**University of Alberta**

# Jack Pine Signalling and Responses to Herbivory

by

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**Abstract**

Intra and inter-plant signalling was investigated in jack pine (*Pinus banksiana*) seedlings in response to jack pine budworm (*Choristoneura pinus*, Lepidoptera: Tortricidae) feeding. Defoliation was followed by a fungal inoculation by blue stain fungus (*Grosmannia clavigera*) to assess resistance. Results from greenhouse experiments showed that: 1) intra-plant signalling was mediated by intensity of larval defoliation, 2) intra-plant signalling was not observed with mechanical wounding 3) seedling resistance to a fungal pathogen depended on type of defoliation before inoculation, and 4) volatile-exposure from defoliated seedlings could mediate resistance to subsequent fungal infection. In mature jack pine stands in Ontario, needle monoterpene concentrations decreased on budworm defoliated and nearby branches. Monoterpene concentration in the phloem of mature trees was higher in trees with high budworm infestation. This research contributed to the understanding of inducible responses and volatile signalling in conifer systems. Effects of herbivory on jack pine were investigated though analysis of volatile and tissue monoterpenes, known to mediate multi-organismal ecological interactions.

**Key Words:** inter-plant communication, conifer defence, volatile organic chemicals, insect-plant interactions, *Pinus banksiana*, *Choristoneura pinus*, *Grosmannia clavigera*

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# Chapter 1

## General Introduction

Conifers inhabit a vast range of biomes on earth, and are widespread in the northern hemisphere. Fossil evidence dates conifers back to the late Carboniferous (Scott and Chalone, 1983). Their longstanding presence on earth and wide distribution underscore their success. A large part of this success is due to their effective defence strategies against many types of biotic and abiotic stressors such as drought, flooding, and a plethora of insect and pathogenic enemies (Franceschi et al., 2005). For this reason, some level of generalized constitutive protection is essential; though it can be a metabolically costly investment. Constitutive defences are constantly present, at any moment ready to help mitigate and inhibit an attack. Induced defences however, are only activated upon attack or stress, and can also be more specific to the stressor (Franceschi et al., 2005). Induced responses may also activate further production of a constitutive response to sufficiently protect the tree (Franceschi et al., 2005).

One important induced response to damage in conifers is resin accumulation in existing resin ducts, or *de novo* production of traumatic resin ducts (Franceschi et al., 2005; Miller et al., 2005). Resin ducts contain oleoresin, present in the needles, stems, and roots (Bohlmann, 2008). Oleoresin is largely composed of terpene chemicals. Of these, the volatile monoterpenes (10 carbon compounds) are the

most abundant. Also present in oleoresin to a lesser extent are sesquiterpenes (15 carbon compounds), as well as the non-volatile diterpenes (20 carbon compounds) that solidify the resin upon exposure to the air (Keeling and Bohlmann, 2006).

Interactions between trees and insects play a significant role in shaping forest dynamics. For example, the jack pine (*Pinus banksiana* Lambert, Pinales: Pinaceae) and jack pine budworm (*Choristoneura pinus* Freeman, Lepidoptera: Tortricidae) system. Jack pine has a large range across North America, east of the Rockies, as well as being an important pulp and timber resource. Its most prevalent defoliator is the jack pine budworm. This essentially monophagous micro-lepidopteran, has a univoltine life cycle: it emerges from overwintering under the bark as first instar larvae in late May, and begins to feed on the pollen cones (Nealis, 1995). The budworm then feeds on fresh needles until pupation in early August, after six or seven instars. Eclosion occurs after one to two weeks and adults mate within a week, laying eggs along needles in clusters of over one hundred eggs (Nealis, 1995). Defoliation by larvae of this pest can infrequently result in top kill, but more importantly, induce susceptibility to tree killing bark beetles such as *Ips grandicollis* Eichhoff (Coleoptera: Scolytidae), and wood-boring beetles (Wallin and Raffa, 1999). This interaction is primarily mediated by induced responses, which result in physiological and anatomical changes, as well as the up-regulation and release of chemical compounds from plant tissues.

The interaction of jack pine budworm on jack pine and tree-killing bark beetles is of particular importance in the northern boreal forest. The impact of defoliation by the jack pine budworm may influence the movement of the mountain pine beetle, (*Dendroctonus ponderosae* Hopkins, Coleoptera: Curculionidae) on jack pine. The jack pine budworm life cycle has some overlap temporally, and spatially with mountain pine beetle's life cycle, and current range (Colgan and Erbilgin, 2010). The recent range expansion of this tree-killing pest into Canada's western jack pine forests (Cullingham et al., 2011) makes these interactions particularly relevant for further research.

Volatile monoterpenes are arguably the most active and ecologically relevant mediators of induced responses in conifers since they give the oleoresin fluidity and therefore mobility while in the ducts, and can also act as semiochemicals. Monoterpenes can deter insects and prevent fungal growth, generally repelling attacking organisms (Raffa and Smalley, 1995; Wallin and Raffa, 1999; Miller et al., 2005; Bonello et al., 2006; Thoss and Byers, 2006). Monoterpenes can also attract herbivores, their parasitoids or predators (Wallin and Raffa, 1999; Hulcr et al., 2006; Raffa et al., 2007). Although at high concentrations these compounds may be repellent, insects such as bark beetles have adapted to metabolize these toxins for use as pheromones (Seybold et al., 2006; Erbilgin et al., 2007; Aukema et al., 2010). For example, the mountain pine beetle can sequester the monoterpene $\alpha$-pinene for the production of an aggregation pheromone (Pitman, 1971; Gries et al., 1990)

On the plant level, monoterpene expression in conifers can be triggered by plant hormones like methyl jasmonate which can initialize terpene synthesis pathways. These pathways and hormones regulate defence genes, which can for example induce the production of proteinase inhibitors which may alter foliar quality (Farmer and Ryan, 1990). These responses can be triggered even by applying methyl jasmonate non-intrusively, which can further induce tree defences against future attacks (Erbilgin et al., 2006). Since monoterpenes are mobile, they can appear in parts of the tree not yet affected by insect attacks (Heijaria et al., 2011). Thus, initial attack induces future resistance, which has been termed systemic induced resistance (Bonello et al., 2001; Erbilgin et al., 2006).

The broad aim of my M.Sc. thesis was to test within and between plant signalling in conifers. Jack pine and jack pine budworm were used as the model species for these studies. The blue-stain fungus (*Grosmannia clavigera* Robinson-Jeffrey R.W. Davidson, Ophiostomatales: Ophiostomataceae), was used to assess resistance in jack pine seedlings. This fungus is a symbiont to the mountain pine beetle: the beetles carry the fungi to a new pine host, while the fungi weaken tree defences, for ease of colonization by the beetle. Testing this mountain pine beetle

associated fungus as a medium to assess jack pine resistance after defoliation by the jack pine budworm is a first step to understanding these complex interactions.

In deciduous trees and other angiosperm species, volatile secondary metabolites, much like monoterpenes, induce resistance in their neighbours (Arimura et al., 2000; Tscharntke et al., 2001; Schmelz et al., 2003; Engelberth et al., 2004; von Dahl et al., 2007). In Sitka willows (*Salix sitchensis*), reduction in leaf quality occurs in defoliated and neighbouring undefoliated trees (Rhoades, 1983). This observation has started a new branch of inducible defence research: inter-plant communication. There is still much unknown about conifer response pathways and inducible resistance. The question remains: can resistance be induced by exposure to volatiles from a damaged neighbour? Chapter 2 of this thesis argues that inter-plant signalling is as likely in conifers as in angiosperms. In Chapter 3, I applied the systemic responses within jack pine seedlings, by monitoring volatile monoterpene emissions from defoliated and foliated branches from the same seedling. I tested two different defoliation intensities, and mechanical defoliation, while simultaneously monitoring the responses of monoterpene emission in neighbouring seedlings. Results showed that intra-plant signalling was mediated by intensity of larval defoliation, though mechanical wounding did not induce higher emissions from branches.

Tree pathogens are often used as a proxy for measuring plant resistance: recently, *G. clavigera* was used to simulate mountain pine beetle attack (Colgan and Erbilgin, 2011; Erbilgin and Colgan, In Press). Chapter 4 encompasses the responses of seedlings to *G. clavigera* after either defoliation, or in relation to proximity to a defoliated neighbour. Volatile responses of seedlings on a whole-tree level were monitored over the course of eight weeks, and tissues from needles and phloem were sampled thereafter. Wound lesions from fungal inoculations were also measured. Results demonstrated that seedling resistance to a fungal pathogen depended on type of defoliation, and volatile-exposure from defoliated seedlings mediated resistance to the fungal infection. In Chapter 5, I tested the effects of

induced responses by herbivory in mature jack pines in a field setting by collecting needles and phloem from trees in stands of different defoliation intensities. A manipulative assay was incorporated to confirm the effects of larval feeding on needle tissues. In that assay, needle monoterpene concentration decreased on defoliated and nearby branches after 3 days of budworm feeding. Monoterpene concentrations in phloem of mature trees were higher in stands with high budworm infestation. Lastly, In Chapter 6, I discuss my results in the broader context of research on conifer induced responses and suggest ideas for future researchers in this field.

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# Chapter 2

# Review: A case for conifer inter-plant communication

## 2.1 Introduction

Research on volatile-induced defences in conifers is still in its infancy, though recent evidence demonstrates induced resistance without physical contact (Hudgins and Franceschi, 2004; Erbilgin et al., 2006). As an introduction to the primary research of this thesis, I provide further evidence that conifers too have the potential for inter-plant signalling.

Interest about inter-plant interactions was triggered by a study that reported reduced leaf quality not only in defoliated Sitka willows (*Salix sitchensis*), but also in their healthy neighbours (Rhoades, 1983). In that same year, the anti-herbivore responses of volatile-exposed poplar (*Populus euroamericana*) and sugar maple (*Acer saccharum*) saplings gave further evidence of inter-plant signalling (Baldwin and Schultz, 1983).

## 2.2 Inter-plant communication

In order to better analyze the concept of inter-plant interactions, I identify the steps, and the critical players in this process. First, a trigger or induction agent (herbivory for example) causes a response in the emitter plant, leading to an induction of volatile organic chemicals (VOC's). The induced response could have immediate consequences to the emitter plant itself, such as reduction of foliar quality; which in turn reduces feeding or slows larval development. For example, significantly fewer Norway spruce trees (*Picea abies*) were killed by *Ips typographus* after priming with a blue stain fungus (*Ceratocystis polonica*), a fungal associate of *I. typographus* (Christiansen and Krokene, 1999; Christiansen et al., 1999). These effects can be long lasting for the emitter plant, as defoliation or fungal infection can reduce the rates of herbivory even one year after the initial damage (Krause and Raffa, 1992). The process whereby induced responses invoke lasting protective effects against pathogens and other organisms is often categorized as systemic acquired resistance (Durrant and Dong, 2004).

The induced response of the emitter plant can also act beyond the limits of the emitter. It exhibits distance signalling effects, which can trigger defence responses in a neighbouring receiver plant. The receiver plant itself activates certain metabolic responses leading to increased VOC's, priming the plant to increase its defences to subsequent threats (Arimura et al., 2000; Schmelz et al., 2003; Engelberth et al., 2004).

The evolutionary benefits of inter-plant signalling for the emitter plant are a topic of debate (Heil and Karban, 2010). On one hand, there is evidence that the receiver plant is simply eavesdropping, or taking advantage of the responses of its damaged neighbour. For example, the emitter benefits directly from emitting deterring insects and preventing fungal growth, generally repelling attacking organisms (Moraes et al., 2001; Shiojiri et al., 2006). The emitter also benefits indirectly from its VOC's by attracting natural enemies of herbivores

(Turlings et al., 1990). VOC's are important for mediating within-plant interactions as well (Rodriguez-Saona et al., 2009). These findings suggest that the response of the receiver plant is a secondary effect that evolved independently from the fitness response of the emitter, and simply as a result of VOC's in the air; or that inter-plant signalling has evolved from within-plant signalling. On the other hand, recent evidence shows that there can be self-recognition in plants: sagebrush plants *Artemisia tridentata*, exposed to volatile cues from genetically identical cuttings of itself were less damaged than those receiving cues from cutting form a non-self sagebrush plant (Karban and Shiojiri, 2009). This evidence is a step towards showing that volatile inter-plant communication may be used for kin selection, improving the overall fitness of direct offspring.

