge tube, 500 µL of dichloromethane (CH_2Cl_2) with 0.1 % tridecane was added to 100 mg of ground tissue. Tridecane was used as an internal standard as it is not present in jack pine tissue. Samples were vortexed for 30 sec, placed in an ultrasonic bath for 10 min, and then centrifuged at 0^{o}C and 13.2 rpm for 15 min. Extract was pipetted into vials and the tissue was extracted for a second time as above. The first and second extracts were combined and stored at – 40^{o}C until analysis using gas chromatography.

Samples (1 µL) were injected in an Agilent 7890A Gas Chromatograph (Agilent Technologies, Palo Alto, CA, USA) with an HP Innowax column (I.D. 0.32 mm, length 30 m) with a helium carrier gas flow of 1.8 mL/min. The temperature program began at 50°C for 2 min, which was increased to 160°C by

5°C/min and then to 250°C by 20°C/min. Peaks were identified using the following standards: Borneol, pulegone, α-terpinene, γ-terpinene, α-terpineol (Sigma-Aldrich, St. Louis, MO, USA), camphor, 3-carene, α-humulene, terpinolene, α-thujone and β-thujone, (–)-α-pinene, (–)-β-pinene, (S)-(–)-limonene, sabinene hydrate, myrcene, (–)-camphene, p-cymene (Fluka, Sigma-Aldrich, Buchs, Switzerland), α-phellandrene, bornyl acetate, ocimene (SAFC Supply Solutions, St. Louis, MO, USA), β-phellandrene (Glidco Inc., Jacksonville, FL, USA), and by comparison of the retention indices (Table 4.2). Sample peaks were integrated and peak area was compared for qualitative and quantitative differences between samples of all seedlings at different time points.

4.2.3 Statistical Analyses

This experiment was designed to compare the responses of challenge treated seedlings to their induction controls, and the differences between the induction-challenge types. The statistical program SAS v 9.1 (SAS Institute, NC, USA) was used for all analyses. Pre-planned pooled comparisons were made between: defoliated control (2L Con + 4L Con) and defoliated-inoculated (2L 3F + 4L 3F) seedlings; inoculated control (3F Con + 6F Con) and inoculated-defoliated (3F 2L + 6F 2L) seedlings; defoliated-inoculated and inoculated-defoliated seedlings; defoliated control and inoculated control seedlings; and inoculated control and wounded control (3W Con + 6W Con) seedlings for all variables where applicable.

JPBW variables pupal mass, adult mass, and wing area were correlated and pupal mass was chosen for the analyses. Proc GLM was used to examine differences in pupal mass among inoculated-defoliated, defoliated-inoculated, and defoliated control seedlings for all pupae and by sex. The feeding damage rate of JPBW was calculated by the number of needle bundles damaged divided by the number of larvae initially put on the seedling divided by the total number of feeding days. This standardized seedlings for differences in crown size, larval mortality, and number of larval feeding days, and created a rate of bundles damaged per larva per feeding day. For missing larvae or those whose date of

death was unknown, I assumed larvae fed for two weeks to underestimate budworm feeding damage on a seedling. Feeding damage rates were transformed (ln) for pooled comparisons, which were conducted using contrast statements. Since sex was not significant in the analysis of pupal mass, it was not tested in the analysis of feeding damage rate.

Lesion lengths could not be easily transformed to meet ANOVA assumptions and instead Proc Npar1way was used to conduct non-parametric Wilcoxon two-sample tests for pooled comparisons listed above.

Total monoterpene concentrations of needles over the experiment (T_0, T_1, T_2) were examined by a repeated measures ANOVA using Proc Mixed with a compound symmetric covariance structure. An lsmeans statement sliced by treatment was used to examine the changes within each treatment. When examined by time period, the initial concentrations of total monoterpenes significantly differed before induction treatments were applied (T_0) (see Results section below). As a result, changes (Δ) in monoterpene concentrations were examined using Proc GLM over the induction stage ($\Delta T_1=[T_1-T_0]/T_0$) and challenge stage ($\Delta T_2=[T_2-T_1]/T_1$). These values were ln(1+x) transformed and pooled comparisons (listed above) were made between challenge treated trees and their induction control for ΔT_1 and ΔT_2.

The change in the concentrations of α-pinene, β-pinene, 3-carene, limonene, and myrcene over the experiment were also examined. These monoterpenes composed over 80% of average total monoterpene concentration and were also measured in a study of JPBW and mature jack pine by Wallin and Raffa (1999). Proc Mixed was used to perform repeated measures ANOVAs to examine the change in the concentration of these monoterpenes within each treatment over the experiment (T_0, T_1, T_2). Similar to the analysis of total monoterpene concentrations, Proc GLM was used to examine the change in individual compounds over the induction (ΔT_1) and challenge stage (ΔT_2). Contrast statements were used to examine the pooled comparisons listed above.

Phloem monoterpene concentrations were transformed (ln) and Proc GLM was used to conduct an ANOVA among treatments and contrast statements were

used to examine pooled comparisons listed above. Since phloem was only sampled after the challenge stage, comparisons of each induction type (e.g. defoliated control, defoliated-inoculated etc.) to the untreated control were also included to observe differences in total monoterpene concentrations.

Canonical correlation analysis (CCA) was used to explore the relationship between the biological and chemical datasets. CCA is a multivariate extension of correlation analysis and a type of gradient analysis used to study the relationship between multivariate explanatory and response datasets, though dependency can be interchangeable. It assumes the normal distributions with no outliers. A variate is constructed from each dataset, using a linear combination of variables, and the correlation between them is maximized. The strength of the relationship between the variates is quantified by the canonical correlation coefficient. Each canonical function, composed of an explanatory and response variate, is orthogonal to the next. The standardized partial correlation of each variable with their canonical variate is a canonical score and can be interpreted in a similar manner to a regression coefficient.

Proc Cancorr was used to produce canonical correlations, with the biological variate consisting of mean pupal mass, mean lesion length, and the natural logarithm of feeding damage rate, and the chemical variate consisting of the change in the concentration of α-pinene, 3-carene, and myrcene over the experiment ($\Delta T=[T_2-T_0]/T_0$). I chose to examine these compounds as they were highly correlated with other important monoterpenes and were present in most samples as CCA omits observations with missing data.

4.3 Results

4.3.1 Jack Pine Budworm

Pupal mass was used in analyses because it was highly correlated with adult mass (r=0.93, $p<0.001$) and wing area (r=0.65, $p<0.001$) and had the highest number of observations. Although some larvae went missing (11 %) or died before pupation (19 %), these were not disproportionally related to treatment (F=0.88, df=5, $p=0.501$ and F=1.36, df=5, $p=0.252$, respectively). Overall, pupal

mass did not vary among treatments with a mean mass (± standard error) of 0.04 ± 0.001 g (F=0.97, df=5, *p*=0.443). Likewise, it did not differ among defoliated-inoculated, inoculated-defoliated, and defoliated control seedlings (F=1.84, df=2 *p*=0.167). When pupae were divided by sex, mass did not differ by treatment for females (F=0.76, df=5, *p*=0.567) nor males (F=0.97, df=5, *p*=0.443). Similarly, when mass was grouped by defoliated-inoculated, inoculated-defoliated, and defoliated control, it did not differ among treatments for female (F=1.15, df=2, *p*=0.330) nor males (F=1.84, df=2, *p*=0.168).

In contrast, average crown defoliation varied with induction type. Crown defoliation was 22.5 ± 2.84 % in defoliated-inoculated, 17.4 ± 1.38 % in inoculated-defoliated, and 16.8 ± 1.56 % in defoliated control seedlings and was standardized by calculating the feeding damage rate. Feeding damage rate significantly differed with treatment (F=16.65, df=5, *p*<0.0001; Fig. 4.1). Larvae damaged significantly more bundles while feeding on inoculated-defoliated seedlings (1.2 ± 0.09 bundles/larva/day) than the defoliated-inoculated (0.6 ± 0.07 bundles/larva/day) and defoliated control (0.7 ± 0.09 bundles/larva/day) seedlings (F=50.18, df=1, *p*<0.0001). There was no difference in damage rate between larvae feeding on defoliated-inoculated and defoliated control seedlings (F=0.35, df=1, *p*=0.558).

4.3.2 Grosmannia clavigera *Lesion Lengths*

Grosmannia clavigera could not be recultured from xylem samples, which is likely because samples were very small and microbes were killed during flame sterilization. As expected, the inoculated control lesions were significantly longer (12.4 ± 1.10 mm) than wounded control lesions (4.9 ± 0.17 mm) (Z=3.97, *p*<0.0001). Lesion length did not differ between three and six inoculations in inoculated-defoliated seedlings (Z=-0.34, *p*=0.735). However, the inoculated-defoliated (11.1 ± 0.67 mm) (Z=4.01, *p*<0.0001) and the inoculated control (Z=3.71, *p*=0.0002) seedlings had significantly longer lesions than the defoliated-inoculated seedlings (7.9 ± 0.18 mm) (Fig. 4.2). Further, lesion lengths were

significantly longer in the inoculated control than the inoculated-defoliated seedlings (Z=1.07, p=0.286).

4.3.3 Jack Pine Monoterpenes

In the repeated measures ANOVA, treatment ($F_{10,88.9}$=2.15, p=0.028), time ($F_{2,178}$=36.28, p<0.0001), and their interaction ($F_{20,178}$=20.35, p<0.0001) were significant in total needle monoterpene concentrations over the experiment (Fig. 4.3). When sliced by treatment, needle monoterpene concentration in all treatments, except the untreated control, change significantly over the experiment (Table 4.3). Since initial monoterpene concentrations in needles differed among treatments ($F_{10,150}$=4.42, p<0.0001), I analyzed the change in the concentration of needle monoterpenes over the induction (ΔT_1) and challenge (ΔT_2) stages.

For induction by JPBW defoliation, over ΔT_1 the total monoterpene concentrations of defoliated-inoculated (2L 3F and 4L 3F) (–2.9 ± 3.62 %) and defoliated control (2L Con and 4L Con) (4.5 ± 2.94 %) seedlings were not different (F=0.64, df=1, p=0.427) nor did they differ from the untreated control (F=0.41, df=1, p=0.521 and F=1.97, df=1, p=0.164, respectively) (Fig. 4.3a). In contrast, over ΔT_2 monoterpene concentrations decreased in defoliated-inoculated seedlings (–17.1 ± 3.61 %) but greatly increased in the defoliated controls (111.6 ± 9.28 %) (F=297.90, df=1, p<0.0001).

For induction by *G. clavigera* inoculation, over ΔT_1 the inoculated-defoliated (3F 2L and 6F 2L) (86.7 ± 36.13 %) and the inoculated control (3F Con and 6F Con) (94.2 ± 19.10 %) seedlings increased total monoterpene concentrations in comparison to the untreated control (F=10.76, df=1, p=0.002 and F= 21.58, df=1, p<0.0001, respectively) and did not differ from one another (F=2.10, df=1, p=0.540) (Fig. 4.3b). Whereas, over ΔT_2 monoterpene concentrations differed (F=63.33, df=1, p<0.0001), continuing to increase in inoculated-defoliated seedlings (54.0 ± 4.11 %) but remaining relatively constant in the inoculated controls (–8.8 ± 3.95 %). The inoculated controls did not differ from the untreated control in ΔT_2 (F=0.61, df=1, p=0.437).