Few studies have examined each step of the complex process of inter-plant signalling. Several plants have been used as subjects to unravel the mechanisms of volatile inter-plant communication. Table 2.1 documents some notable research on angiosperm inter-plant signalling in chronological order, blank spots indicate elements which were not covered by the referenced studies . There are many similarities between types of induced chemicals emitted and received. Table 2.2 introduces what is known about conifer responses to various induction agents. Note the similarities in certain monoterpenoid compounds, and the common octadecanoid pathway.

### 2.2.1 The emitter

Upon induction by herbivory, pathogen infection, mechanical wounding or application of plant hormones such as methyl jasmonate, activation of response in the emitter leads to VOC emission. In general, the response is fairly specific to plant and elicitor combinations, as is demonstrated in conifers and angiosperms alike (Dicke and Hilker, 2003; Mumm and Hilker, 2006). In corn, VOC's emitted are specific to the larval stage of the defoliator, which can benefit parasitoids

using these cues for host location (Takabayashi et al., 1995). Some responses remain general however, and can be simulated with mechanical wounding (Dicke and Hilker, 2003). The mechanism of induction is well known in angiosperms. In general, the wounding event or induction causes metabolic changes in the plant that activate plant hormones through signal transduction. For example methyl jasmonate (the methyl ester of jasmonic acid) triggers the metabolic cascade through the octadecanoid pathway; and methyl salicylate (methyl ester of salicylic acid) through the shikimic pathway (Dicke and Hilker, 2003). The pathway induced depends on the type of wound or trigger (Karban and Baldwin, 1997). These pathways and hormones regulate defence genes, which can also induce the production of proteinase inhibitors. These inhibitors alter the foliar quality (Farmer and Ryan, 1990), and in turn activate genes that stimulate volatile emission, including terpenes (Figure 2.1).

In conifers, there are still gaps with regards to the mechanisms of signal transduction for the formation and accumulation of terpenoids. However, recent research indicates that the octadecanoid pathway and its metabolites, together with the ethylene pathway play major roles, much like in angiosperms (Hudgins and Franceschi, 2004; Phillips et al., 2006). A review paper by Phillips et al. (2006) compiled possible system of induction in conifer emitters, modelled in Figure 2.2. Note the similarity to the angiosperm induction diagram in Figure 2.3.

Research in conifer defence responses thus far is largely driven by experiments applying methyl jasmonate, a plant hormone associated with conifer responses to insect feeding and pathogen attack (Martin et al., 2002; Hudgins et al., 2003). This non-intrusive stimulation method causes resin duct formation, as well as defence response *de novo* in Norway spruce (Martin et al., 2002; Erbilgin et al., 2006; Krokene et al., 2008). In the Pinaceae, the octadecanoid and ethylene pathways are the likely responders to the non-intrusive stimuli of methyl jasmonates, as well as insect and pathogen stimuli (Bohlmann, 2008; Miller et al., 2005).

Terpenes are an important component of conifer defence. For example, they are present in the oleoresin of pines, the fluid responsible for the physical barriers and pitch tubes. The oleoresin can literally force out invading organisms, and it is composed largely of volatile monoterpenes (Langenheim, 2006). Figure 2.4 shows the major terpenes in conifers, including those present in resin, and the general pathways from which they are derived. Although Green Leaf Volatiles (GLV's) are the general volatiles emitted from angiosperms, these compounds share terpene synthases in common with conifers. GLV's are C6 aldehydes, alcohols, and their esters and may also present in the volatile vocabulary of the coniferous trees. Norway spruce trees *Picea abies* emit a blend of GLV's in their mini-seedling stage (Pettersson et al., 2008). Figure 2.1 provides some examples of the signalling network of herbivore damaged angiosperm leaves. Volatile emissions in conifers and angiosperms are very much alike. Both respond to the methyl jasmonate, a key volatile in the process of induction response, and both share similar pathways to VOC emission.

### 2.2.2 The signal

The next step in inter-plant communication is the transmission of a volatile signal from a damaged plant to the neighbouring plant. This signal must have some specific properties, considering the magnitude of random molecules perpetually floating in the air. Plants must differentiate among volatiles usually emitted from their neighbours to identify something worthwhile enough to trigger a response. Modelled by the criteria for hypothetical within-plant transduction signals from Karban and Baldwin (1997), the following criteria are proposed for a hypothetical airborne plant to plant signal:

1. generated by plant stress or damage
2. travels as an airborne molecule
3. recognized by the neighbouring conspecific

4. acts within a relevant time after induction

5. elicits a response in the neighbour

A small molecule such as ethylene is a likely signalling compound (Arimura et al., 2000; Dolch and Tscharntke, 2000; Tscharntke et al., 2001; Schmelz et al., 2003; Engelberth et al., 2004; von Dahl et al., 2007). Although small and can easily diffuse, it may be limited to within plant, branch to branch signalling, or between trees with touching canopies (Baldwin et al., 2006). Ethylene emissions peak in black alder (*Alnus glutinosa*) when attacked by leaf beetles (Tscharntke et al., 2001). In tobacco (*Nicotiana attenuata*), a significant burst of ethylene is also induced, and plants with their ethylene-perceiver gene removed are more susceptible than those with the gene (von Dahl et al., 2007).

Conifers are also prime candidates to receive ethylene signals, in Douglas-fir (*Pseudotsuga menziesii*) and Giant Redwood (*Sequoiadendron giganteum*) ethylene induces a response similar to that from methyl jasmonate induction (Hudgins and Franceschi, 2004). Ethylene and jasmonic acid pathways are often associated in response to stress, yet it is still unclear in what way. Ethylene production can be activated by methyl jasmonate, yet the inverse activation has not yet been observed in conifers (Phillips et al., 2006).

With regards to the possibility of monoterpenes acting as signal molecules, a study on *Arabidopsis* showed induction by $\alpha$- and $\beta$-pinene as well as myrcene (Godard et al., 2008). These compounds may be candidates for signal molecules in conifers as well. Pines are well known to produce $\alpha$- and $\beta$-pinene consistently, and in abundance. However, signal could also be distinguished by a particular ratio of different compounds or short bursts which change concentration ratios of a single compound with regards to the rest (Mumm et al., 2003; Mumm and Hilker, 2005). Larger molecules such as methyl jasmonate or methyl salicylate would be more likely to induce greater volatile plumes, and may therefore be better signals

for longer distances (Baldwin et al., 2006). Signalling molecules in angiosperms and conifers are most likely different, since conifers emit mostly monoterpenes, and angiosperms release mostly GLV's. In both cases, these two groups emit volatiles which could satisfy the criteria for being a signal.

### 2.2.3 The receiver

Finally, it is important to understand the mechanisms of reception of the signal molecule. It is for this reason that this section is the most speculative, since the mechanisms of perception are not certain either in angiosperms or gymnosperms. The most likely hypothesis is that stomata are the key to signal reception (Baldwin et al., 2006). Stomatal opening is triggered by environmental changes, such as humidity, photoperiod or atmospheric pressure, and allows for airborne chemical intake. In *Arabidopsis*, calcium concentration was associated with responses from volatile cues (Asai et al., 2009). This is significant since guard cells of stomata are mediated in part by active transport by Calcium ATPase pumps, and a higher concentration of calcium ions would contribute to stomatal closing (Kinoshita et al., 1995). By this proposed mechanism, induction by a volatile signal would cause an increase of calcium ions in the stomata which cause stomatal closing, and keep foliage-protecting compounds better contained as a result. The influx of calcium ions is shown theoretically in conifers (Figure 2.2), and angiosperms (Figure 2.1). Both groups possess stomatal openings with guard cells for water regulation in their foliage, and open or close them via the same mechanisms. If we assume that stomata are the structures mediating reception of volatile signals for both groups, it is likely that the mechanisms of between plant signalling are the similar as well.

## 2.3 Next steps for conifer inter-plant signalling

Mechanisms of induction for the emitter are similar in conifers and angiosperms: for example, the induction of the octadecanoid pathway, and responses

to hormone triggers like methyl jasmonate. Terpene synthases are enzymes that produce volatiles in both conifers and angiosperms, and in response to herbivory increase production of VOC's. There is evidence for ethylene and methyl jasmonate as signalling molecules (Hudgins and Franceschi, 2004), though additional research is needed to identify the volatile responses, and mechanisms of conifer defence responses in general. The next step could be to test whether different concentrations or ratios of monoterpenes can trigger a defence response in conifer seedlings, or cuttings for example. Further research into the physical receiving mechanisms is especially needed in both conifers and angiosperms. Finally, it is worthwhile to test ethylene as a signal in conifer responses. Looking into the relationship between ethylene and jasmonic acid pathways in conifers could also be beneficial for better understanding conifer response mechanisms to herbivores or airborne cues.

| | *EMITTER* | | | *RECEIVER* | |
|---|---|---|---|---|---|
| **Plant** | **Trigger for induction** | **Induction** | **Specific Trigger of response** | **Response** | **Reference** |
| Poplar (*Populus euroamericana*) | Mechanical wounding | Increased production of phenolics | Ethylene (proposed) | Increased production of phenolics | Baldwin and Schultz, 1983 |
| Maple (*Acer saccharum*) | Mechanical wounding | Increased production of phenolics | Ethylene (proposed) | Increased production of phenolics | Baldwin and Schultz, 1983 |
| Willow (*Salix sitchensis*) | Natural herbivory | Decreased nutrient richness in foliage (implied) | | Decreased nutrient richness in foliage | Rhoades, 1983 |
| Lima bean (*Phytoseiulus persimili*) | Spider mite (*Tetranychus urticae*) | Sesquiterpenes, homo-terpenes | Ethylene, GLV's | Volatiles increased as high in infested plant | Arimura et al., 2000 |
| Alder (*Alnus glutinosa*) | Leaf beetle (*Agelastica alni*) | Monoterpenes, sesquiterpenes, homo-terpenes, fatty acid derivatives and aromatic compounds, methyl salicylate, indole, ethylene | Suggested triggers: ethylene, monoterpenes, sesquiterpenes, GLV's and possibly methyl jasmonate | | Tschtarntke et al., 2000 & 2001 |
| Corn (*Zea mays*) | Beet armyworm on corn (*Spodoptera exigua*) | Ethylene, sesquiterpenes, indole | GLV's, ethylene | Slight stimulation of volatile release by receiver plants, and ethylene | Engelberth, 2004, Schmelz et al., 2003 |
| Arabidopsis (*Arabidopsis thaliana*) | | | GLV's and monoterpenes | promoted transient increases in [Ca2+], Increased levels of methyl jasmonate | Asai et al., 2009, Kishimoto et al. 2005, Godard et al., 2008 |
| Tobacco (*Nicotiana attenuata*) | Tobacco hornworm (*Manduca sexta*) | Ethylene | Ethylene | inhibited plant response to ethylene | Dahl et al., 2007 |

Table 2.1: Examples of volatile plant communication in some angiosperm species arranged by processes in the emitter and receiver plants. Left columns deal with the agent of induction, mechanism of induction and induction chemicals of the emitter while the columns on right specify the response triggers, mechanisms and responses of the receivers. Blank spaces indicate areas not covered by the study mentioned.

| | *EMITTER* | | |
|---|---|---|---|
| **Plant** | **Trigger for induction** | **Induction** | **Reference** |
| Ponderosa pine | insect attack and mechanical wounding | alpha and beta pinene, delta-3-carene, limonene, myrcene | Litvak et al., 1998 |
| Sitka spruce | white pine weevils | α-pinene, myrcene, β-pinene, sabinene, terpinolene, linalool, farnesene, bisabolene | Miller et al., 2005 |
| Norway spruce seedlings | Mechanical wounding | hexenol and hexenal, α-pinene and limonene | Pettersson et al., 2008 |
| Grand fir | insect attack and mechanical wounding | camphene, phellandrene, terpinolene, limonene, pinene, myrcene | Bohlmann et al, 1999<br>Steele et al., 1998 |
| Loblolly Pine | Mechanical wounding | α-pinene, camphene, limonene, myrcene, 3-carene, β-phellandrene | Phillips et al. 1999 |
| Scotch pine | insect attack and mechanical wounding | α-pinene, camphene, limonene, myrcene, 3-carene, β-phellandrene, terpinolene | Sadof & Grant 1997 |

Table 2.2: Examples of volatile plant responses in some conifer species, arranged by the induction agent, mechanism of induction and responses of the emitter only. Receivers of volatile signals are still unknown in conifer systems.

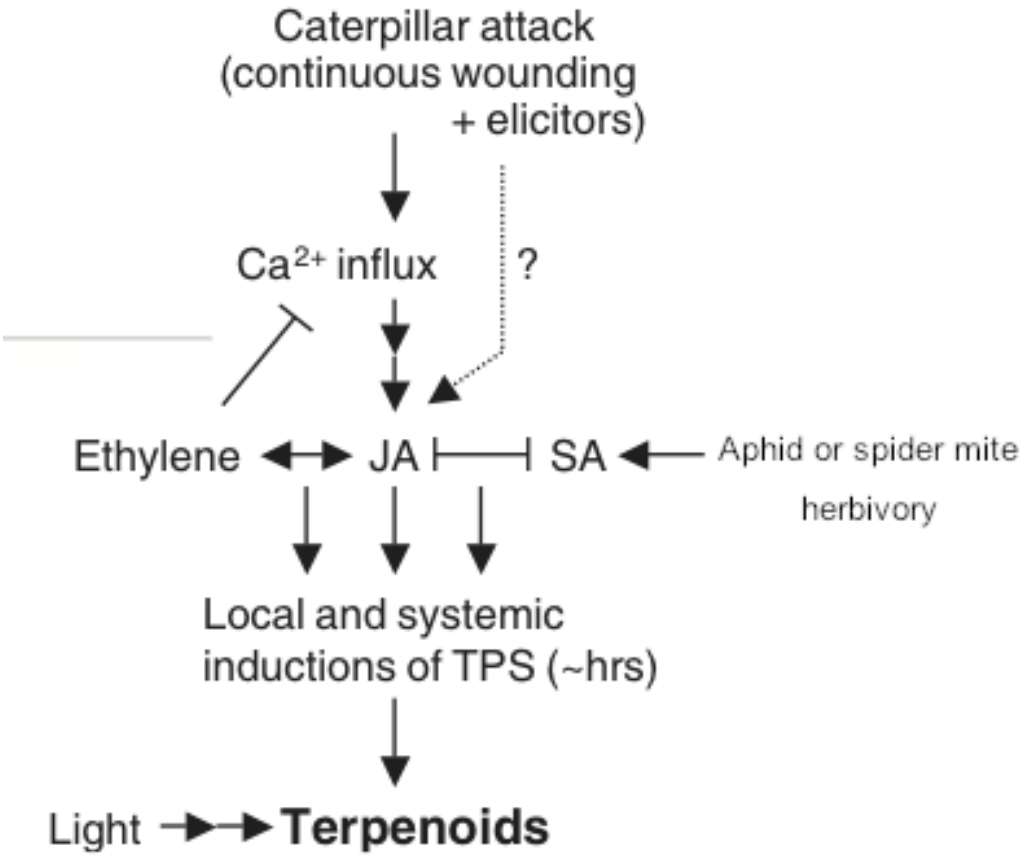


Figure 2.1: Model of the signalling network required for terpenoid biosynthesis in chewing arthropod-damaged leaves and sucking arthropod-damaged leaves. Images of the change in leaf calcium ion influx following insect damage are shown. Arrows and bars indicate positive and negative interactions, respectively. The overall scenario may differ in certain plant taxa. JA, jasmonic acid; SA, salicylic acid (Arimura et al., 2009).

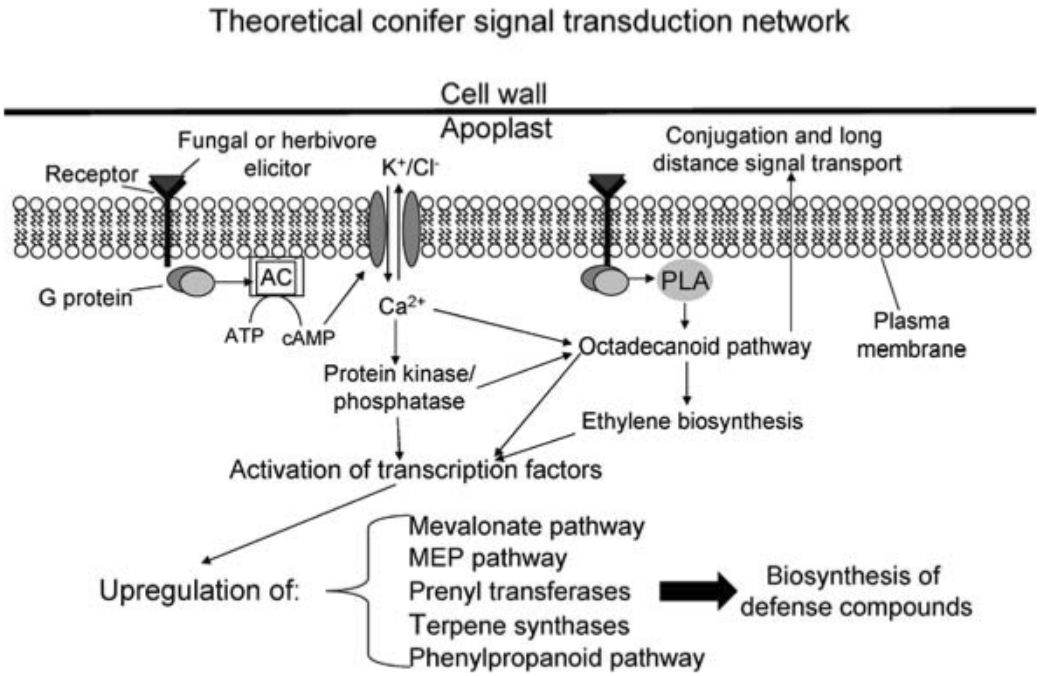


Figure 2.2: Outline of a possible signalling network in the formation of induced conifer defences based on evidence from angiosperm systems and reports cited in this chapter. Abbreviations: AC, adenyl cyclase; PLA, phospholipase A. Adapted from Phillips (2006).

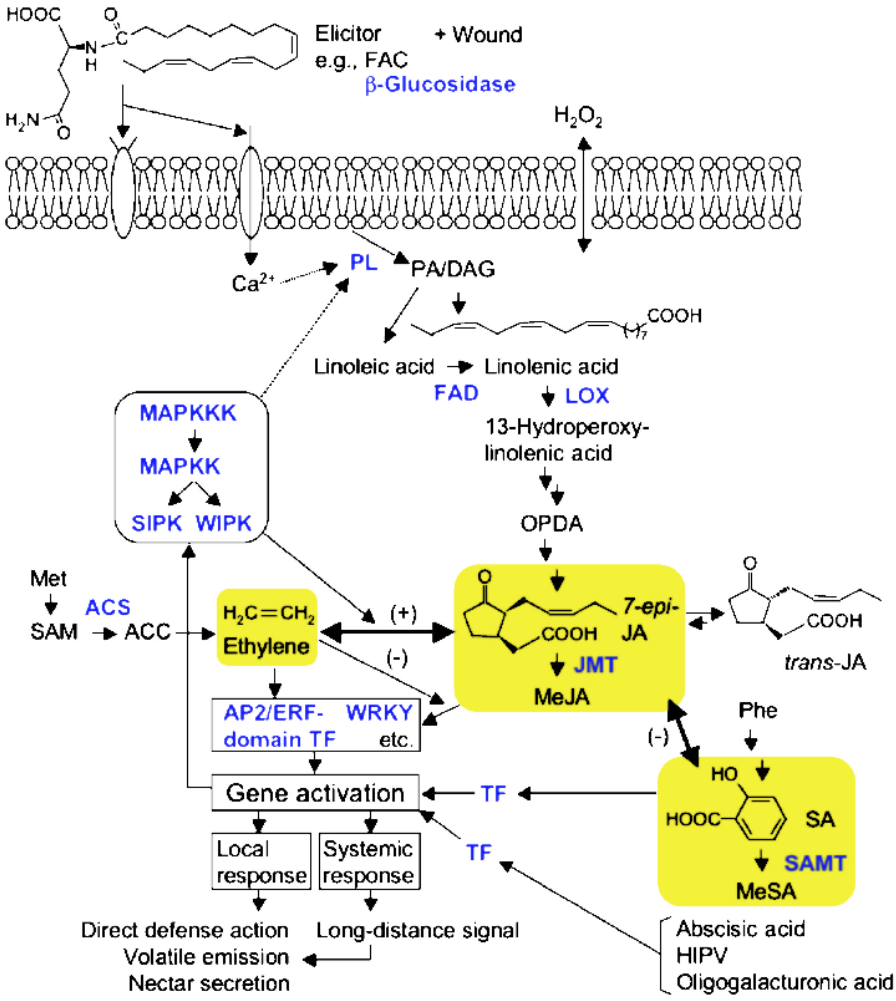


Figure 2.3: Schematic representation of the signalling pathways required for herbivore-induced responses in plants. This scheme merges the evidence obtained from several plant taxa. The overall scenario may differ in certain plants; in particular the existence and the extent of synergistic and antagonist interaction between pathways may vary significantly. Elements in blue represent enzymes. Broken arrows indicate possible steps not yet described. Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ACS, ACC synthase; DAG; diacylglycerol; FAC, fatty acid-amino acid conjugate; FAD, N-3 fatty acid desaturase; HIPV, herbivore-induced plant volatiles; JA, jasmonic acid; JMT, JA carboxyl methyl transferase; LOX, lipoxygenase; MAPK, mitogen-activated protein kinase; MeJA, methyl JA; MeSA, methyl SA; OPDA, 12-oxophytodienoic acid; PL, phospholipase; PA, phosphatidic acid; SA, salicylic acid; SAM, S- adenosyl-methionine; SAMT, SA carboxyl methyl transferase; TF, transcription factor (Arimura et al., 2005).

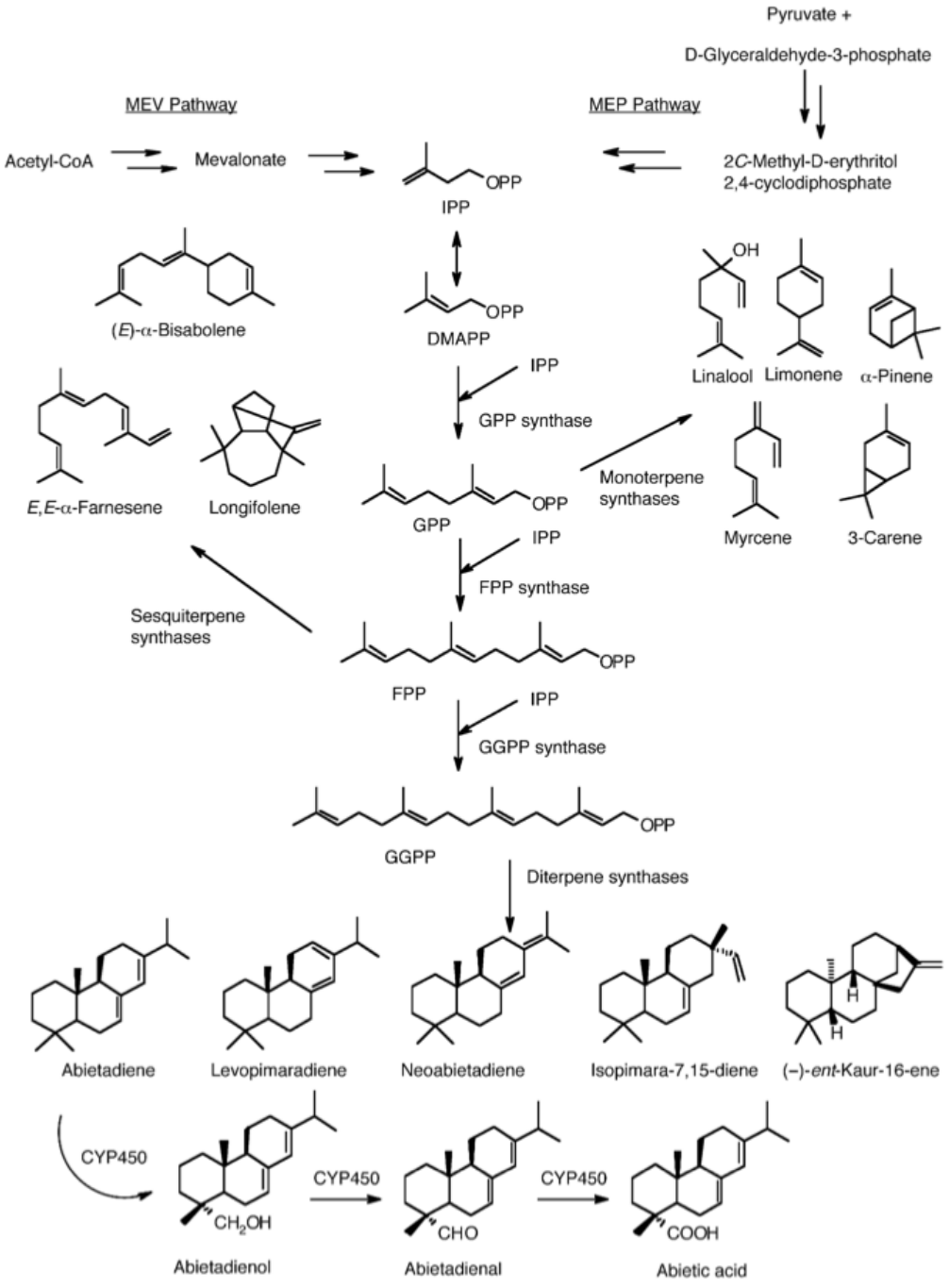


Figure 2.4: Biosynthesis of terpenes. Prenyl transferases condense one or more isopentenyl diphosphates (IPPs) with dimethylallyl diphosphate (DMAPP) from the mevalonate (MEV) or methyl-erythritol 4- phosphate (MEP) pathways to produce geranyl diphosphate (GPP), farnesyl diphosphate (FPP), or geranylgeranyl diphosphate (GGPP). Terpene synthases then use these diphosphates as substrates to form the various terpenes. Additional enzymes, such as CYP450s, can further functionalize these terpenes (Keeling and Bohlmann, 2006).