When monoterpene concentrations were compared between inoculated-defoliated and defoliated-inoculated seedlings at the end of study, I detected a surprising outcome with defoliated-inoculated seedlings having lower concentrations (1374.0 ± 124.88 μg/g) than the inoculated-defoliated seedlings (2359.9 ± 155.39 μg/g) (Fig. 4.3c). These differences were apparent in ΔT_1 and ΔT_2. In ΔT_1, total monoterpene concentrations remained constant in the defoliated-inoculated seedlings but increased in inoculated-defoliated seedlings (F=22.87, df=1, p<0.0001). In ΔT_2, concentrations continued to increase in the inoculated-defoliated seedlings but decreased in the defoliated-inoculated seedlings (F=134.72, df=1, p<0.0001).

Likewise, monoterpene responses of seedlings varied between defoliated-control and inoculated-control treatments. Monoterpene concentrations increased in both the defoliated and inoculated controls over the study, but in different stages. In ΔT_1, concentrations remained relatively constant in the defoliated control but increased in the inoculated control (F=22.65, df=1 p<0.0001). In ΔT_2, concentrations greatly increased in the defoliated controls but slightly decreased in the inoculated controls (F=158.54, df=1, p<0.0001). At the end of the experiment, the concentrations of monoterpenes in the defoliated controls were higher than the inoculated controls.

Monoterpene response in seedlings differed between *G. clavigera* inoculated and wounded controls. In ΔT_1, concentrations increased more in inoculated (94.2 ± 19.10 %) than wounded control seedlings (42.1 ± 15.11 %) (F=5.71, df=1, p=0.019). Wounded controls also differed from the untreated control (F=7.26, df=1, p=0.008). In ΔT_2, concentrations were also different as wounded controls decreased (–47.4 ± 2.50 %) but the inoculated controls did not decrease significantly (–8.8 ± 3.95 %) (F=53.23, df=1, p<0.0001). Again, wounded controls differed from the untreated controls (F=45.41 df=1, p<0.0001).

Although phloem was sampled only after the challenge stage, total monoterpene concentrations differed among the induction types (F=3.42, df=10, p=0.0008) (Fig. 4.4). Total monoterpenes in the defoliated-inoculated seedlings (5027.9 ± 331.3 μg/g) were higher than inoculated-defoliated seedlings (4034.8 ±

322.4 µg/g) (F=4.50, df=1 p=0.037). Similarly, the defoliated controls (5524 ± 418.1 µg/g) had a higher concentrations than the inoculated controls (3293.6 ± 322.3 µg/g) (F=15.33, df=1, p=0.0002). The defoliated-inoculated seedlings had similar phloem monoterpene concentrations to the defoliated controls (F=0.62, df=1, p=0.435) as did the inoculated-defoliated seedlings to the inoculated controls (F=2.36, df=1, p=0.128). Since phloem was sampled once, comparisons to the untreated control (5689.19 ± 502.90 µg/g) were included. Defoliated-inoculated seedlings had a similar monoterpene concentrations (F=0.90, df=1, p=0.346), whereas the inoculated-defoliated seedlings had significantly lower concentrations (F=5.26, df=1, p=0.024) than the untreated control. The concentrations of the inoculated controls did not differ from the wounded controls (F=0.01, df=1, p=0.912) and the wounded controls (3293.6 ± 322.26 µg/g) were also lower than the untreated controls (F=10.80, df=1, p=0.002).

The concentrations of α-pinene, β-pinene, 3-carene, myrcene, and limonene in needles were examined over T_0, T_1, and T_2 within a treatment (Table 4.4) and their changes over the experiment (ΔT) are presented in Figure 4.5. The general trend is a decrease in monoterpene proportions for defoliated-inoculated seedlings and large increases in inoculated-defoliated, especially for α-pinene, β-pinene and limonene. In ΔT_1, the change in concentration of α-pinene and β-pinene differed significantly in most of the pooled comparisons (Table 4.5a). In ΔT_2, almost all compounds differed significantly in the pooled comparisons (Table 4.5b). The monoterpene proportions for every compound tested for phloem and needle tissue are present in Appendix B.

4.3.4 Relationship among Jack Pine Monoterpenes, Grosmannia clavigera*, and Jack Pine Budworm*

Canonical correlation analysis was used to explore the relationship between the proportional change (ΔT) in the concentration of major monoterpenes from T_0 to T_2 and the biological data that were observed. The changes in α-pinene, β-pinene, limonene, myrcene, and 3-carene over the experiment were correlated (Table 4.6). Myrcene, α-pinene, and 3-carene were chosen for the chemical

variate as they were present in almost all trees and data were sufficient to produce a good model. Mean pupal mass, mean lesion length, and the natural logarithm of feeding damage rate were included as the biological variables.

The correlation for the most significant canonical function was 0.640 (p=0.0005). The results of the canonical correlation analysis are listed in Table 4.7 and the canonical scores for the significant canonical functions are listed in Table 4.8. When the two significant canonical functions were combined, the chemical variates explained 69.7 % of the variability in the chemical variables and 25.9 % of the variability in the biological variables. The two significant biological variates explained 63.2 % and 22.6 % of the variability in the biological and chemical variables, respectively. When the first canonical function was graphed, seedlings grouped according to their induction-challenge treatments, although there appeared to be more variance in the inoculated-defoliated seedlings (Fig. 4.6). Examining the canonical scores revealed that defoliated-inoculated seedlings generally had smaller lesions, lower feeding damage rates, and larger proportional change in concentration of α-pinene and 3-carene. In contrast, inoculated-defoliated seedlings had longer lesions, a higher feeding damage rate, and larger proportional change in the concentration of myrcene.

4.4 Discussion

JPBW defoliation induced resistance in jack pine to *G. clavigera*, demonstrated by shorter lesions on defoliated-inoculated trees. A similar pattern was observed in Austrian pine (*Pinus nigra* Arnold) where prior defoliation by the European sawfly (*Neodiprion sertifer* Geoffory) resulted in smaller lesions *Sphaeropsis sapinea* (Fr.:Fr.) Dyko and Sutton (Eyles et al. 2007). Likewise, Scots pine was more resistant to the beetle *Tomicus piniperda* (L.) after defoliation by the sawfly *Diprion pini* (L.), even when only 10% foliage remained (Annila et al. 1999). In contrast, Wallin and Raffa (2001) observed that when jack pine was ≥ 26 % defoliated by JPBW, trees were more susceptible to colonization by the pine engraver (*I. pini*) and a cerambycid beetle *Monochamus carolinensis* (Olivier). This discrepancy could be because my experiment had lower levels of

defoliation or because of differences in ontogeny (Karban and Niiho 1995), phenology (Wallin and Raffa 1999), or the potential for different intensities of damage and biological agents to induce varying levels of defence (Karban and Baldwin 1997). Additionally, *G. clavigera* inoculations were used to mimic MPB colonization and the results may have been different if live beetles were used. Since the monoterpene concentrations in the wounded controls differed from the *G. clavigera* inoculated controls, it is possible that the response to real beetles in mature trees could be different from the responses observed in this study.

In contrast, induction by *G. clavigera* inoculation did not influence the growth of JPBW in the challenge; however, larvae fed more relative to defoliated control and this suggests possible compensatory feeding by JPBW. Host quality could have decreased as monoterpenes increased by 86.7 ± 36.13 % during the inoculation stage and 54.0 ± 4.11 % during defoliation stage and larvae increased their feeding by 71 % compared to the defoliated control. Compensatory feeding has been observed in many species in response to low nitrogen levels (Berner et al. 2005), including the JPBW (McCullough and Kulman 1991), and high levels of allelochemicals (Barbehenn et al. 2009). The current study provides evidence that supports JPBW larvae increasing feeding rates on jack pines with prior *G. clavigera* inoculation, although whether this change in host quality is due to a reduction in nutrients and/or an increase in plant secondary metabolites needs to be more thoroughly investigated.

Differences in jack pine's responses to JPBW and *G. clavigera* could be due to their evolutionary histories (Tollrain and Harvell 1999): jack pine has evolved with JPBW but not with MPB and its fungal associates. Alternatively, the results may reflect technical constraints as fungal inoculations on the stem do not inflict the same type of damage as budworm larvae feeding on needles. Nevertheless, the chemical responses of the induction controls (defoliated or inoculated) and induction-challenge (defoliated-inoculated or inoculated-defoliated) seedlings in this study reveal three important conclusions:

First, trees solely inoculated with *G. clavigera* or defoliated by JPBW increased the monoterpene concentration of their needles. This confirms the

results of earlier research of monoterpene response induction in the jack pine (Raffa and Smalley 1995; Wallin and Raffa 1999) as well as widely reported results in conifers (see review by Eyles et al. 2009).

Second, the monoterpene responses differed in magnitude and direction depending on the order of JPBW and *G. clavigera* in the induction and challenge stages. Trees that were initially inoculated and then defoliated increased their monoterpene concentrations by 129.0 ± 15.03%, whereas trees that were initially defoliated then inoculated decreased their monoterpene concentrations by 34.4 ± 6.89 %. The canonical correlation analysis demonstrated that the biological characteristics of the organisms and the chemical changes in the seedlings separate according to whether they were inoculated then defoliated or defoliated then inoculated. These results suggest that the order of attack can be important between species interactions because the first organism can change host plant quality and affect the relationship of the host with subsequent organisms (Hatcher et al. 2004). There are many examples of change in conifer resistance after damage (Wright et al. 1984; Raffa et al. 1998; Annila et al. 1999; Christiansen and Krokene 1999; Krokene et al. 1999, 2000; Kosaka et al. 2001; Wallin and Raffa 2001).

Third, jack pine trees inoculated with *G. clavigera* showed an increase in needle monoterpene concentration nine weeks after inoculation, whereas trees defoliated by JPBW showed an increase thirteen weeks after defoliation. This observed lag in the monoterpene increase in defoliated trees demonstrates that induction occurred over a longer time scale than anticipated. Heavily defoliated trees can have a slower or less evident induction than lightly or non-defoliated trees (Wallin and Raffa 1999) and response can vary within tissues and temporally (Wallin and Raffa 1998). Consequently, experimental design is crucial in characterizing monoterpene responses. The experiment may have been too short to observe total response induction and sampling may not have been frequent enough to fully describe monoterpene response. This is further complicated by differences in responses to the level of damage by an induction and by the organ of induction (Eyles et al. 2007, 2009). For example, Austrian

pines exhibited resistance when inoculated with *S. sapinea* or *Diplodia scrobiculata* de Wet, Slippers and Wingfield at the base and then challenge inoculated with the pathogen *S. sapinea* in the upper stem, as well as when induced in the upper stem and challenged at the base (Blodgett et al. 2007). However, when inoculated at the base and then challenged in the shoots, trees were more susceptible to *S. sapinea*. I cannot determine how much response induction was achieved relative to a tree's tolerance of damage and their threshold of SIR and SIS (Bonello et al. 2006).