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# Chapter 3

# Greenhouse Experiment Part I: Monitoring Systemic and Inter-plant Signalling in Jack Pine Seedlings with Monoterpene Emissions

## 3.1 Introduction

Plants produce chemicals *de novo* in response to herbivory, injury or stress (Karban and Baldwin, 1997). Induced responses can directly deter insects and pathogens (Franceschi et al., 2005; Bonello et al., 2006) but can also increase resistance to subsequent attacks on the same plant at later time (Agrawal, 1998; Krokene et al., 2003). For example, prior contact with a pathogenic fungus significantly reduced fungal lesion size in comparison to unprimed controls in Monterey pine (*Pinus radiata*) (Bonello et al., 2001; Erbilgin et al., 2009). Systemically induced defences are byproducts of initial stimulation after wounding or infection, and can be offset from the initial place or time of damage (Franceschi et al., 2005; Howe and Schaller, 2008).

Induction agents such as herbivory activate plant hormones and other metabolic changes through signal the octadecanoid or shikimic acid pathways.

Methyl jasmonate and methyl salicylate are two such hormones (Dicke and Hilker, 2003). These pathways and hormones regulate defence genes, which induce production of proteinase inhibitors that can alter foliar quality (Farmer and Ryan, 1990), and in turn activate genes that stimulate volatile emission. Induction of volatile organic compounds (VOCs) is shared among the plant kingdom. Terpenoids (primarily mono and sesquiterpenes), as well as green leaf volatiles are well known VOCs that increase in response to insect herbivory (McKay et al., 2003; Howe and Schaller, 2008).

Many studies demonstrate the multifaceted benefits of induced chemical resonses to plants. For example, volatile compounds released from plants upon herbivory can attract natural enemies of herbivores in indirect defence (Turlings et al., 1990; Kost and Heil, 2006; Mumm and Hilker, 2006). Inter-plant communication also results from induced chemical defences. The VOCs produced by a plant upon herbivory have the capacity to prime a conspecific neighbouring plant against current or future attacks (Yi et al., 2010). Communication between injured and uninjured plants reduces herbivory in field crops like corn (*Zea mays*), lima bean (*Phytoseiulus persimili*) and tobacco (*Nicotiana attenuata*) (Arimura et al., 2000; Schmelz et al., 2003; Engelberth et al., 2004; von Dahl et al., 2007) and also in trees like poplar (*Populus euroamericana*), willow (*Salix sitchensis*) and maple (*Acer saccharum*) (Baldwin and Schultz, 1983; Rhoades, 1983; Dolch and Tscharntke, 2000). For example, in black alder (*Alnus glutinosa*), defoliation and oviposition by leaf beetles (*Agelastica alni* Coleoptera: Chrysomelidae) was significantly lower in most proximal neighbours of manually defoliated trees. This observation was attributed to the emissions of ethylene and an array of mono-, sesqui- and homoterpenes by the neigbouring defoliated alders (Tscharntke et al., 2001). Nevertheless, after more than thirty years of research concerning inter-plant communication in angiosperms, it remains an unexplored phenomenon in conifers.

Several studies have identified chemical compounds that elucidate a response in neighbouring plants such as ethylene (Arimura et al., 2000; Dolch and Tscharntke,

2000; Tscharntke et al., 2001; Schmelz et al., 2003; Engelberth et al., 2004; von Dahl et al., 2007). Since ethylene is highly volatile and can easily diffuse, it was proposed that ethylene can function both within and between plant communications (Baldwin et al., 2006). The most prevalent VOCs in conifers are monoterpenes and sesquiterpenes (Kishimoto et al., 2005; Keeling and Bohlmann, 2006). These are present in the resin, which conifers use as physical and chemical defence against intruders (Franceschi et al., 2005). There have been several advancements in understanding which pathways lead to a defence response, and VOC emission in conifers. The octadecanoid pathway and its metabolites, together with the ethylene pathway play major roles in conifers (Hudgins and Franceschi, 2004; Phillips et al., 2006), much like in angiosperms. Although signalling molecules in angiosperms and conifers may differ, a study on *Arabidopsis* showed induction of methyl jasmonate accumulation and changes in its transcriptome by exposure to $\alpha$- and $\beta$-pinene (Godard et al., 2008): two common volatiles emitted by many conifer species.

Research about induced responses is still developing, and can already be observed without physical contact in conifers (Hudgins and Franceschi, 2004; Erbilgin et al., 2006). For example, in Scots pine (*Pinus sylvestris*) L., monoterpene emissions were stimulated systemically from intact foliage as well as at the damaged site (Heijaria et al., 2011).

In the present study, jack pine (*Pinus banksiana* Lambert, Pinales: Pinaceae) seedlings were used to test the hypotheses of within and between plant communication. Since communication among and within plants is a complex and multiphase process, our preliminary work with jack pine was focused specifically on the monoterpene emissions from jack pine seedlings. It was confirmed in several studies that monoterpenes are important components of induced responses of jack pine (McCullough and Kulman, 1991; Raffa and Smalley, 1995; Wallin and Raffa, 1999).

Systemic induced response was also observed by monitoring monoterpenes in jack pine foliage and phloem after herbivory by the jack pine budworm (JPBW)

(*Choristoneura pinus* Freeman, Lepidoptera: Tortricidae) (Colgan and Erbilgin, 2011). Building on that previous work, this chapter is focused specifically on the volatile emissions of jack pine seedlings. This study focused on two main study questions: 1) How does defoliation type (mechanical or larval) and intensity (low: two larvae or high: six larvae) affect jack pine responses over time? 2) Is there communication within or between jack pine seedlings? We monitored monoterpene emissions from defoliated and foliated (unchallenged) branches on jack pine seedlings and we observed changes in monoterpene emission in the healthy, foliated neighbours of defoliated seedlings.

## 3.2 Methodology

### 3.2.1 Treatments and volatile collection

Two-year-old jack pine seedlings from Boreal Horticultural Services Ltd. in Bonnyville, Alberta, were planted in the greenhouse by mid March 2010. They were planted in 4L pots, with planting substrate containing five parts peat, one part perlite and one half-part clay particles. Seedlings were fertilized with (N: 15% P: 30% K: 15%) bi-weekly to exclude confounding factors such as nutrient deficiency (Zhao et al., 2008) and watered regularly until the experiment began mid June, 2010. The average height of the seedlings was 31.2cm $\pm$4.6. The average temperature in the greenhouse was 27.6°C $\pm$1.8, with day length of 18h (supplemented when necessary with artificial lighting) and 60% relative humidity during the course of the experiment.

Early instar jack pine budworm larvae, acquired from jack pine stands in Ontario, Canada were allowed to feed on artificial diet bought from McMorran Diet from Insect Production Services, Great Lakes Forest Research Centre in Sault Ste. Marie, Ontario. This diet is used as an alternative to the pollen diet of the early instars. After $3^{rd}$ instar, the larvae usually begin to feed on needles.

Four treatments were set up with either no larvae (control), two or six jack pine budworms per seedling or mechanical defoliation. Larval treatments had two larvae on each branch, thus for the treatment with six larvae, three branches were used. The larvae were left to feed on needles for two weeks within mesh bags. Volatile collections were made from one defoliated and one foliated (adjacent) branch from each seedling once per week. Larvae were removed during the sampling period. Mechanical defoliation was administered by snipping a 2-5 needles daily around the base-midpoint of the needle to mimic defoliation by herbivory.

Each seedling was paired with a unchallenged (foliated) seedling, designed to assess whether defoliation intensity (0, 2 or 6 larvae) or type of defoliation (mechanical or larval), play any role in volatile emission and response from the neighbouring seedlings. Two pairs of seedlings with the same treatment were air-isolated from the rest by vapour barrier plastic sheets with a slightly open top, to avoid moisture accumulation and unwanted microbial growth on trees. There were five such enclosures (0.4m x 0.4m x 1.0m) for each treatment, totalling 80 trees, see greenhouse placement diagram (Figure 3.1).

Volatile collections were made from the same branch of each seedling for all weeks except if the larvae were relocated to a new branch after 100% branch defoliation. After the two week defoliation period, volatiles of whole seedlings were collected from one pair of each enclosure. Volatiles were collected by enclosing the whole seedlings (or just a branch) with an oven bag (LOOK®). The oven bags were cut down to a quarter of their size and heat-sealed at the edges prior to use for branch collections. The open end of the bag was gently tied around the branch. An absorbent tube (Porapak Q (OD 6mm, length 110mm; absorbent: front layer 150 mg, back up layer 75 mg; separated by glass wool) SKC Inc., Eighty Four, Pennsylvania, USA) was positioned through a small hole in the upper corner of each oven bag. Volatile samples were collected through Porapak tubes and tubing for one hour using small pumps at an airflow rate of 0.4L/min. Porapak tubes were

sealed with Teflon caps immediately after volatile collection and extracted directly or frozen at -40°C until extraction.

At a given sampling time, ten pairs were sampled, from two seedlings from each treatment type. The same sets of seedlings were always sampled at the same time, in order to maintain a comparable set of data over time, with an interval of one week between each sample collection (including the control run, prior to defoliation). Herbivory was measured after two weeks by assessing the average percentage defoliation per branch from each seedling, divided by the total number of branches.

### 3.2.2 Chemical analysis

Porapak Q tubes were extracted with 1mL of dichloromethane (Sigma-Aldrich, St. Louis, Missouri, USA) spiked with 0.01% (v/v) tridecane (Sigma-Aldrich, St. Louis, Missouri, USA) as surrogate standard (since its retention time is within the timeframe of this GC-MS method, and does not interfere with extracted sample compounds) and subsequent extraction was stored at -40°C before GC/MS analysis. Extracts (1$\mu$L) were injected in an Agilent 7890A/5062C Gas Chromatograph/ Mass Spectrometer (Agilent Technologies, Santa Clara, California, USA) equipped with an HP Innowax (Agilent Technologies) column (I.D. 0.25 mm, length 30m). The helium carrier gas flow was set at 1.0 mL/min and the following temperature programme was applied: 50°C for 2 min, increased to 60°C by 1°C per min and then ramped up to 250°C by 20°C.

The following standards were used to determine concentrations of individual compounds: Borneol, pulegone, $\alpha$-terpinene, $\gamma$-terpinene, $\alpha$-terpineol (Sigma-Aldrich, St. Louis, Missouri, USA), camphor, 3-carene, $\alpha$-humulene, terpinolene, $\alpha$-thujone and $\alpha$-thujone, (-)-$\alpha$-pinene, (-)-$\beta$-pinene, (S)-(-)-limonene, sabinene hydrate, myrcene, (-)-camphene, p-cymene (Fluka, Sigma-Aldrich, Buchs, Switzerland), bornyl acetate and cis-ocimene (SAFC Supply Solutions, St. Louis, Missouri, USA), $\beta$-phellandrene (Glidco Inc., Jacksonville, Florida, USA).

### 3.2.3 Statistical analyses

Analyses were performed using R statistical software (R, 2010) or SPSS version 17.0 (SPSS, 2008.). If data was not normally distributed, it was log transformed for a normal distribution. For all comparisons, Shapiro-Wilk tests for normality and Levene's test for homogeneity of variances were performed to ensure that data met standard assumptions for mixed effects models. The 'nlme' package was used in R (Pinheiro et al., 2012). F-values were reported followed by numerator and then denominator degrees of freedom along with associated p-values. Post-hoc tests were reported with p-values and reported from mixed model outputs in R. Total monoterpene emissions from whole seedlings were modelled with the dependent variable as total monoterpene concentration, and the factor included in the model was treatment type. Treatment type was separated into unchallenged (foliated) and defoliated branches (or seedlings). Since these seedlings were contained in enclosures, the enclosure was used as a random factor in the analysis. This same model was used for individual monoterpenes as well as the total monoterpenes emitted from whole seedlings. Other models were compared (each seedling nested within the enclosure) using the Akaike Information Criteria (AIC), but the one using only enclosure as random factor accounted for the most variation with the fewest variables.

For comparisons of branches on the same seedling over time, data was not normally distributed and failed to meet assumption of normality even with logarithmic transformations, so the Friedman test for repeated measures was used with post-hoc comparisons with Wilcoxon Signed-Rank tests. Time was used as the repeated measure, and comparisons were made within treatment type and corrected for with the Bonferroni adjustment. For these analyses, $\chi^2$ values are reported with degrees of freedom in parentheses, followed by associated p-values.

## 3.3 Results

The average defoliation per seedling for seedlings defoliated by two larvae was 11.5% ±1.8 , 38.4% ±4.4 in seedlings treated by six larvae and 22.7% ±2.9 of the seedling was defoliated by mechanically defoliated seedlings.

### 3.3.1 Within-seedling systemic communication

Overall, monoterpene emission from branches fed on by by two-larvae were significantly different from controls over the course of the two week defoliation period ($\chi^2(4) = 12.7$, p= 0.013, Figure 3.2-A). In the first week of defoliation, both defoliated and foliated branches of seedlings emitted a higher concentration of monoterpenes than branches prior to defoliation (p=0.038, p=0.036, respectively). By the second week however, the monoterpenes emitted from all branches decreased, with no significant differences from emissions observed from the branches prior to defoliation (defoliated branch vs. control: p=0.515, foliated branch vs. control: p=0.066).

For the six-larvae treatment, there was a significant difference in monoterpene emissions from the branches over the course of the two week defoliation period ($\chi^2(4) = 14.000$, p= 0.007, Figure 3.2-B). In the first and second week of defoliation, both defoliated and foliated branches of seedlings emitted more monoterpenes than branches prior to defoliation; at week 1, foliated branches vs. control: p=0.022; defoliated branches vs. control: p=0.009; and week 2: foliated branches vs. control: p=0.017; defoliated branches vs. control: p=0.05.

Regarding the mechanically defoliated seedlings, there were no differences in monoterpene emissions from branches ($\chi^2(4) = 6.3$, p= 0.178, Figure 3.2-D), though the pattern of emission is similar to the 6-larvae treatment. In the first and second weeks of mechanical defoliation, the defoliated branches remained similar

to controls (n=8): week 1 (p=0.093, week 2: p=0.161). The branches that remained unchallenged (foliated) over the two week period also maintained similar concentrations of monoterpene emissions to branches prior to defoliation (week 1: p=0.484, week 2: p=0.263). Differences were noted only between unchallenged (foliated) and defoliated branches on the same tree at week one (p=0.037). Branches of mechanically defoliated seedlings remained similar in monoterpene emissions throughout the two-week sampling period ($\chi^2(2) = 3.176$, p= 0.204, Figure 3.2-C). A summary table showing sample sizes with means ±standard error is shown in Table 3.1

### 3.3.2 Treatment intensity and inter-plant communication

Total monoterpene emissions from whole-seedling volatile collections were significantly higher in treated seedlings than untreated control seedlings ($F_{(6,14)}$=9.6382, p<0.001, Figure 3.3) two larvae vs. control: p= 0.0047, six larvae vs. control: p=0.0008, mechanical vs. control: p=0.0018). There were no differences in monoterpene emissions between neighbours of treated seedlings and controls: two larvae neighbour vs. control p=0.1090; six larvae neighbour vs. control: p=0.9061; and mechanical-neighbour vs. control: p= 0.3307. There were also no differences between emissions of two larvae and six larvae defoliated seedlings (p=0.3639), two larvae and mechanically treated seedlings (p=0.6284) or mechanically treated seedlings and six larvae treated seedlings (p=0.665).

The six most abundant monoterpenes contributing to 97% of the total volatile emissions were: $\alpha$-pinene, $\beta$- pinene, 3-carene, limonene, $\beta$-phellandrene and camphene. Their ratios with regards to the total emissions for each treatment are shown in Table 3.2. $\alpha$- and $\beta$-pinene were the majority of the volatile blend released (Fig. 3.4 A-B). There were no significant differences in emission of 3-carene from any of the defoliated seedlings, or their neighbours (Fig. 3.4 C). Defoliated seedlings differed in their concentration of limonene emission from controls in emissions in

two larvae treated seedlings (p=0.0396), and from mechanically defoliated seedlings (p=0.0303, Fig. 3.4 D). None of the neighbouring seedlings differed from controls in terms of limonene emission, thus specific monoterpenes of exposed neighbours were not induced by the release of volatiles from defoliated seedlings.

Two minor monoterpenes which contributed up to 5% of the total monoterpenes were $\beta$-phellandrene and camphene. All defoliated seedlings emitted significantly higher concentrations of $\beta$-phellandrene compared to controls ($F_{(6,14)}$=6.5800, p=0.0018). Similarly, all defoliated seedlings emitted higher concentrations of camphene compared to controls, ($F_{(6,14)}$=6.6135 p=0.0018). However, there was no significant difference in concentrations of $\beta$-phellandrene or camphene emissions in neighbouring seedlings compared to controls. Both $\beta$-phellandrene and camphene increased most drastically in terms of ratio to total monoterpenes in the mechanically defoliated seedlings compared to controls ($p<0.001$).

## 3.4 Discussion

### 3.4.1 Within-plant communication in jack pine

Increased monoterpene emissions from undamaged branches of damaged jack pine seedlings in both two-larvae and six-larvae treatments suggests systemic or within-plant induction response in jack pine. Furthermore, systemic induction appears to be mediated by an activation threshold based on defoliation intensity. This is demonstrated by the decrease in emissions after two weeks in the low intensity (two-larvae) treatment, while at the high intensity (six-larvae), the increased monoterpene emission persisted for at least two weeks in the foliated and defoliated branches of seedlings defoliated by herbivory (Fig. 3.2). It is interesting to note however, that the magnitude of response was greater in the two larvae treated branches than the ones treated with six larvae. One possible theory is that defoliation by larvae can be recognized by the plant as a threat. Low intensities

of herbivory may be tolerated by plant tissues by extra emissions of monoterpenes for a short time. Tolerance can be expressed as overcompensation of plant primary defences (Agrawal, 2000). Testing whether over-compensation may be possible in terms of secondary metabolites in response to low levels of herbivory may be worthwhile for interpretation of these results. The effects of these responses on herbivores themselves would also help in clarification of these findings.

In the current study, mechanical defoliation did not induce a different response from untreated control seedlings, but larval defoliation did significantly increase monoterpene emissions compared to controls. This suggests that the mechanism responsible for monoterpene release is triggered based on damage type. This interpretation is consistent with a study on lodgepole pine (*Pinus contorta*), in which herbivory by tiger moth larvae (*Halisdota ingens* Lepidoptera: Arctiidae) resulted in a significantly larger response in monoterpene cyclase activity than with mechanically induced wounding (Litvak and Monson, 1998). Another possibility is that a systemic response as observed with natural herbivory is only elicited by mechanical defoliation at higher intensities than were tested in this study.

Although the exact mechanism of systemic induction is not known in jack pine trees, signalling through vascular tissue as well as by volatile communication should be considered. Some proposed mechanisms in conifers include: induced lignification or accumulation and growth of polyphenolic parenchyma cells, and mobilization of secondary metabolites such as glycosidic lignin precursors (Martin et al., 2002; Bonello and Blodgett, 2003). Thus far, several studies have mostly demonstrated anatomical responses of pines to applied methyl jasmonate or ethylene (Martin et al., 2002; Hudgins and Franceschi, 2004; Miller et al., 2005; Krokene et al., 2008). The concept of a mobile wound signal is much better understood in angiosperm systems, especially the tomato (*Solanum lycopersicum*). Grafting experiments in tomato demonstrate that systemic signalling requires the ability to recognize a signal in remote tissues (Ryan and Moura, 2002; Schilmiller and Howe, 2005). Although some studies suggest that the octadecanoid pathways for jasmonic acid signalling

are active in conifers (Miller et al., 2005; Phillips et al., 2006) these mechanisms in conifers have not yet been described in response to real insect or pathogen attack. A follow up experiment in jack pine would be useful to test the mechanism of systemic communication and the role of monoterpenes in this process.

Release of volatiles following herbivory in conifers is not extensively studied, though in mature jack pine, volatile response to herbivory can be measured consistently after one week of defoliation (Wallin and Raffa, 1999). In our study, two weeks of elevated volatile emissions were observed from seedlings defoliated by six larvae, and at least one week from two larvae defoliated seedlings. In some angiosperm species, release of terpenes can be as quick as one to two hours after injury (Degenhardt, 2008). There may be other key chemicals induced within this time frame which were undetected by our sampling method such as ethylene (Hudgins and Franceschi, 2004), a molecule known to be released within the first three hours in some plants (von Dahl et al., 2007); or methanol, recently found to be systemically released in holly oak (*Quercus ilex*)(Seco et al., 2011).

### 3.4.2 Inter-plant communication

Conifers are well known to produce monoterpenes consistently, these compounds may act as background scent rather than a signal of warning. In our study, $\alpha$- and $\beta$-pinene, 3-carene and limonene were found to be the most abundant monoterpenes in the volatile array of jack pine, confirming a recent publication in this system (Lusebrink et al., 2011). If monoterpene responses in the neighbouring seedlings are activated in the short term, we may have overlooked these signals by measuring volatiles on the whole-seedling level only after two weeks of defoliation. One hypothesis to investigate in the future is that inter-plant signalling may be triggered by a quantitative ratio shift of several particular chemical compounds or possibly a sudden peak in emission of one compound relative to another. Likewise, Mumm and Hilker (2005) suggested that a signal is not necessarily the most

abundant compound in the array, but may merely differ from the usual array of the undisturbed plant. In the jack pine system, candidates may be the slightly less abundant but variable 3-carene, or the even less abundant compounds such as $\beta$-phellandrene.

In our trials, untreated neighbouring seedlings had similar emissions of monoterpenes to controls, suggesting that monoterpenes may not be a practical detection measure for inter-plant communication. This finding is inconclusive with regards to priming due to two reasons: (1) I monitored emitted responses of neighbouring seedlings using the same protocol as the treated seedlings themselves, so it is possible that the neighbours are receiving communication, yet express their response in a different time-frame or by releasing different chemicals, which were not measured in this assay; (2) the seedlings in this study were obtained from a nursery with no history of genetic similarity. Plants receiving volatiles from genetically identical foliage have stronger resistance than those exposed to foliage cues from genetically different cuttings of the same plant species (Karban and Shiojiri, 2009; Ishizaki et al., 2011). Further, a recent study by Lusebrink et al. (2011) also noted that jack pine from the western prairies (the origin of seedling stock in the current study) may have a higher chance of introgression with lodgepole pine, and may have more variabilty in levels of monoterpenes, like 3-carene. This is notable since the variability of genotypes and chemotypes may be high in our trials, and thus may affect the consistency in seedling emissions- if inter-plant communication is mediated by similarity in genotype. Using clones or seedlings from a single seed-source would be ideal for future research testing the theory of kin selection in volatile plant communication.

In conclusion, the jack pine seedlings did respond differently according to defoliation type and intensity. Jack pine seedlings did exhibit within-plant communication expressed by volatile monoterpenes released from unchallenged (foliated) branches on seedlings treated by herbivory. Intensity of herbivory mediated the length of seedling response. Mechanical wounding did not induce

differential responses from branches, yet mechanical wounding at higher intensities may be needed in order to induce a response in jack pine seedlings. Concentration of monoterpenes in emissions of seedlings were similar in controls as in neighbouring unchallenged seedlings, thus inter-plant communication can not be concluded from this study.