Furthermore, inoculated trees had a lower concentration of phloem monoterpenes than the untreated or defoliated control trees. Considering plant responses at the tree level is important as chemical responses in one tissue can produce correlated but not predicable changes in others (Latta et al. 2000). Wallin and Raffa (1999) found a greater increase in phloem rather than foliar monoterpenes after JPBW defoliation. Jack pine phloem monoterpenes can rapidly increase after inoculation (Raffa and Smalley 1995), measurable only three days after induction (Wallin and Raffa 1999), and level off after an initial peak (Raffa and Smalley 1995). My ability to draw conclusions about phloem response is limited because it was sampled once at the end of the challenge stage. Additional studies are required to allow frequent sampling in order to gain a finer resolution of how defence responses change at the tree level over time.

Although needle monoterpene concentrations increased after induction by *G. clavigera* inoculation and continued to increase during the defoliation challenge, trees were not better defended against JPBW. Larvae increased their feeding rate and their pupal mass suggests they would be as fecund as those in other treatments. Increasing the quantity of allelochemicals does not necessarily better protect a plant but rather some compounds are more effective in defence (Berenbaum 1995). Individual monoterpenes differed between the induction and induction-challenge types over the induction and challenge stages. There was a larger change in the concentration of myrcene in seedlings that were inoculated then defoliated, as shown in the CCA. This may have affected JPBW feeding as JPBW prefers tissues with low myrcene content (Wallin and Raffa 1998). Jack

pines that were defoliated then inoculated had a larger change in α-pinene and smaller lesions than seedlings that were inoculated then defoliated. The change in the concentration α-pinene was highly correlated with that of β-pinene and Wallis et al. (2008) found lesions of *Diplodia pinea* in Austrian pine were marginally ($P<0.10$) negatively correlated with β-pinene. *Grosmannia clavigera* growth may also be affected by individual monoterpenes. Although monoterpenes were the focus of this paper, host suitability for herbivores and pathogens can also be affected by other defensive compounds, such as phenolics (Wallis et al. 2008) and alkaloids (Franceschi et al. 1998, 2005; Mumm and Hilker 2006).

Climate change is likely to impact inter-specific interactions as it is facilitating species movement beyond their historical ranges (Parmesan and Yohe 2003; Parmesan 2006) and is affecting insect and pathogen population dynamics and host tree characteristics (Ayres and Lombardero 2000). This study explored the novel interaction among JPBW, *G. clavigera*, and jack pine and I found that JPBW defoliation increased jack pine resistance to *G. clavigera*, whereas *G. clavigera* inoculation had no adverse effects on JPBW. The timing and sequence in which these organisms attack jack pine in the boreal will be important in determining their success and depending on the nature of their interaction (e.g. synergistic or antagonistic) they could have very different effects on the ecosystem (Chapter II). Additional field experiments are necessary to verify my findings as predicting defence is difficult because many variables affect each individual host's response (Bonello 2010). Range-expanding insect species in new ecosystems, like MPB, will likely impact the dynamics of new ecosystems and a thorough understanding of plant-induced responses is required to forecast and manage their impacts.

4.5 References

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Table 4.1. The two stage design of the experiment: an induction stage with one organism and a challenge stage with the other. Seedlings in control groups were not treated in the challenge stage but were left to grow for that period. Con = Control; F = *Grosmannia clavigera* inoculations; L = Jack pine budworm larvae; W = Inoculation wound.

	Defoliated-Inoculated		Defoliated Control		Inoculated-Defoliated		Inoculated Control		Wounded Control		Untreated Control
Induction Stage	2L	4L	2L	4L	3F	6F	3F	6F	3W	6W	Untreated
Challenge Stage	3F	3F	Con	Con	2L	2L	Con	Con	Con	Con	Con
n	12	12	12	11	12	11	6	6	6	6	6

Table 4.2. Retention times for gas chromatograph.

Compound	Retention Time (min)
α-pinene	4.339
Camphene	4.996
β-pinene	5.724
3-carene	6.616
α-phellandrene	6.902
Myrcene	6.926
α-terpinene	7.214
Limonene	7.662
β-phellandrene	7.846
Ocimene	8.511
γ-terpinene	8.741
p-cymene	9.341
Terpinolene	9.631
Tridecane	10.046
α-thujone	12.967
β-thujone	13.416
Sabinene hydrate	14.008
Camphor	14.967
Linalool	16.049
Bornyl acetate	16.735
Pulgeone	18.203
α-humulene	18.785
α-terpineol	19.389
Borneol	19.454

Table 4.3. Results of repeated measure ANOVAs of total needle monoterpene concentrations sliced by treatment before the induction (T_0), after the induction (T_1), and after the challenge (T_2) stages. Con = Control; F = *Grosmannia clavigera* inoculations; L = Jack pine budworm larvae; W = Inoculation wound.

Treatment	NDF	DDF	F Value	*p*
2L 3F	2	178	4.10	*0.016*
4L 3F	2	178	5.13	*0.006*
3F 2L	2	178	40.75	*<0.0001*
6F 4L	2	178	50.04	*<0.0001*
2L Con	2	178	42.62	*<0.0001*
4L Con	2	178	47.4	*<0.0001*
3F Con	2	181	4.26	*0.016*
6F Con	2	178	28.76	*<0.0001*
3W Con	2	178	14.85	*<0.0001*
6W Con	2	178	16.63	*<0.0001*
Untreated	2	178	1.73	*0.171*

Table 4.4. The change in the concentrations of α-pinene, β-pinene, 3-carene, myrcene, and limonene in jack pine needles from before induction (T_0) to after the challenge stage (T_2) ($\Delta T_2 = [T_2 - T_0]/T_0$). Values are means and with standard errors below in smaller font. Con = Control; F = *Grosmannia clavigera* inoculations; L = Jack pine budworm larvae; W = Inoculation wound.

Treatment	α-pinene	β-pinene	3-carene	Myrcene	Limonene
2L 3F	-0.170* 0.06	-0.073 0.06	-0.217* 0.09	-0.097* 0.05	0.079 0.23
4L 3F	-0.186 0.05	-0.211 0.04	-0.198 0.07	-0.270* 0.03	-0.084 0.04
3F 2L	0.943* 0.30	1.380* 0.27	1.106* 0.90	0.657* 0.12	2.195* 0.67
6F 2L	1.683* 0.93	3.390* 1.86	0.492 0.18	1.017* 0.36	2.277 0.71
2L Con	1.165* 0.18	1.082* 0.13	0.729 0.19	1.048* 0.157	1.294* 0.10
4L Con	1.414* 0.167	1.491* 0.23	0.571 0.22	0.959* 0.07	1.482 0.18
3F Con	0.304 0.28	0.453 0.23	-0.133 0.24	0.021 0.10	0.337 0.17
6F Con	1.215* 0.21	1.707* 0.27	0.038 0.26	0.341 0.13	1.725 0.54
3W Con	-0.407 0.11	-0.290 0.15	-0.761 0.05	-0.448* 0.04	0.275* 0.10
6W Con	-0.258* 0.08	0.166* 0.12	-0.276* 0.37	-0.317* 0.05	-0.009 0.09
Untreated	-0.140 0.14	-0.146 0.13	0.117 0.38	-0.125 0.07	-0.232 0.06

* indicates the repeated measure ANOVA of log(1+Δ monoterpene concentration) changed significantly ($p < 0.05$) within a treatment over initial (T_0), induction (T_1), and challenge (T_2) measurements.

Table 4.5a. Pooled comparisons of the change in individual needle monoterpene concentrations from before induction (T_0) to after the induction stage (T_1) ($\Delta T_1=[T_1-T_0]/T_0$). Data were transformed (ln) for analysis. Con = Control; Def = Defoliation by jack pine budworm; Inoc = Inoculation with Grosmannia clavigera.

		α-pinene	β-pinene	3-carene	Myrcene	Limonene
Def-Inoc vs. Def Con	F	0.58	0.00	0.07	0.82	0.62
	p	*0.449*	*0.958*	*0.797*	*0.369*	*0.433*
Inoc-Def vs. Inoc Con	F	7.80	3.18	4.82	0.01	1.22
	p	*0.006*	*0.078*	*0.031*	*0.930*	*0.272*
Def Con vs. Inoc Con	F	19.13	30.50	0.17	1.79	0.32
	p	*<0.0001*	*<0.0001*	*0.680*	*0.185*	*0.572*
Def-Inoc vs. Inoc-Def	F	7.23	21.29	8.54	5.89	0.02
	p	*0.009*	*<0.0001*	*0.005*	*0.017*	*0.895*
Inoc Con vs Wounded Con	F	8.07	7.43	0.08	0.20	2.16
	p	*0.006*	*0.008*	*0.780*	*0.655*	*0.145*

Table 4.5b. Pooled comparisons of the change in individual needle monoterpene concentrations from after induction (T_1) to after the challenge stage (T_2) (ΔT_2=[T_2-T_1]/T_1). Data were transformed (ln) for analysis. Con = Control; Def = Defoliation by jack pine budworm; Inoc = Inoculation with *Grosmannia clavigera*.

		α-pinene	β-pinene	3-carene	Myrcene	Limonene
Def-Inoc vs. Def Con	F	213.87	199.46	26.91	309.40	1.49
	p	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*0.225*
Inoc-Def vs. Inoc Con	F	44.73	39.65	18.84	49.41	3.88
	p	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*0.052*
Def Con vs. Inoc Con	F	145.66	140.74	10.68	126.12	4.20
	p	*<0.0001*	*<0.0001*	*0.002*	*<0.0001*	*0.043*
Def-Inoc vs. Inoc-Def	F	64.94	53.74	42.83	155.34	1.26
	p	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*0.264*
Inoc Con vs. Wound Con	F	32.03	31.74	15.73	79.79	0.11
	p	*<0.0001*	*<0.0001*	*0.0002*	*<0.0001*	*0.744*

Table 4.6. Spearman correlation of the change in monoterpene concentrations from before induction to after the challenege stage ($\Delta T=[T_2-T_0]/T_0$) in the induction-challenge treated seedlings (n=48). Listed as Spearman correlation coefficient and p value below in italics.

	β-pinene	3-carene	Myrcene	Limonene
α-pinene	0.5754 *<0.0001*	0.3782 *0.012*	0.7173 *<0.0001*	0.2418 *0.118*
β-pinene		0.3222 *0.035*	0.6979 *<0.0001*	0.4016 *0.008*
3-carene			0.4457 *0.003*	0.0900 *0.566*
Myrcene				0.6604 *<0.0001*

Table 4.7. Results of canonical correlation analysis of biological (mean pupal mass, lesion length, and ln of damage rate) and chemical variables (change in α-pinene, myrcene, and 3-carene over the experiment ($\Delta T=[T_2-T_0]/T_0$).

Canonical Function	Canonical Correlation	Squared Canonical Correlation	Eigenvalue	Proportion	Cumulative	F	*p*
1	0.640	0.410	0.695	0.696	0.696	3.75	*0.001*
2	0.482	0.233	0.303	0.304	1.000	2.62	*0.042*
3	0.002	0.000	0.000	0.000	1.000	0.00	*0.992*

Table 4.8. Canonical scores (standardized coefficients) for the significant canonical functions from the canonical correlation analysis. Monoterpenes listed are the change in monoterpene concentration from before the induction stage (T_0) to after the challenge stage (T_2) (ΔT=[T_2-T_0]/T_0).