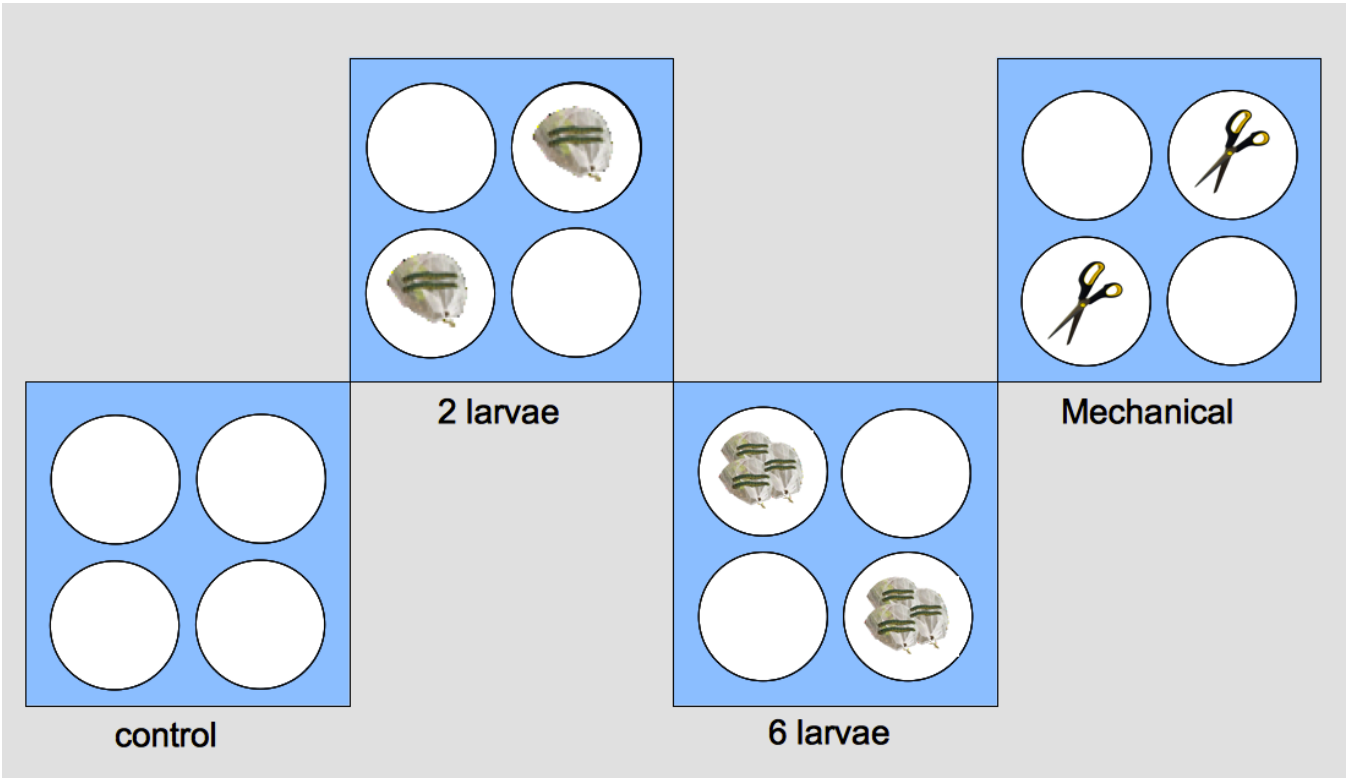


Figure 3.1: Example of greenhouse bench setup, grey area represents part of the greenhouse bench, this setup is repeated five times along bench length, with treatment enclosures changing order of placement to remove spacial bias. Pots of jack pine (*Pinus banksiana*) seedlings are represented by circles, with treatments shown inside. Larvae of *Choristoneura pinus* were used in herbivore treated seedlings and mechanical defoliation was administered with scissors daily to mimic larval herbivory. Blank circles represent unchallenged seedlings. Squares represent enclosures sided with vapour barrier plastic.

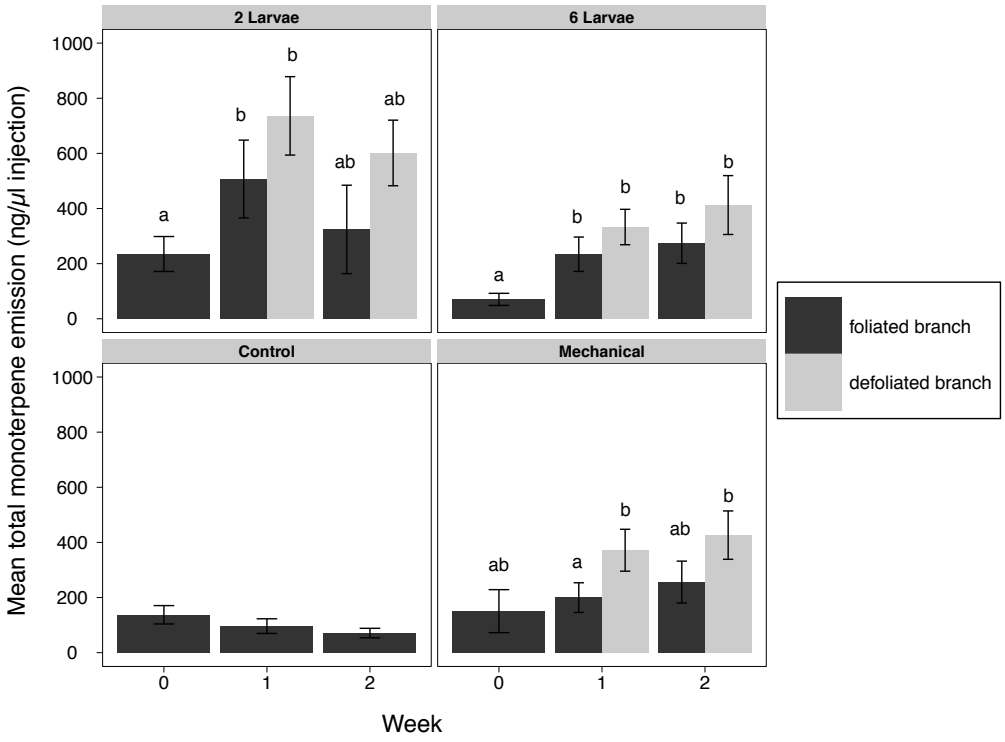


Figure 3.2: Mean total monoterpene concentration emitted (ng/$\mu$l injection) from foliated and defoliated branches of the same jack pine (*Pinus banksiana*) seedling before treatment (0) and after 1 and 2 weeks of herbivory by *Choristoneura pinus*, for each treatment type. Post hoc analyses were conducted for each branch type by collection date within each treatment type. Friedman test for repeated measures was used with post-hoc comparisons with Wilcoxon Signed-Rank tests within each treatment type. Date was used as the repeated measure, and comparisons were corrected for with the Bonferroni adjustment. Different letters indicate statistical significance at $p<0.05$ within each treatment type plot.

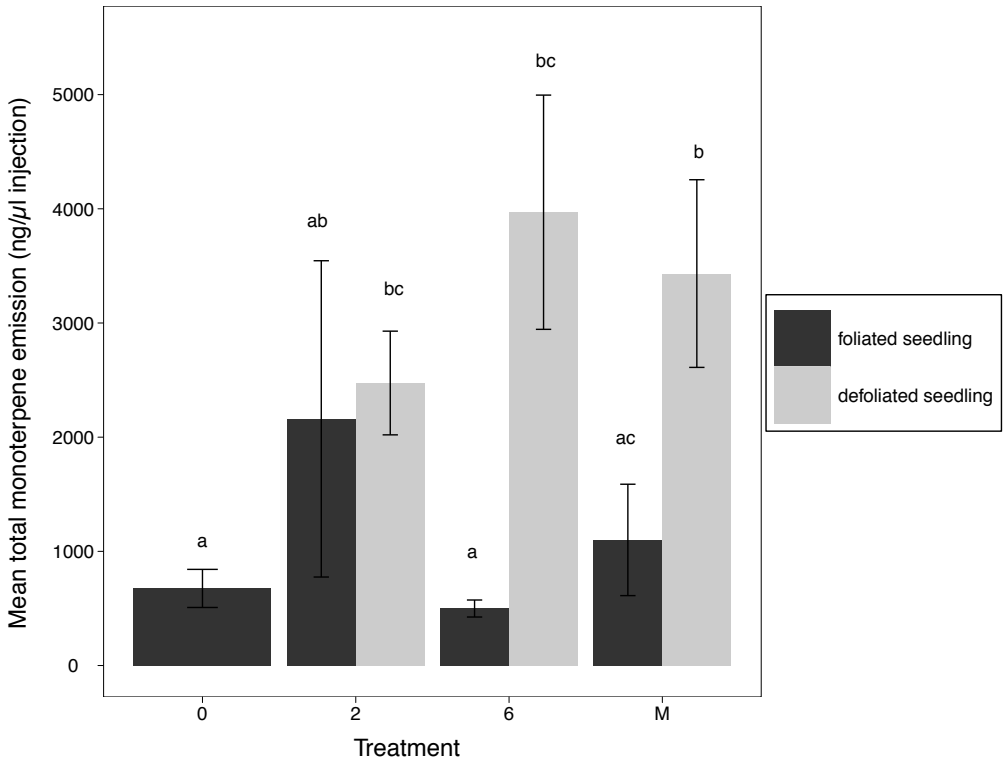


Figure 3.3: Mean total monoterpene concentration emitted (ng/$\mu$L injection) per jack pine (*Pinus banksiana*) seedling 2 weeks after defoliation by *Choristoneura pinus*, by treatment type: 0=control, 2= two larvae defoliated, 6= six larvae defoliated, M= mechanical defoliation. Post-hoc comparisons were made between foliated and defoliated seedlings of each treatment type and compared among treatment types (using treatment and foliated status to categorize each seedling), seedling enclosure was used as a random factor in the mixed model analysis. Different letters indicate statistical significance at $p<0.05$.

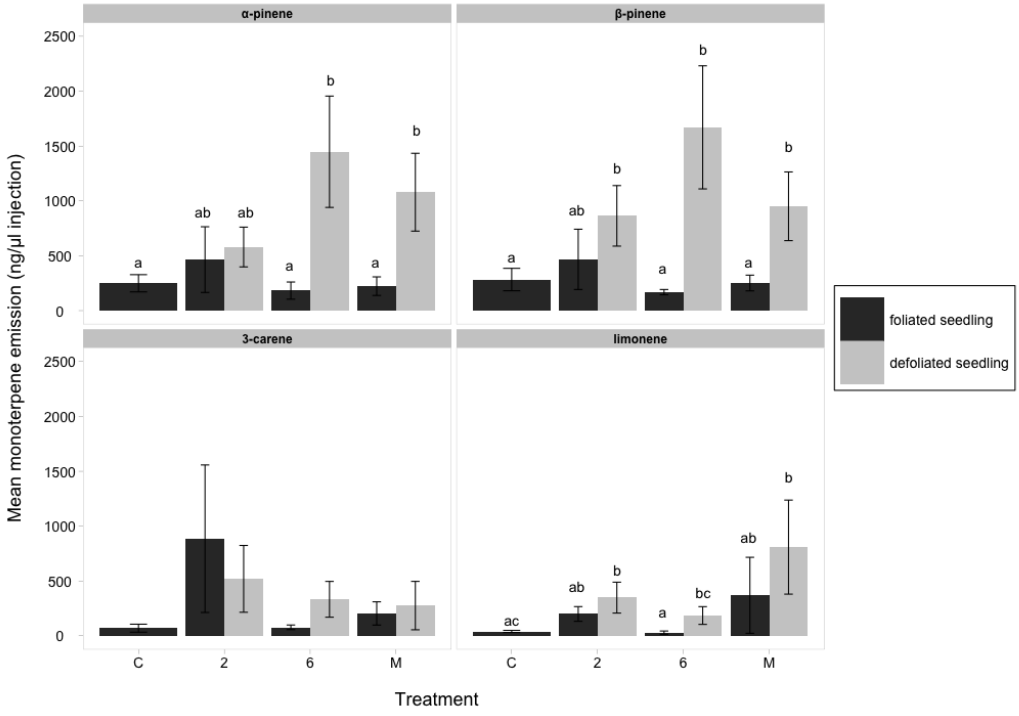


Figure 3.4: Mean concentrations (ng/$\mu$L injection) of four major monoterpenes emitted by whole jack pine (*Pinus banksiana*) seedlings, C=control, 2= two larvae defoliated, 6= six larvae defoliated, M= mechanical defoliation. Foliated seedlings were paired with the defoliated neighbours in enclosures. Post-hoc comparisons were made between foliated and defoliated seedlings of each treatment type and compared among treatment types (using treatment and foliated status to categorize each seedling), seedling enclosure was used as a random factor in the mixed model analysis. Different letters indicate statistical significance at $p<0.05$, within each monoterpene type plot.

| | Control | | | 2 Larvae | | | | | 6 Larvae | | | | | Mechanical | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | foliated (unchallenged) | | | foliated branch (unchallenged) | | | defoliated branch | | foliated branch (unchallenged) | | | defoliated branch | | foliated branch (unchallenged) | | | defoliated branch | |
| Week | 0 | 1 | 2 | 0 | 1 | 2 | 1 | 2 | 0 | 1 | 2 | 1 | 2 | 0 | 1 | 2 | 1 | 2 |
| N | 20 | 18 | 17 | 9 | 10 | 10 | 8 | 10 | 10 | 10 | 10 | 10 | 10 | 8 | 10 | 10 | 10 | 10 |
| Mean | 137.5 | 96.3 | 71.1 | 235.0 | 507.0 | 324.1 | 736.1 | 601.5 | 70.4 | 234.2 | 274.1 | 332.9 | 412.6 | 150.7 | 199.7 | 256.1 | 371.6 | 426.4 |
| ±SE | 33.3 | 26.6 | 17.3 | 63.4 | 141.1 | 160.4 | 142.2 | 119.0 | 22.0 | 62.3 | 73.1 | 64.2 | 106.8 | 78.0 | 53.9 | 76.0 | 76.0 | 87.7 |

Table 3.1: Mean total monoterpene emission (ng/$\mu$L injection) is reported as well as sample size (N) used in analyses of jack pine (*Pinus banksiana*) seedling branches for each treatment at three time points: before experimental defoliation (0), one week after continued larval defoliation or daily mechanical clipping (1) and two weeks after continued defoliation or daily mechanical clipping (2) from branches which were defoliated as well as unchallenged (foliated) branches on the seedling.

| | Treatment groups<br>mean % total monoterpene concentration (±SE) | | | | | | |
|---|---|---|---|---|---|---|---|
| **Monoterpene** | **Control** | **2 Larvae-neighbour** | **2 Larvae** | **6 Larvae-neighbour** | **6 Larvae** | **Mechanical-neighbour** | **Mechanical** |
| **α-pinene** | 36.8 (14.5) | 22.8 (3.6) | 22.5 (6.9) | 31.8 (20.7) | 33.0 (11.1) | 22.5 (5.9) | 32.3 (13.3) |
| **β-pinene** | 39.0 (18.1) | 26.1 (15.4) | 34.8 (19.7) | 35.9 (15.7) | 39.6 (11.5) | 32.0 (21.5) | 27.5 (8.8) |
| **3-Carene** | 11.7 (17.2) | 31.0 (18.9) | 21.6 (24.7) | 18.9 (13.4) | 13.1 (15.8) | 26.1 (26.3) | 9.6 (17.5) |
| **Limonene** | 7.7 (5.6) | 14.5 (10.3) | 14.7 (13.4) | 5.7 (5.8) | 6.0 (7.5) | 14.2 (24.9) | 20.0 (23.6) |
| **β-phellandrene** | 1.5 (0.8) | 1.6 (1.1) | 3.4 (3.5) | 3.8 (4.5) | 3.8 (2.7) | 0.9 (1.1) | 4.5 (3.9) |
| **Camphene** | 1.9 (1.4) | 1.7 (1.0) | 1.2 (0.4) | 1.8 (0.6) | 2.4 (1.6) | 1.6 (0.9) | 3.9 (3.8) |

Table 3.2: Percentages of individual monoterpenes emitted from whole jack pine (*Pinus banksiana*) seedlings (originally units of ng/$\mu$L injection) with regards to the total measured volatile organic chemicals grouped by treatment type. Measurements on a whole seedling level were taken after two weeks of defoliation.

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# Chapter 4

# Greenhouse Experiment Part II: Prior Defoliation and Inter-plant Signalling Affect Responses of Jack Pine Seedlings to a Subsequent Challenge

## 4.1 Introduction

Conifers exhibit inducible responses to biotic and abiotic stress. These responses may be physical as well as chemical, and can deter insects and pathogens on site of attack (Franceschi et al., 2005; Bonello et al., 2006). Induced responses may even protect plants from future attacks by the same or different organism (Agrawal, 1998; Krokene et al., 2003; Eyles et al., 2010). These responses may be byproducts of the initial stimulation after wounding or infection, and offset spatially or temporally from the initial place and time of damage (Franceschi et al., 2005; Howe and Schaller, 2008). For example, priming(by prior inoculation) of Monterey pine (*Pinus radiata*) by pathogenic pitch canker fungus results in shorter lesion size (fungal wound) than uninoculated trees (Bonello et al., 2001; Erbilgin et al., 2009). Responses to future attacks may even be triggered by induction resulting from attack by an organism of a

completely different kingdom. In a recent study by Colgan and Erbilgin (2011), jack pine seedlings (*Pinus banksiana*) were shown to have shorter lesion lengths from blue stain fungus *Grosmannia clavigera* after prior herbivory from jack pine budworm (*Choristoneura pinus pinus* Freeman, Lepidoptera: Tortricidae) than fungal infected seedlings without herbivory.

Induced responses may benefit plants in many ways, for example, the volatile organic compounds (VOCs) released upon herbivory can attract natural enemies of herbivores in indirect defence (Turlings et al., 1990; Kost and Heil, 2006; Mumm and Hilker, 2006). Inter-plant communication is another consequence of induced chemical responses. The VOCs produced from initial herbivory have the capacity to prime conspecic neighbouring plants against current or future attacks (Yi et al., 2010). Communication between injured and neighbouring healthy trees reduces herbivory in poplar (*Populus euroamericana*), willow (*Salix sitchensis*) and maple (*Acer saccharum*)(Baldwin and Schultz, 1983; Rhoades, 1983; Dolch and Tscharntke, 2000). For example, in black alder (*Alnus glutinosa*), defoliation and oviposition by leaf beetles (*Agelastica alni*) was signicantly lower in most proximal neighbours of manually defoliated trees. This observation was attributed to emissions of ethylene and an array of monoterpenes (10 carbon compounds) and sesquiterpenes (15 carbon compounds) by the neighbouring, defoliated alders (Tscharntke et al., 2001).

Nevertheless, inter-plant communication has not yet been observed in conifers. Several studies using pine model systems have identied monoterpenes as commonly emitted volatiles from damaged or stressed trees (Litvak and Monson, 1998; Phillips et al., 1999; Faldt, 2000; Mumm and Hilker, 2005; Lusebrink et al., 2011). Though the physiological costs and benefits of these emissions are still being investigated. For example, in Chapter 3 of this thesis, I investigated airborne responses to herbivory in jack pine by monitoring monoterpene emissions from defoliated jack pine seedlings and healthy neighbours of these defoliated seedlings. The volatile monoterpene emissions of healthy neighbours of defoliated seedlings did not differ from those emitted from the seedlings without defoliated neighbours.

To build on that work, and to further explore inter-plant signalling, the study question is explored: How does defoliation or exposure to volatiles from defoliated neighbours impact jack pine responses or resistance to a subsequent challenge? The challenge administered was the pathogenic fungus *G. clavigera*. This is an ophiostomatic fungus which detoxifies conifer defences and kills trees by disrupting the flow of nutrients to the crown (DiGuistini et al., 2011).

In several studies, the monitoring of lesions on wood made by fungal inoculation have been used to assess resistance. For example, systemic induced resistance in Monterey pine was observed by measuring lesion lengths of the pitch canker *Fusarium circinatum* (Storer et al., 1999; Bonello et al., 2001; Gordon et al., 2011), and in the Austrian pine, lesion length (by pathogen *Sphaeropsis sapinea*) was found to be negatively correlated with lignification, which gives it further support as a defensive indicator (Blodgett et al., 2007; Eyles et al., 2007). *Grosmannia clavigera* has been incorporated in recent research on jack pine since this fungus is intimately tied to the most recent host and range expansion of the mountain pine beetle *Dendroctonus ponderosae* (Coleoptera: Curculionidae) in western Canada's jack pine forests (Rice et al., 2007; Lusebrink et al., 2011). In these studies, *G. clavigera* was used to simulate the mountain pine beetle attack on jack pine trees because experiments with live beetles are not permitted in jack pine forests. This simulation is a logical proxy, as this symbiotic fungus is transported by the beetle to a host and aids in the eventual death of the tree. Understanding of the natural mechanisms of jack pine responses, even in the seedling stages, helps researchers and foresters to better understand how jack pine stands may respond to an invasive pest, and forecast risk.

Jack pine-dominated ecosystems are considered to have evolved with periodic jack pine budworm defoliation (McCullough, 2000). Budworm defoliation can cause significant damage to the stand structure and even tree death in times of outbreak (McCullough, 2000). Jack pine budworm relies heavily on pollen cones in the early part of the life cycle. The life cycle of jack pine budworm temporally and spatially

overlaps with the life cycle of mountain pine beetle (Colgan and Erbilgin, 2010). Weakened, senescent or stressed trees can be a perfect breeding ground for future mountain pine beetle colonization (Paine and Baker, 1993; Wallin and Raffa, 1999), and thus jack pine budworm cycles could be an important factor in creating an environment suitable for colonization.

The resistance of jack pine seedlings to *G. clavigera* was reported after prior budworm herbivory by Colgan and Erbilgin (2011); so in the current study, I built on those results further by uncovering the effects of defoliation intensity and type of defoliation (larval herbivory and mechanical defoliation) on seedling resistance to pathogenic attack. I also measured the volatile responses from both challenged and neighbouring unchallenged seedlings over time. Finally, I quantified resistance in these seedlings by comparing their lesion wounds eight weeks after the inoculation challenge.

## 4.2 Methodology

### 4.2.1 Treatments and volatile collection

Two-year-old jack pine seedlings from Boreal Horticultural Services Ltd. in Bonnyville, Alberta, were planted in the greenhouse by mid March 2010. They were planted in 4L pots, with planting substrate containing five parts peat, one part perlite and one half-part clay particles. Seedlings were fertilized with (N: 15% P: 30% K: 15%) bi-weekly to exclude confounding factors such as nutrient deficiency (Zhao et al., 2008) and watered regularly until the experiment began mid June, 2010. The average height of the seedlings was 31.2cm $\pm$4.6. The average temperature in the greenhouse was 27.6°C $\pm$1.8, with day length of 18h (supplemented when necessary with artificial lighting) and 60% relative humidity during the course of the experiment.

Early instar jack pine budworm larvae, acquired from jack pine stands in Ontario, Canada were allowed to feed on artificial diet bought from McMorran Diet from Insect Production Services, Great Lakes Forest Research Centre in Sault Ste. Marie, Ontario. This diet is used as an alternative to the pollen diet of the early instars. After $3^{rd}$ instar, the larvae usually begin to feed on needles.

Four treatments were set up with either no larvae (control), two or six jack pine budworms per seedling or mechanical defoliation. Larval treatments had two larvae on each branch, thus for the treatment with six larvae, three branches were used. The larvae were left to feed on needles for two weeks within mesh bags. Volatile collections were made from one defoliated and one foliated (adjacent) branch from each seedling once per week. Larvae were removed during the sampling period. Mechanical defoliation was administered by snipping a 2-5 needles daily around the base-midpoint of the needle to mimic defoliation by herbivory.

Each seedling was paired with a unchallenged (foliated) seedling, designed to assess whether defoliation intensity (zero, two or six larvae) or type of defoliation (mechanical or larval), play any role in volatile emission and response from the neighbouring seedlings. Two pairs of seedlings with the same treatment were air-isolated from the rest by vapour barrier plastic sheets with a slightly open top, to avoid moisture accumulation and unwanted microbial growth on trees. There were five such enclosures (0.4m x 0.4m x 1.0m) for each treatment, totalling 80 trees.