	Canonical Function 1	Canonical Function 2
	Chemical 1	Chemical 2
α-pinene	-0.6439	1.1137
Myrcene	1.3610	-0.3424
3-carene	-0.1482	0.2035
	Biological 1	Biological 2
Mean pupal mass	0.1841	-0.4484
Mean lesion length	0.8025	-0.8081
Ln of feeding damage rate	0.3666	1.0810

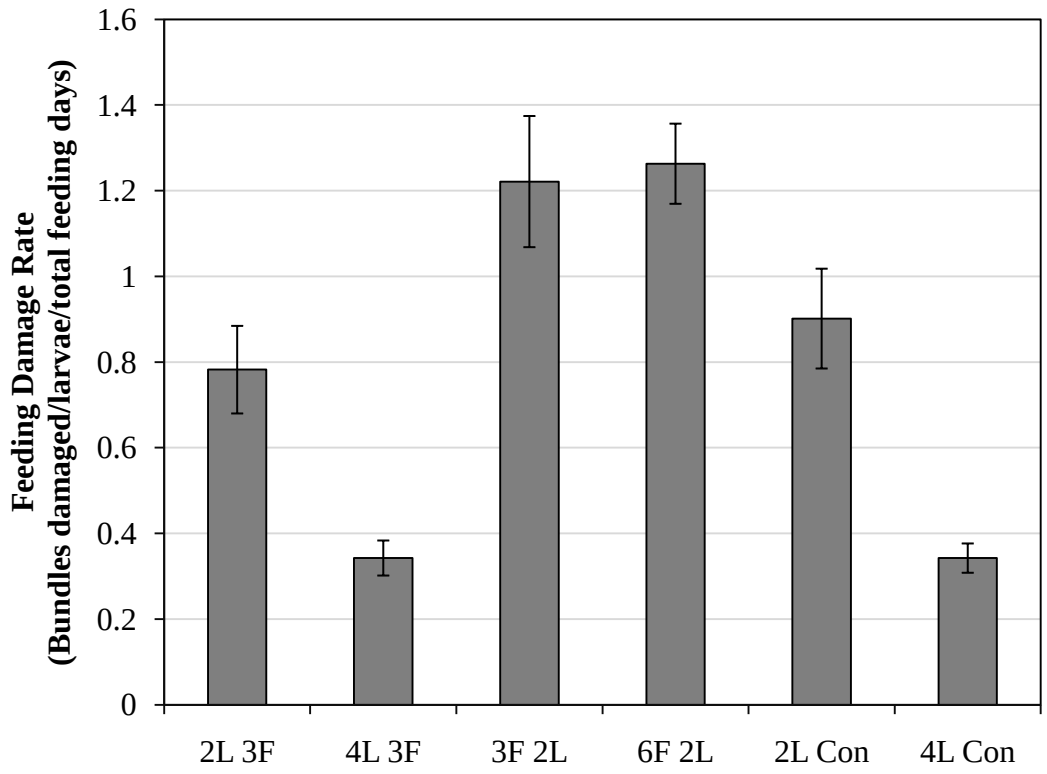

Figure 4.1. The feeding damage rate of jack pine budworm (mean ± standard error). Feeding damage rate was transformed (ln) for the analysis. Larvae on defoliated then inoculated seedlings (3F 2L + 6F 2L) fed at significantly higher rates than the other treatments (2L 3F + 4L 3F + 2L Con + 4L Con) ($F=50.18$, $df=1$, $p<0.0001$). Con = Control, F = *Grosmannia clavigera* inoculations; L = Jack pine budworm larvae.

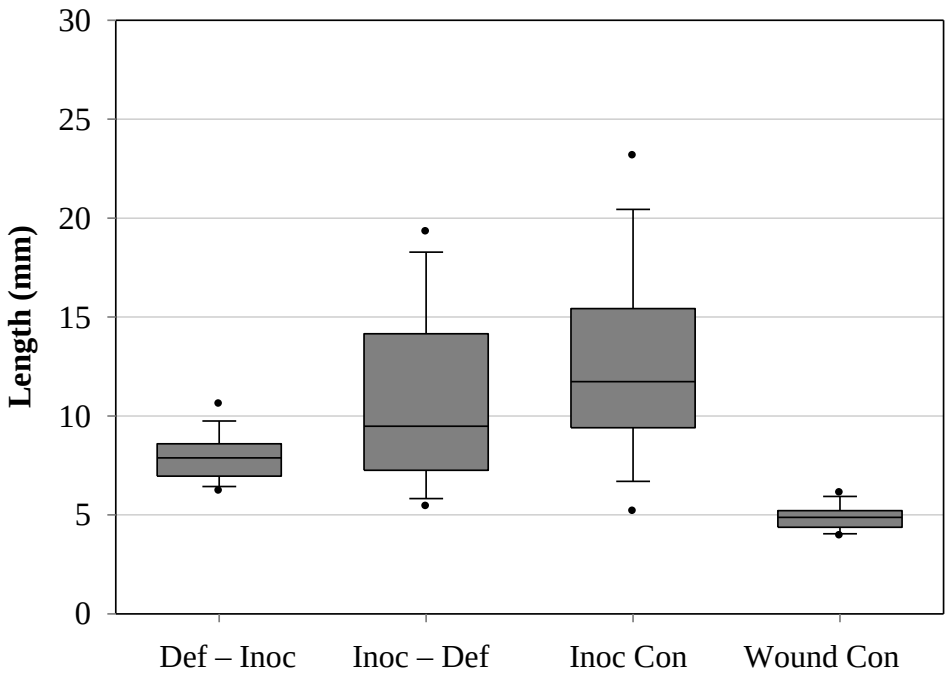

Figure 4.2. Box plot of lesion lengths grouped according to induction-challenge type. Box indicates the 25th and 75th percentile, the line through the box is the median, the capped lines indicate the 10th and 90th percentile, and the dots are the 5th and 95th percentile. Inoc –Con had longer lesions than Wound Con (χ^2=67.24, p<0.0001). Inoc Con and Inoc – Def had longer lesions than the Def – Inoc seedlings (χ^2=37.27, p<0.0001 and χ^2=18.91, p<0.0001, respectively). Con = Control; Def = Jack pine budworm defoliation; Inoc = *G. clavigera* inoculation

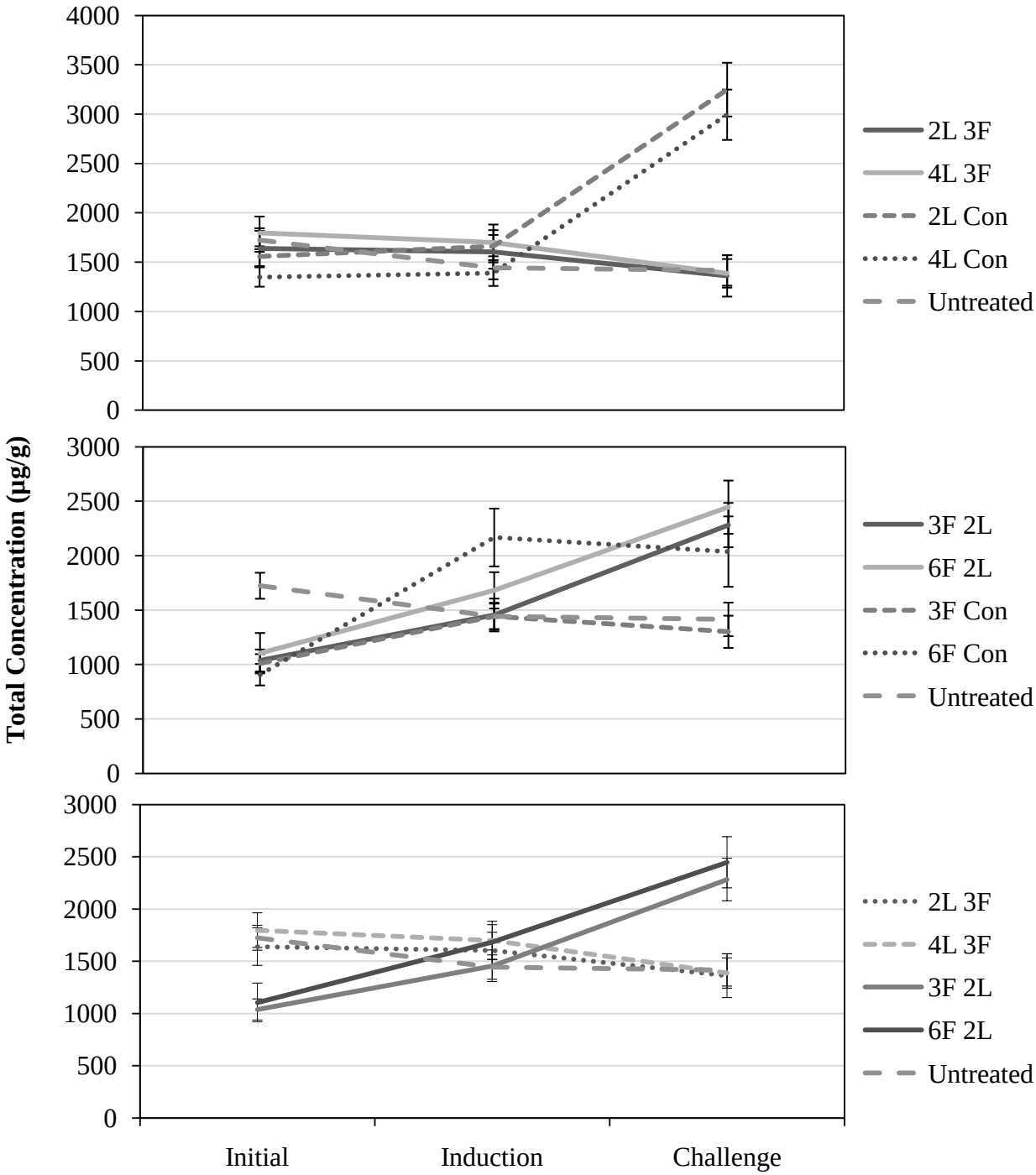

Figure 4.3. Total monoterpene concentration (mean ± standard error) before the induction stage (T_0), after the induction stage (T_1), and after the challenge stage (T_2). Seedlings that received (a) jack pine budworm defoliation induction and the untreated control, (b) *Grosmannia clavigera* inoculation induction and the untreated control, and (c) an induction and challenge treatment and the untreated control. Con = Control, F = *Grosmannia clavigera* inoculations; L = Jack pine budworm larvae.

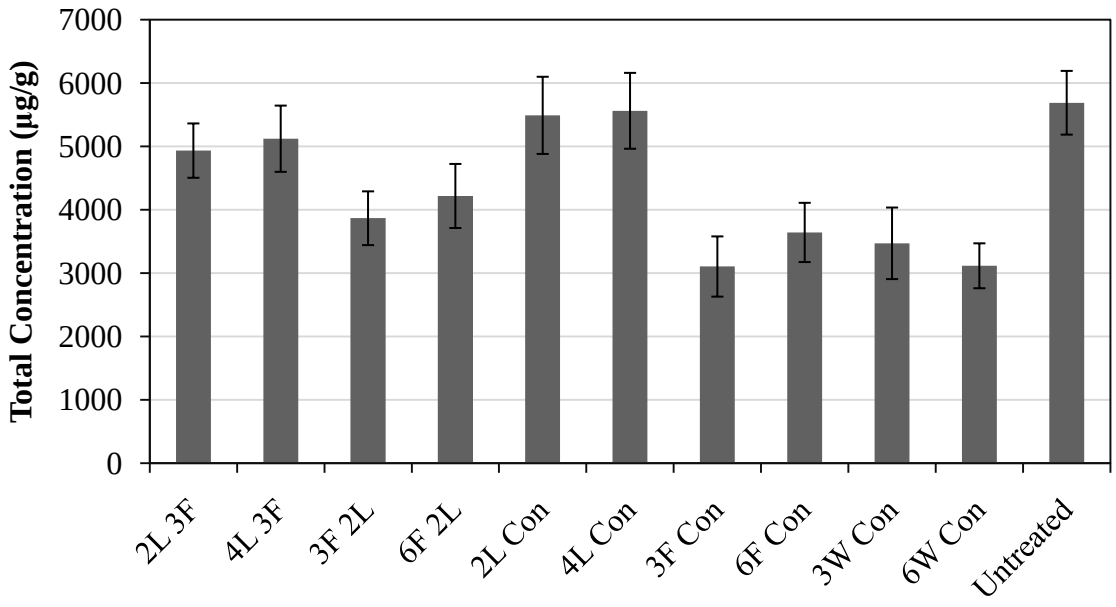

Figure 4.4. Total monoterpene concentration of phloem tissue after the challenge stage (T_2) (mean ± standard error). Con = Control, F = *Grosmannia clavigera* inoculations; L = Jack pine budworm larvae.

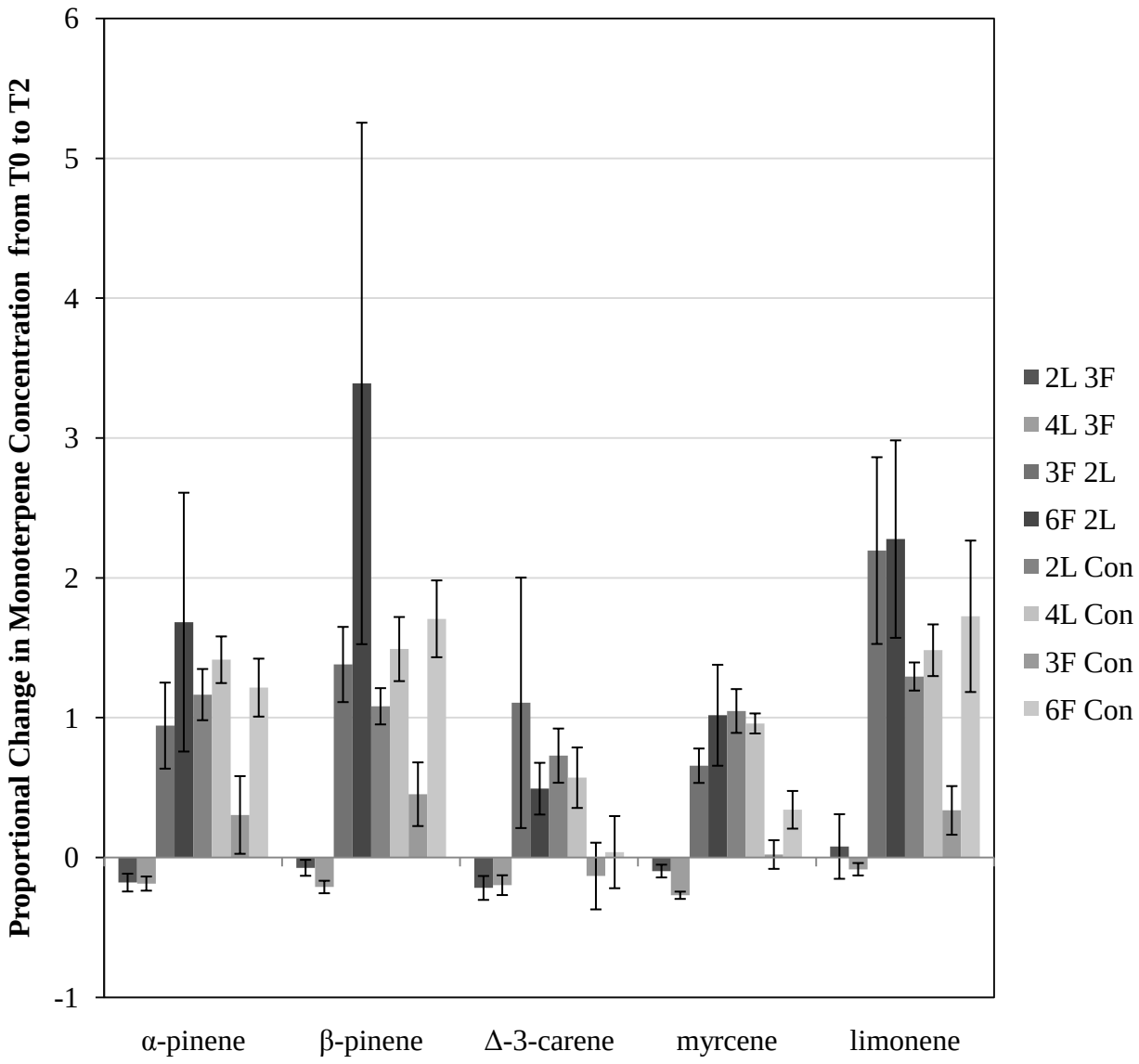

Figure 4.5. Change in the concentration of α-pinene, β-pinene, 3-carene, myrcene, and limonene monoterpene from before induction (T_0) to after the challenge stage (T_2) (ΔT=[T_2-T_0]/T_2) (mean ± standard error). Con = Control, F = *Grosmannia clavigera* inoculations; L = Jack pine budworm larvae.

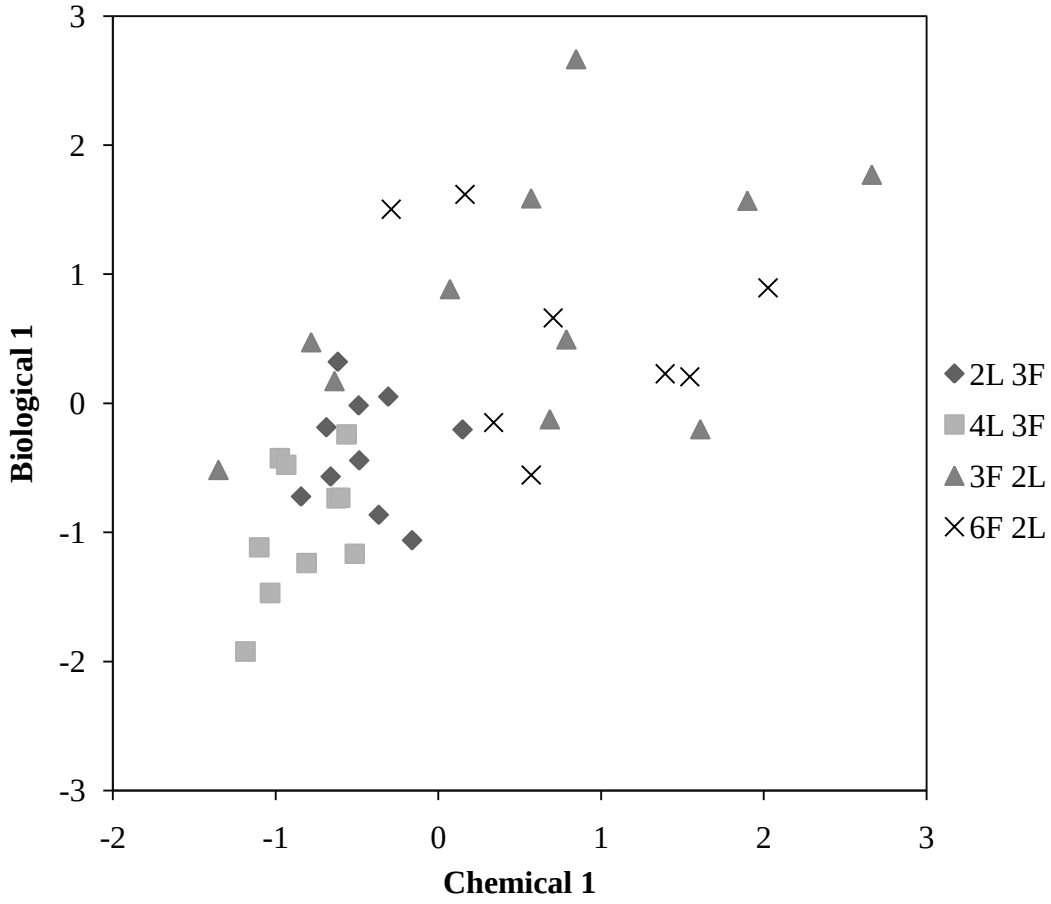

Figure 4.6. Plot of the results of the first canonical function from the canonical correlation analysis. Biological 1 is a variate composed of mean jack pine budworm pupal mass, mean *Grosmannia clavigera* lesion length, and ln of feeding damage rate. Chemical 1 is a variate composed of the change in α-pinene, 3-carene, and myrcene from before the induction stage (T_0) to after the challenge stage (T_2) ($\Delta T=[T_2-T_0]/T_2$).

V. GENERAL DISCUSSION

For my Master of Science research, I conducted experiments to evaluate monoterpene response induction in jack pine. I examined jack pine response to different types of induction in seedlings in a greenhouse experiment and mature trees in a field experiment (Chapter III). The concentration and composition of monoterpenes were measured in needles and phloem of seedlings and phloem of mature trees. I also investigated cross-induction of jack pine budworm (JPBW) and *Grosmannia clavigera* on jack pine seedlings in a greenhouse experiment (Chapter IV). I quantified changes in monoterpene composition in response to induction by a single organism and both organisms in succession and linked these to changes observed in jack pine resistance.

The results of my research strongly support the concept that monoterpenes are part of the inducible response system in jack pine, concurring with earlier research (Raffa and Smalley 1995; Wallin and Raffa 1999; Jost et al. 2008). Further, the resulting information indicates that jack pine has different chemical defence strategies against single or multiple agents.

Monoterpene concentrations in needles and phloem of seedlings did not differ between treated and untreated control trees, with the exception of higher concentrations in the phloem after methyl jasmonate application. I suspect that the amount of damage inflicted by mechanical defoliation or *G. clavigera* inoculation may not have been strong enough to observe induction only three weeks after treatment. For example, elevated monoterpenes were observed eight and a half weeks after inoculation in mature phloem and nine weeks after inoculation in seedling phloem in Chapter IV. Alternatively, defensive compounds other than monoterpenes like phenolics may have been induced, but they were not measured here. Further, although mechanical defoliation did not induce monoterpene responses compared to untreated seedlings, concentrations in needles increased after JPBW defoliation. This result supports that mechanical foliage removal does not have the same effects on jack pine as insect feeding, which has been observed in other systems (Haukioja 1990; Alborn et al. 1997; Litvak and Monson 1998).

Monoterpene concentrations in needles of methyl jasmonate treated seedlings were higher than those of *G. clavigera* inoculation and mechanical defoliation and were also significantly higher in phloem of methyl jasmonate treated than untreated mature trees. This suggests jasmonic acid is involved in signalling of defence induction in jack pine as it is in other conifers (Davis et al. 2002; Hudgins et al. 2003; Erbilgin et al. 2006) but more research of biochemical signalling of defence in jack pine is needed to verify this result. Methyl salicylate did not affect monoterpenes but other defences may have been involved in seedlings, as it has been shown to play significant role in conifer defences, particularly in the induction of phenolic compounds (Kozlowski et al. 1999; Davis et al. 2002). These results require further investigations to understand the relationship between phytohormone application and real damage.

Mature trees had lower monoterpene concentrations but higher magnitudes of induction than juveniles, as measured by the proportional change from untreated controls. The concentrations and magnitudes of induction correspond to the levels reported in other studies of Scots pine (*Pinus sylvestris* L.) seedlings (Heijari et al. 2005) and mature Norway spruce (*Picea abies* Karst.) (Erbilgin et al. 2006) after methyl jasmonate application. These results suggest that juvenile and mature trees have different strategies for allocating resources between defences and growth. It appears that juvenile trees rely more on constitutive defences whereas mature trees rely heavily on induced defences. I suspect that this is because constitutive defences are costly to maintain and induced responses may be synthesized by mature trees when needed (Franceschi et al. 2005). Juvenile trees lack storage organs for carbon and nutrients to support synthesis of additional defensive compounds and there is a greater cost of lost tissue compared to mature trees; therefore, juveniles would be expected to show a greater investment in constitutive defences and less investment in induced defences. Alternatively, mature trees have well-developed physical defences and may rely more heavily on these for defence from organism attack. The evolution of plant defences is thought to be constrained by multiple trade-offs (Bazzaz et al. 1987; Zangerl and Bazzaz 1992; Mole 1994) and this study added further complexity to

the cost-defence relationship between juvenile and mature trees of same species. Additional studies of this system should focus on trade-offs between different types of defences (Berenbaum 1995) in both juvenile and mature jack pine trees and the comparability between them.

In the cross-induction study in Chapter IV, I found that JPBW defoliation increased jack pine resistance to *G. clavigera*, but *G. clavigera* inoculation did not change jack pine resistance to JPBW defoliation. Although there were systemic increases in monoterpenes after inoculation, larvae compensated for a potential change in host quality by increasing their feeding rate. The differences in jack pine response to a challenge organism could be due to differences in evolutionary history between the organisms and jack pine (Tollrain and Harvell 1999) as jack pine have co-evolved with JPBW unlike MPB fungal associates.

This experiment had constraints that could have influenced its outcome. First, the experiment had to be staggered so JPBW could feed at the same time and inoculations did not occur in the late summer when MPB would be colonizing new hosts. This could be important as there can be seasonal changes in tree chemistry (Paine et al. 1997; Wallin and Raffa 2001). Second, fungal inoculations do not inflict the same physical damage as MPB larvae feeding on the phloem. Third, systemic responses to multiple organisms can be inconsistent between experiments repeated in different years. For example, Eyles et al. (2007) reported that inoculation with a foliar pathogen, *Sphaeropsis sapinea* reduced growth of sawfly *Neodiprion sertifer* in Austrian pine in 2005 but not 2006, and induction by *N. sertifer* reduced lesions length of *S. sapinea* in 2006 but not in 2005.

To better understand the interaction among JPBW, *G. clavigera*, and jack pine, future studies should incorporate both defensive compounds and the nutritional status of jack pine tissues. Examining physical and anatomical defences in jack pine would also help to further characterize this relationship as they are important in bark beetle colonization. Frequent sampling of future experiments will more accurately depict jack pine defences. Ideally, *in situ* studies in northern Alberta would be conducted and include stands with JPBW defoliation

with trees naturally infested or baited for MPB, which would eliminate many of the design issues described above.

In summary, these studies demonstrated that jack pine defences are inducible and monoterpenes can have an important role in indirect interactions between organisms. Further, jack pine-mediated interactions depend on the order of organisms attacking (e.g. Bonello et al. 2006) and resistance may also be influenced by the timing and lag between attacking organisms (Wallin and Raffa 1999). Experimental design and technical limitations explained above could have influenced the results of these experiments and may not have fully characterized the interaction of JPBW and *G. clavigera*.

As suggested in Chapter II, research of jack pine defence to different biotic agents and MPB could be used to build a simulation model of ecosystem dynamics. This model should factor in numerous variables including weather, site conditions, insect dynamics, characteristics of the non-native species, tree physiology, induced responses, climate change, and disturbance regimes. Unknown variables in this model largely pertain to jack pine responses to different organisms. The results reported in Chapter IV and similar studies with multiple agents of induction could be used in such a model. An ecosystem model would provide a more accurate picture of MPB expansion in boreal ecosystems and help entomologists to make recommendations to forest managers for management of the boreal with the new challenge of MPB expansion. For example, it could be used to identify stands and characteristics of stands that are at higher risk for MPB colonization.

Researching tree-mediated interactions will provide a more thorough understanding of indirect relationships between organisms and the effects of a species invasion on ecosystem dynamics. Range expansions facilitated by climate change will continue to challenge forest management plans. Invasion of insect pest species, like the MPB, into new ecosystems will impact the structure of the new community and their rate of spread may be affected by induced responses. Research of induced responses and resistance will allow a better understanding of

tree-mediated interactions and the invasion potential of a new species, and could be used in pest management plans in the future.

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APPENDIX A: PROPORTIONS OF INDIVIDUAL MONOTERPENES FROM CHAPTER III EXPERIMENTS

Table A.1. Proportion of individual monoterpenes of total monterpene concentration (%) in mature tree phloem (mean above, standard error below).

	Methyl Jasmonate		*G. clavigera* Inoculation		Wounded Control		Un-treated
	25 mM	100 mM	12 F	36 F	12 W	36 W	
α-pinene	55.69 5.89	59.88 8.33	59.55 8.64	64.98 6.83	52.70 8.80	59.21 6.11	53.86 6.60
β-pinene	5.90 1.40	6.91 1.43	3.09 0.80	4.90 0.77	6.98 2.39	4.75 0.87	4.16 0.91
Limonene	26.25 7.36	17.02 6.43	28.29 8.08	18.41 7.07	30.54 10.07	30.60 6.93	28.15 8.59
3-carene	4.77 2.67	5.88 4.15	4.46 3.10	6.57 3.73	4.32 2.88	0.00 0.00	10.28 4.74
Myrcene	5.35 0.84	3.61 0.72	2.73 0.88	2.66 0.65	3.80 1.62	5.13 2.06	1.82 0.77
β-phellan-drene	0.60 0.23	5.07 3.06	1.28 1.11	0.46 0.28	0.36 0.20	0.07 0.07	0.40 0.21
Camphene	0.47 0.17	0.65 0.14	0.16 0.11	0.62 0.21	0.42 0.23	0.25 0.11	0.00 0.00
Terpinolene	0.76 0.76	0.82 0.47	0.45 0.35	0.77 0.48	0.00 0.00	0.00 0.00	1.34 0.89

	Methyl Jasmonate		*G. clavigera* Inoculation		Wounded Control		Un-treated
	25 mM	100 mM	12 F	36 F	12 W	36 W	
Cis-ocimene	0.00 0.00	0.11 0.11	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00
Bornyl acetate	0.21 0.21	0.00 0.00	0.00 0.00	0.59 0.39	0.88 0.68	0.00 0.00	0.00 0.00
Gamma-terpinene	0.00 0.00	0.04 0.04	0.00 0.00	0.03 0.03	0.00 0.00	0.00 0.00	0.00 0.00
p-cymene	0.00 0.00	0.01 0.01	0.00 0.00	0.01 0.01	0.00 0.00	0.00 0.00	0.00 0.00

* α-terpinene, camphor, α-thujone, linalool, α-terpineol, sabinene hydrate, α-humulene, borneol, and pulegone were also included as standards in gas chromatography but proportions were 0.

Table A.2. Proportion of individual monoterpenes of total monterpene concentration (%) in needles of greenhouse seedlings (mean above, standard error below).

	Mechanical Defoliation			Methyl Jasmonate			Methyl Salicylate			*G. clavigera* Inoculation			Wounded Control			Un-treated
	10%	25%	40%	10 mM	25 mM	50 mM	10 mM	25 mM	50 mM	1 F	3 F	6 F	1 W	3 W	6 W	
α-pinene	38.17 4.79	33.02 6.85	34.19 5.77	24.75 5.00	27.44 4.93	39.97 6.76	38.92 4.95	41.70 7.07	39.56 6.15	29.71 5.93	34.76 6.33	35.37 5.08	38.11 8.78	37.91 8.07	34.38 9.96	37.36 7.22
β-pinene	23.59 4.09	25.20 4.43	24.38 4.94	25.89 3.50	22.27 3.99	29.03 4.34	29.35 5.79	24.12 4.44	23.38 4.80	28.23 3.40	20.92 5.07	27.45 5.61	31.25 8.40	27.35 5.80	25.48 5.09	29.66 5.84
Limonene	12.36 6.06	23.54 7.64	24.17 8.50	38.20 7.38	27.36 8.28	13.81 6.15	19.94 7.80	15.78 7.02	22.70 7.94	19.73 6.11	29.83 8.99	13.41 6.28	17.48 14.76	15.31 7.00	24.10 13.38	18.13 9.50
3- carene	6.97 2.87	5.32 3.46	0.95 0.79	1.02 1.02	6.47 3.44	2.66 1.83	2.04 1.96	2.27 1.87	0.49 0.31	6.02 2.50	1.22 1.13	7.83 3.66	4.31 3.97	7.14 5.18	4.92 4.49	1.60 1.60
Myrcene	3.78 0.22	3.27 0.16	3.37 0.25	3.02 0.14	3.61 0.24	3.27 0.19	2.88 0.14	3.22 0.20	3.36 0.26	3.42 0.16	3.10 0.19	3.51 0.22	2.94 0.16	3.22 0.51	3.17 0.35	3.11 0.25
β-phell-andrene	5.66 1.68	3.84 1.68	6.00 3.19	1.66 0.57	3.13 1.09	5.49 1.74	2.59 0.71	5.88 2.07	2.38 1.08	6.60 1.58	4.20 1.98	5.01 1.27	1.66 0.42	4.06 1.73	2.55 0.61	3.34 1.10
Camph-ene	2.27 0.41	1.64 0.45	1.95 0.44	1.55 0.41	2.45 0.76	1.73 0.28	1.40 0.21	2.10 0.50	2.23 0.31	1.61 0.33	1.67 0.49	2.02 0.29	1.44 0.29	1.45 0.37	1.65 0.39	2.03 0.64
Terpin-olene	0.69 0.33	0.38 0.25	0.06 0.06	0.08 0.08	0.49 0.29	0.13 0.13	0.17 0.17	0.12 0.12	0.10 0.10	0.40 0.31	0.07 0.07	0.55 0.25	0.24 0.24	0.53 0.53	0.28 0.28	0.00 0.00

	Mechanical Defoliation			Methyl Jasmonate			Methyl Salicylate			*G. clavigera* Inoculation			Wounded Control			Un-treated
	10%	25%	40%	10 mM	25 mM	50 mM	10 mM	25 mM	50 mM	1 F	3 F	6 F	1 W	3 W	6 W	
Cis-ocimene	0.55 0.24	0.13 0.10	0.32 0.25	0.08 0.06	0.07 0.07	0.33 0.19	0.21 0.15	0.15 0.10	0.43 0.24	0.14 0.14	0.00 0.00	0.33 0.20	0.23 0.23	0.19 0.19	0.49 0.49	0.14 0.14
Bornyl acetate	5.97 1.45	3.66 1.08	4.58 1.20	3.74 1.24	6.70 2.83	3.57 0.92	2.51 0.70	4.66 1.41	5.38 1.05	4.14 1.06	4.24 1.78	4.48 0.94	2.33 0.47	2.84 1.25	2.96 1.19	4.62 2.04
Borneol	0.00 0.00	0.00 0.00	0.04 0.04	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.05 0.05	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00

* α-humulene, α-terpineol, α-thujone, α-terpinene, sabiene-hydrate, pulgeone, camphor, linalool, p-cymene, and gamma-terpinene were also included as standards in gas chromatography but proportions were 0.

Table A.3. Proportion of individual monoterpenes of total monterpene concentration (%) in phloem of greenhouse seedlings (mean above, standard error below).

	Mechanical Defoliation			Methyl Jasmonate			Methyl Salicylate			*G. clavigera* Inoculation			Wounded Control			Un-treated
	10%	25%	40%	10 mM	25 mM	50 mM	10 mM	25 mM	50 mM	1 F	3 F	6 F	1 W	3 W	6 W	
α-pinene	43.72 4.81	36.38 7.40	38.45 4.95	28.95 3.85	30.68 4.81	43.41 4.10	44.24 5.07	47.61 5.98	44.18 5.70	39.39 3.76	42.20 5.80	41.77 4.85	43.36 6.99	47.62 6.27	40.16 8.09	46.20 4.66
β-pinene	25.83 3.15	24.16 3.18	25.01 3.93	26.82 2.82	23.42 2.47	33.05 2.08	25.81 4.15	27.03 3.12	24.01 2.75	26.14 2.23	24.01 3.17	27.45 3.35	27.81 5.27	26.55 5.14	25.33 3.14	24.71 6.27
Limonene	12.04 5.83	25.15 8.11	24.7 8.92	36.02 6.20	25.62 7.32	11.28 5.11	21.70 8.14	14.02 6.21	22.62 7.84	17.73 5.46	24.88 7.91	12.36 5.95	11.80 8.85	10.61 6.91	20.63 11.02	16.74 8.67
3- carene	11.11 4.59	7.02 3.31	4.15 3.02	9.53 4.36	2.04 1.67	11.28 4.43	2.12 1.84	10.05 4.70	4.90 2.59	2.46 2.38	3.03 2.57	2.46 2.01	9.84 9.57	8.16 7.89	7.43 6.57	6.77 3.36
Myrcene	3.28 0.15	2.95 0.14	2.89 0.14	3.06 0.13	3.42 0.19	3.11 0.12	2.82 0.12	3.03 0.15	3.02 0.07	3.07 0.21	2.82 0.13	3.00 0.15	3.15 0.29	3.07 0.23	2.93 0.18	2.57 0.09
β-phellan-drene	1.80 0.30	2.70 1.15	3.50 2.11	1.65 0.59	2.30 0.76	2.26 0.15	1.59 0.36	3.58 1.59	2.35 0.90	2.49 1.04	2.00 0.77	1.77 0.31	2.00 0.15	2.21 0.23	1.50 0.45	1.24 0.55
Camph-ene	0.82 0.09	0.65 0.12	0.67 0.09	0.59 0.08	1.14 0.34	0.95 0.09	0.86 0.12	0.89 0.11	0.84 0.12	0.77 0.10	0.99 0.24	0.91 0.08	0.84 0.12	0.86 0.12	0.91 0.18	0.80 0.13
Terpin-olene	0.99 0.42	0.71 0.40	0.28 0.2	0.38 0.26	0.95 0.48	0.33 0.18	0.21 0.21	0.51 0.26	0.29 0.26	0.53 0.26	0.08 0.08	0.81 0.36	0.55 0.55	0.62 0.62	0.42 0.42	0.51 0.22

	Mechanical Defoliation			Methyl Jasmonate			Methyl Salicylate			*G. clavigera* Inoculation			Wounded Control			Un-treated
	10%	25%	40%	10 mM	25 mM	50 mM	10 mM	25 mM	50 mM	1 F	3 F	6 F	1 W	3 W	6 W	
Gamma-terpinene	0.11 0.05	0.06 0.04	0.03 0.03	0.03 0.02	0.10 0.06	0.02 0.02	0.02 0.02	0.03 0.03	0.00 0.00	0.02 0.02	0.00 0.00	0.10 0.04	0.08 0.08	0.00 0.00	0.00 0.00	0.00 0.00
Bornyl acetate	0.15 0.08	0.11 0.06	0.28 0.14	0.25 0.06	2.24 1.37	0.60 0.26	0.24 0.10	0.24 0.09	0.15 0.09	0.22 0.06	0.95 0.84	0.44 0.10	0.26 0.12	0.30 0.30	0.55 0.43	0.41 0.17
α-Terp-ineol	0.03 0.02	0.01 0.01	0.00 0.00	0.05 0.03	0.01 0.01	0.02 0.02	0.03 0.03	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.02 0.02	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00
Borneol	0.00 0.00	0.08 0.06	0.00 0.00	0.05 0.03	0.02 0.02	0.04 0.04	0.02 0.02	0.03 0.03	0.02 0.02	0.00 0.00	0.02 0.02	0.07 0.05	0.00 0.00	0.00 0.00	0.00 0.00	0.06 0.06
Cis-ocimene	0.11 0.08	0.00 0.00	0.00 0.00	0.00 0.00	0.05 0.05	0.03 0.03	0.00 0.00	0.00 0.00	0.06 0.06	0.10 0.10	0.00 0.00	0.00 0.00	0.31 0.31	0.00 0.00	0.15 0.15	0.00 0.00
p-cymene	0.00 0.00	0.01 0.01	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00

* α-thujone, sabinene-hydrate, camphor, linalool, α-humulene, pulgeone, and α-terpinene were also included as standards in gas chromatography but proportions were 0.

APPENDIX B: PROPORTIONS OF INDIVIDUAL MONOTERPENES FROM CHAPTER IV EXPERIMENT

Table B.1. Concentration (µg/g) of individual monoterpenes in needles over the greenhouse experiment (mean above, standard error below). T_0 is before the induction stage, T_1 is after induction but before the challenge stage, and T_2 is after the challenge stage.

		2L 3F	4L 3F	3F 2L	6F 2L	2L CON	4L CON	3F CON	6F CON	3W CON	6W CON	Un-treated
α-pinene	T_0	370.94	433.83	283.62	218.74	405.16	270.48	248.03	198.53	392.15	272.17	338.97
		91.09	90.87	45.30	40.23	60.42	38.20	44.71	45.98	128.59	64.42	77.87
	T_1	347.34	407.09	337.83	329.17	413.21	271.88	355.09	476.53	341.68	412.49	278.93
		90.43	88.18	59.09	75.04	63.77	39.01	74.35	84.11	73.33	108.30	46.06
	T_2	274.80	320.01	525.20	486.71	831.76	630.86	298.06	426.56	178.87	200.07	253.92
		62.50	62.51	99.35	124.31	120.11	79.66	49.67	88.32	45.05	51.65	27.06
β-pinene	T_0	237.77	328.70	198.81	181.75	280.97	299.93	245.34	169.26	321.30	136.38	141.71
		49.98	64.24	52.29	43.02	75.08	88.84	68.07	33.12	51.90	63.31	50.17
	T_1	229.00	316.93	244.94	292.52	290.59	315.83	379.36	546.59	532.83	209.24	126.43
		48.64	67.77	44.58	49.61	88.81	109.75	93.17	111.80	186.29	68.26	50.17
	T_2	201.31	253.20	368.76	409.34	553.71	695.96	326.96	453.13	247.74	92.73	113.33
		42.15	47.47	66.45	70.44	154.78	200.70	71.44	90.65	77.37	34.69	39.73
Limonene	T_0	428.32	386.19	163.87	223.67	319.48	261.59	14.86	135.82	178.12	326.94	405.31
		125.00	152.31	63.30	82.46	123.18	103.31	9.74	66.33	110.18	105.78	124.24
	T_1	433.20	428.73	322.40	470.80	319.46	284.53	44.85	428.31	191.31	626.65	341.09
		123.49	185.61	106.73	161.59	124.12	109.25	10.12	207.48	105.13	200.73	103.35
	T_2	380.35	349.63	527.34	634.58	693.72	598.19	46.29	430.64	110.13	328.01	310.51
		117.79	139.56	174.82	205.71	267.51	214.99	7.68	220.70	65.15	98.80	92.86

		2L 3F	4L 3F	3F 2L	6F 2L	2L CON	4L CON	3F CON	6F CON	3W CON	6W CON	Un-treated
3-carene	T_0	158.26 41.18	235.47 103.62	198.65 54.53	301.60 130.91	159.82 66.81	144.30 49.84	303.73 121.07	181.46 50.22	462.76 322.41	232.77 85.16	343.93 156.81
	T_1	183.96 39.48	188.16 81.56	233.59 109.45	255.72 113.14	211.10 80.14	134.08 54.27	350.01 164.00	276.69 170.78	226.11 137.78	255.72 98.52	259.58 130.11
	T_2	114.92 24.85	162.64 63.73	351.68 135.56	446.49 177.79	311.71 118.27	259.38 99.95	330.86 166.15	249.94 144.77	107.73 73.75	112.84 45.33	237.50 95.09
Myrcene	T_0	115.74 10.82	122.70 11.22	88.58 4.34	87.98 7.41	109.09 5.66	98.05 6.43	92.82 4.81	94.70 7.29	99.07 10.08	97.22 8.49	129.61 14.80
	T_1	124.51 8.53	113.99 11.19	98.47 7.89	111.00 8.59	125.59 7.94	99.05 8.47	99.74 5.98	127.23 15.00	97.54 5.63	123.46 11.05	103.32 11.97
	T_2	104.94 12.20	88.68 7.20	143.50 9.27	158.11 12.61	218.91 14.41	193.83 15.96	93.22 6.51	129.17 18.82	53.09 2.49	64.94 5.08	111.64 13.00
Camphene	T_0	79.73 19.05	59.95 21.38	11.16 5.84	4.98 3.34	74.51 14.15	56.11 14.84	0.00 0.00	6.60 6.60	14.23 9.22	0.00 0.00	113.02 28.46
	T_1	73.28 18.24	53.41 18.34	58.79 14.73	50.87 12.98	81.24 14.68	64.55 16.46	40.17 18.17	73.58 15.95	30.00 16.87	46.33 9.59	103.19 26.67
	T_2	90.46 31.63	59.61 18.33	105.38 24.12	73.94 15.36	214.39 44.54	181.78 32.83	47.36 17.85	83.38 25.16	20.17 7.04	27.17 5.89	141.10 36.08

		2L 3F	4L 3F	3F 2L	6F 2L	2L CON	4L CON	3F CON	6F CON	3W CON	6W CON	Un-treated
Borneol	T_0	105.37 21.98	86.64 20.73	58.53 16.70	42.66 13.01	79.05 12.30	92.48 20.96	26.87 11.46	66.90 27.44	76.43 21.96	74.32 15.19	82.47 20.29
	T_1	84.86 15.38	67.92 15.14	72.50 18.31	65.61 17.48	77.04 11.98	81.67 18.00	40.83 13.28	90.87 32.27	89.21 21.24	84.02 18.44	67.99 17.17
	T_2	42.66 10.39	30.52 7.09	108.73 29.31	87.94 24.28	105.03 16.31	119.34 26.33	34.85 11.98	91.61 32.74	44.96 8.73	42.02 8.99	43.53 8.52
Bornyl acetate	T_0	72.24 20.65	52.81 20.35	10.17 3.64	5.73 2.03	68.16 16.32	59.94 18.04	6.55 3.05	3.44 3.44	3.86 2.44	1.53 1.53	118.65 33.80
	T_1	65.29 18.39	46.71 17.15	49.61 11.80	46.33 12.19	73.89 15.52	66.38 18.63	41.79 15.46	61.63 15.40	27.57 16.07	32.81 5.23	111.21 32.15
	T_2	83.96 34.26	56.36 18.89	92.17 23.66	61.42 11.09	204.50 49.29	169.54 35.70	41.80 18.72	76.10 25.69	15.53 7.44	20.92 3.98	157.63 47.44
β-phellan-drene	T_0	5.74 2.63	7.53 2.70	2.19 1.00	3.33 1.42	5.00 1.69	5.86 1.80	6.86 1.45	5.01 2.64	1.83 0.55	5.28 2.18	4.09 2.40
	T_1	5.23 2.28	6.81 2.52	2.34 0.88	5.30 1.97	5.61 1.91	6.18 1.87	8.27 1.83	7.78 3.07	2.31 0.96	6.37 2.37	3.82 2.04
	T_2	5.70 2.69	5.35 1.99	3.87 1.46	7.73 2.96	9.16 2.99	11.90 3.66	7.44 1.87	8.22 3.48	1.12 0.39	3.30 1.28	3.57 1.84

		2L 3F	4L 3F	3F 2L	6F 2L	2L CON	4L CON	3F CON	6F CON	3W CON	6W CON	Un-treated
Ocimene	T_0	9.78 5.30	9.03 7.02	0.00 0.00	0.00 0.00	9.00 4.81	5.41 3.65	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	4.22 4.22
	T_1	7.20 3.93	5.04 3.41	5.55 3.74	0.00 0.00	8.13 4.28	6.80 4.57	0.00 0.00	0.00 0.00	5.68 5.68	0.00 0.00	5.51 5.51
	T_2	5.52 3.13	5.64 4.08	7.84 5.43	0.00 0.00	12.78 8.70	16.01 8.84	0.00 0.00	4.96 4.96	3.22 3.22	0.00 0.00	3.39 3.39
Terpino-lene	T_0	1.55 1.10	3.30 2.28	2.37 1.72	4.36 2.57	1.49 1.49	1.89 1.27	4.19 2.80	0.97 0.97	6.38 6.38	3.21 2.03	5.37 3.44
	T_1	1.53 1.09	2.25 1.52	4.63 3.25	4.31 2.50	2.19 1.77	2.11 1.43	5.92 3.76	3.90 3.90	3.11 3.11	5.88 4.38	4.98 3.15
	T_2	0.95 0.64	2.05 1.43	8.83 5.75	6.52 3.84	3.31 2.49	3.96 2.73	5.67 3.61	3.27 3.27	1.70 1.70	3.91 3.08	3.83 2.43
α-terpineol	T_0	1.39 1.00	1.87 0.99	0.00 0.00	0.00 0.00	0.51 0.51	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00
	T_1	1.57 0.83	1.00 0.68	1.20 1.20	2.16 1.45	0.78 0.78	0.00 0.00	3.03 2.03	2.90 1.93	0.00 0.00	0.00 0.00	1.19 1.19
	T_2	3.91 2.11	4.53 2.63	1.28 1.28	2.52 1.73	4.66 2.17	4.74 2.85	1.68 1.68	3.28 2.09	0.00 0.00	0.69 0.69	3.28 1.07

		2L 3F	4L 3F	3F 2L	6F 2L	2L CON	4L CON	3F CON	6F CON	3W CON	6W CON	Un-treated
α-humu-lene	T_0	0.53 0.53	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.42 0.42	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00
	T_1	0.46 0.46	0.00 0.00	0.69 0.69	0.00 0.00	0.82 0.55	0.45 0.45	0.00 0.00	1.18 1.18	0.00 0.00	0.00 0.00	0.00 0.00
	T_2	0.33 0.33	0.25 0.25	0.92 0.92	0.80 0.80	0.68 0.68	0.00 0.00	0.00 0.00	2.23 1.42	0.00 0.00	0.00 0.00	0.00 0.00
Gamma-terpinene	T_0	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00
	T_1	0.00 0.00	0.00 0.00	1.29 0.87	0.70 0.70	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	1.18 1.18
	T_2	0.00 0.00	0.28 0.28	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	1.31 1.31	0.00 0.00	0.00 0.00	0.00 0.00
Pulgeone	T_0	0.00 0.00	0.64 0.64	0.00 0.00	0.00 0.00	0.84 0.84	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00
	T_1	0.00 0.00	0.00 0.00	0.59 0.59	0.00 0.00	0.74 0.74	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00
	T_2	0.00 0.00	0.00 0.00	1.00 1.00	0.00 0.00	1.49 1.49	1.19 1.19	0.00 0.00	0.00 0.00	0.00 0.00	0.64 0.64	0.00 0.00

* α-thujone, β-thujone, sabinene hydrate, camphor, p-cymene, α-terpinene, and linalool were also included as standards in gas chromatography but concentrations were 0.

Table B.2. Concentration (µg/g) of individual monoterpenes in phloem over the greenhouse experiment (mean above, standard error below).

	2L 3F	4L 3F	3F 2L	6F 2L	2L CON	4L CON	3F CON	6F CON	3W CON	6W CON	Un-treated
α-pinene	1513.24 218.45	1783.42 208.53	1313.78 167.24	1236.48 169.16	2055.43 293.56	1877.12 322.56	1046.81 199.67	1232.14 170.33	1171.47 252.60	983.49 148.89	1374.41 150.96
β-pinene	1214.85 99.62	1376.14 230.78	1056.48 124.00	1174.64 119.92	1272.06 160.84	1583.05 429.69	807.25 162.57	1104.28 236.74	1171.44 426.52	715.77 112.38	871.10 114.92
Limonene	976.73 319.61	752.45 257.95	614.09 174.40	665.99 217.30	993.57 384.52	893.82 306.41	69.62 7.29	485.94 190.62	251.48 157.74	560.10 111.49	1898.03 725.75
3-carene	564.07 370.99	667.17 376.73	515.86 286.40	754.03 457.04	462.49 265.99	702.49 384.02	916.35 545.89	507.75 349.17	608.42 408.08	610.66 392.74	1120.42 575.94
Myrcene	205.72 15.35	184.53 17.42	133.56 6.78	143.14 14.85	201.45 21.90	214.29 19.94	110.64 10.61	139.50 16.20	122.75 13.99	118.21 9.11	217.02 25.61
Camphene	136.44 26.46	125.67 13.86	91.04 14.12	103.60 11.13	208.93 86.08	107.35 17.02	59.70 10.12	76.96 11.95	66.16 12.46	55.88 6.32	76.17 6.21
Bornyl acetate	81.39 36.20	39.77 13.56	36.62 12.87	35.72 10.65	158.77 129.67	26.49 9.10	10.54 3.67	9.25 4.43	8.50 2.86	8.07 3.66	14.90 5.48
Camphor	22.92 5.01	18.64 6.13	11.13 4.96	18.70 4.78	12.75 5.56	7.46 5.03	4.32 4.32	5.84 3.70	0.00 0.00	7.27 4.62	6.84 6.84

	2L 3F	4L 3F	3F 2L	6F 2L	2L CON	4L CON	3F CON	6F CON	3W CON	6W CON	Un-treated
β-phellan-drene	15.46 7.49	11.28 3.41	4.32 0.33	4.56 0.52	6.14 0.67	7.90 1.41	3.92 0.52	4.33 0.75	4.52 1.20	3.19 0.35	5.28 0.66
Terpinolene	13.73 6.64	13.30 7.75	7.43 3.67	12.75 6.91	17.10 10.26	10.62 5.98	18.83 9.93	9.66 6.18	14.66 8.99	11.70 7.40	20.91 9.29
Alpha-terpineol	13.15 3.61	16.49 5.25	6.09 2.51	9.52 2.86	5.00 1.96	10.01 4.93	9.09 5.88	5.53 2.64	1.58 1.58	5.86 3.88	9.02 4.45
Borneol	13.15 4.16	13.78 5.35	11.42 11.42	4.12 2.92	24.51 13.53	12.70 5.75	3.12 3.12	3.73 3.73	2.56 2.56	2.01 2.01	8.35 8.35
Gamma-terpinene	2.25 1.52	2.16 1.48	1.91 1.32	3.08 1.66	3.02 2.08	1.97 1.32	3.14 1.99	1.35 1.35	3.32 2.21	2.88 1.83	4.56 2.90
Alpha humulene	1.86 1.27	5.32 2.03	3.11 1.13	6.33 1.50	5.85 1.29	6.25 1.59	4.32 2.27	1.98 1.33	3.29 2.11	2.52 1.64	5.27 2.36
ocimene	15.83 11.30	8.39 8.39	0.00 0.00	0.00 0.00	2.83 2.83	20.54 10.80	0.00 0.00	11.12 11.12	0.00 0.00	0.00 0.00	0.00 0.00
Beta-thujone	2.17 2.17	1.78 1.78	0.83 0.83	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00
sabinene	2.02 2.02	0.00 0.00	1.89 1.89	3.51 2.36	3.55 3.55	3.55 2.38	0.00 0.00	2.98 2.98	0.00 0.00	0.00 0.00	5.39 5.39

	2L 3F	4L 3F	3F 2L	6F 2L	2L CON	4L CON	3F CON	6F CON	3W CON	6W CON	Un-treated
Linalool	0.00 0.00	0.00 0.00	1.86 1.86	0.81 0.81	0.00 0.00	4.74 2.51	1.68 1.68	1.27 1.27	0.00 0.00	0.00 0.00	4.01 4.01
Pulgeone	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	1.67 1.67	0.76 0.76	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00
p-cymene	0.00 0.00	0.00 0.00	2.88 2.88	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00
α-terpinene	0.00 0.00	0.00 0.00	13.60 13.60	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.00 0.00

* α-thujone was also included as a standard in gas chromatography but concentrations were 0.