After the two week defoliation period, larvae were removed and volatiles of whole seedlings were collected from one pair of each enclosure. Following volatile collection, all seedlings except controls were inoculated with *G. clavigera*. The fungus was propagated in the dark, ten days prior in Petri dishes on 50% malt agar. A corkborer (0.4mm in diameter, as per methods in Colgan & Erbilgin 2011) was used to inoculate seedlings and each seedling received two inoculations just under the bark. At 24 hours, one week, three weeks and eight weeks after inoculation, one pair from each enclosure was sampled for volatiles on a whole tree level. The

seedlings were enclosed with an oven bag (LOOK®) with the open end of the bag gently tied around the stem. An absorbent tube (Porapak Q (OD 6mm, length 110mm; absorbent: front layer 150 mg, back up layer 75 mg; separated by glass wool) SKC Inc., Eighty Four, Pennsylvania, USA) was positioned through a small hole in the upper corner of each bag. Volatiles were collected for 3 hours using small air pumps at a flow rate of 0.4L/min. Porapak tubes were sealed with Teflon caps immediately after volatile collection and extracted directly or frozen at -40°C until extraction.

Eight weeks after inoculation, all seedlings were sampled destructively by peeling bark to measure lesion lengths. Needle and phloem were also sampled at this time and stored at -40°C until extraction. Phloem samples from the length of the stem were ground by mortar and pestle with liquid nitrogen prior to chemical extraction. Needle samples from defoliated and foliated branches of the same tree were kept separate and ground using the same method.

### 4.2.2 Chemical analysis

Ground samples were extracted using the methods outlined for tissue extraction outlined by Lusebrink et al. (2011) with 1mL of dichloromethane (Sigma-Aldrich, St. Louis, Missouri, USA) spiked with 0.01% (v/v) tridecane (Sigma-Aldrich, St. Louis, Missouri, USA) as surrogate standard and subsequently stored at -40°C before Gas Chromatograph/ Mass Spectrometer (GC/MS) analysis. Samples (1$\mu$l) were injected in a GC/MS (Agilent Technologies 7890A/5062C , Santa Clara, California, USA) equipped with an HP Innowax (Agilent Technologies) column (I.D. 0.25 mm, length 30m). The helium carrier gas flow was set at 1.0mL/min and the following temperature programme was applied: 50°C for 2 min, increased to 60°C by 1°C per min and then ramped up to 250°C by 20°C.

The following standards were used to determine sample concentrations: Borneol, pulegone, $\alpha$-terpinene, $\gamma$-terpinene, $\alpha$-terpineol (Sigma-Aldrich, St. Louis,

Missouri, USA), camphor, 3-carene, $\alpha$-humulene, terpinolene, $\alpha$-thujone, (-)-$\alpha$-pinene, (-)-$\beta$-pinene, (S)-(-)-limonene, sabinene hydrate, myrcene, (-)-camphene, p-cymene (Fluka, Sigma-Aldrich, Buchs, Switzerland), bornyl acetate and cis-ocimene (SAFC Supply Solutions, St. Louis, Missouri, USA), $\beta$-phellandrene (Glidco Inc., Jacksonville, Florida, USA).

### 4.2.3 Statistical analyses

Analyses were performed using R statistical software (R, 2010) or SPSS version 17.0 (SPSS, 2008.). If data was not normally distributed, it was log transformed for a normal distribution. For all comparisons, Shapiro-Wilk tests for normality and Levene's test for homogeneity of variances were performed to ensure that data met standard assumptions for mixed effects models. The 'nlme' package was used in R (Pinheiro et al., 2012). F-values were reported followed by numerator and then denominator degrees of freedom along with associated p-values. Post-hoc tests were reported with p-values and reported from mixed model outputs in R. Total monoterpene emissions from whole seedlings were modelled with the dependent variable as total monoterpene concentration, and the factor included in the model was treatment type. Treatment type was separated into unchallenged (foliated) and defoliated branches (or seedlings). Since these seedlings were contained in enclosures, the enclosure was used as a random factor in the analysis. This same model was used for testing individual monoterpenes as well as the total monoterpenes emitted from whole seedlings. Other models were compared (each seedling nested within the enclosure) using the Akaike Information Criteria (AIC), but the one using only enclosure as random factor accounted for the most variation with the fewest variables.

For comparisons of seedling emissions at different time points, the data was not normally distributed, and logarithmic transformations did not help to conform data to meet standard assumptions for parametric tests. Kruskal-Wallis non-parametric

tests were performed using treatment as a factor and monoterpene concentration as the dependent variable. The post-hoc comparisons of treatment groups (for the volatile responses of whole seedlings over time) were tested for each time period separately (twenty-four hours, one week, three weeks, eight weeks). Non-parametric comparisons were achieved with nparcomp package for R (Konietschke, 2011). Relative percent change was calculated by taking the concentration at twenty-four hours, one week, three weeks, or eight weeks subtracting concentration before inoculation, dividing by the concentration before inoculation and multiplying by one hundred. This transformation was made for ease of visual comparisons among treatments.

Lesion lengths were compared first between neighbours and controls, and data from these categories was normally distributed after log transformation, though for comparisons of defoliated seedlings to controls, data failed to meet normality assumptions and Kruskal-Wallis non-parametric tests were performed. For all statistics reported in this chapter, where F tests are reported, the mixed effects model was used as mentioned above, and $\chi^2$ values are reported when Kruskal-Wallis tests were performed.

## 4.3 Results

### 4.3.1 Needle monoterpenes

Needle monoterpene concentration was investigated only after eight weeks of inoculation since needle collection is a destructive sampling process which would have confounded our results from volatile collections over the course of the fungal inoculation period (see section on 'whole seedling volatiles over time').

Seedlings which were not inoculated had generally lower needle monoterpene concentrations than inoculated seedlings ($F_{(7,81)}$=3.18, p=0.005). However, the only

noteworthy significant increase in monoterpene concentration was detected between defoliated branches of two-larvae seedlings and not inoculated controls (p=0.0017) (Fig. 4.1). Needle monoterpenes of foliated branches on defoliated seedlings did not differ from each other (Fig. 4.1). Monoterpene concentration from needles of unchallenged neighbouring jack pine seedlings remained similar to the controls which were not inoculated eight weeks after inoculation ($F_{(5,28)}$=0.743, p=0.598, Fig. 4.2).

### 4.3.2 Phloem monoterpenes

Phloem of defoliated jack pine seedlings did show differences among treatments overall ($F_{(4,40)}$=2.88, p=0.034, Fig. 4.3). However, in post hoc comparisons, only the two-larvae defoliated seedlings had higher tissue monoterpene concentrations than mechanically defoliated seedlings (p=0.022). The controls did not significantly differ from neighbours of treated seedlings ($F_{(4,41)}$=0.239, p=0.9142).

### 4.3.3 Whole seedling volatiles over time

Generally, defoliated seedlings decreased in volatile monoterpenes of the after inoculation compared to untreated controls (Fig. 4.4). Overall, after three weeks, there were no differences among treatments ($F_{(6,25)}$=1.715, p=0.159). Significant differences were observed only after 24 hours ($F_{(6,28)}$=6.898, p<0.001), one week ($F_{(6,28)}$=4.355, p=0.003), and eight weeks ($F_{(6,27)}$=9.524, p<0.001).

### 4.3.4 Lesion lengths

Neighbouring seedlings (receiving exposure from volatiles) had longer lesion lengths than in controls ($F_{(3,35)}$=3.3273, p=0.0306, Fig. 4.5). Specifically, neighbours of two and six-larvae defoliated seedlings (represented as 'foliated' seedlings in the figure) had significantly longer lesions than controls (p=0.0185).

Mechanically defoliated seedlings and their neighbours had significantly longer lesion lengths than control seedlings ($\chi^2(4) = 11.5$, p= 0.0219, p= 0.007 and p=0.012 respectively).

## 4.4 Discussion

Our goal in this study was to quantify how defoliation and exposure to volatiles from defoliated neighbouring jack pine seedlings affected the seedling resistance to a subsequent challenge (*G. clavigera*). In defoliated seedlings, resistance to a fungal pathogen depended on type of defoliation, as demonstrated by the shorter lesion lengths on seedlings defoliated by larvae, and longer lesions on those previously defoliated mechanically. In phloem tissues, seedlings defoliated by two-larvae had significantly higher monoterpenes, yet a short lesion; compared to the mechanically defoliated seedlings, which had lower phloem monoterpenes, and longer lesions. This suggests that herbivory can trigger responses in plants that mechanical defoliation can not. These observations are consistent with a study on lodgepole pine, in which monoterpene cyclase activity was 2.5 fold higher in trees with tiger moth herbivory than mechanical wounding (Litvak and Monson, 1998). However, the relationship between lesion lengths and monoterpene concentrations should be taken with caution, because chemicals such as phenolic glycosides in addition to monoterpenoids can be important in determining lesion development in trees (Franceschi et al., 2005; Heijari et al., 2005; Eyles et al., 2010).

Intensity of herbivory played a role in the resistance of neighbouring seedlings receiving volatile exposure. This was demonstrated by the shorter lesion lengths on seedlings adjacent to the six-larvae defoliated seedlings, and longer lesions in those adjacent to two-larvae or mechanically defoliated seedlings. This suggests that the intensity of volatile exposure a neighbouring seedling receives is inversely related to its susceptibility to future pathogenic infection.

Systemic induced susceptibility is not commonly studied; however, it has been demonstrated in another conifer system. In Austrian pine (*Pinus nigra*), expression of resistance was organ dependent; initially inoculated at the needles and then challenged on the stem resulted in susceptibility, while inoculating and challenging the stem on different locations resulted in resistance (Blodgett et al., 2007). These results represent the first case of systemic induced susceptibility resulting from airborne signalling, suggesting that expression of resistance may also be modality (type of elicitation) and intensity dependent.

Systemic induced susceptibility was also observed in *Arabidopsis thaliana* (Cui et al., 2005), this result was attributed to interactions between different signalling pathways. Generally, wounding and herbivory are associated with the jasmonic acid pathway, and pathogen attack triggers the salicylic acid pathway. These pathways may be interchangeable, depending on the nature of the elicitor and plant (Walters, 2011). So far, the salicylic and jasmonic acid pathways are mainly studied in herbaceous plants; and are responsible for mediating systemic induced resistance (Eyles et al., 2010). The pathways may also cross-communicate in a synergistic or antagonistic way when overlapping or consecutive stressors are present (Pieterse et al., 2009; Colgan and Erbilgin, 2011). In this system, defoliation may trigger the jasmonic acid pathway in defoliated seedlings, yet neighbouring seedlings receiving volatile cues from these are subsequently inoculated with a fungus, this switch in treatment may create a mixed signal in the neighbours resulting longer lesions. When volatile signals are received, the pathways may be triggered in the receiver depending on the strength or length of the volatile signal. The volatile cues from a seedlings defoliated with six larvae may have been enough to trigger the correct pathway as well, thus reducing lesion length. This induction of susceptibility also invokes an interesting question about whether these responses would be different if the seedlings were genetically related or identical. A follow up experiment testing the significance of kin selection in responses to volatile exposure would be worthwhile to better understand the evolutionary context of these responses.

After inoculation, emissions of volatile monoterpene increased less from previously defoliated seedlings as from seedlings that did not experience previous herbivory. After eight weeks of inoculation, the previously defoliated seedlings actually began to reduce monoterpene emissions compared to before inoculation. This result was most significant in the six-larvae treatment. Reduction in volatile emissions from initially defoliated and then fungal inoculated jack pine trees is not surprising as it is generally accepted that production of secondary chemicals is metabolically expensive and can drain resources from plants (Franceschi et al., 2005; Keeling and Bohlmann, 2006); thus the initial release of secondary metabolites from herbivory, or rather allocation to needle tissues for defence against herbivory may have depleted a portion of resources for subsequent release after inoculation.

There were no differences between controls and exposed neighbours in terms of monoterpene emissions at any volatile collection point after inoculation of seedlings by blue stain fungus *G. clavigera*. This observation was also true in the assessment of needle and phloem monoterpenes after the eight week fungal growth period. The lack of differences between the control and neighbour treatments is consistent with the study from Chapter 3. Furthermore, longer lesions were observed in the neighbours of seedlings defoliated by two-larvae compared to controls, as well as longer lesions in neighbours of mechanically treated seedlings- even though these seedlings did not differ from controls in terms of phloem monoterpenes (albeit only at eight weeks post inoculation). It is possible that phloem monoterpenes may have responded to treatments at earlier time points post-inoculation, but unfortunately destructively sampling at earlier times would have confounded volatile emission data at those time-points.

To conclude, inter-plant communication is observed in this conifer system, though the mechanisms of these responses require further investigation. By testing larval and mechanical defoliation, I observed that resistance response to subsequent pathogen attack depends on type of defoliation. However, resistance caused by volatile priming is determined by intensity of herbivory in the neighbouring seedling.

Future research is needed in order to determine which signals trigger this response in the neighbours as well as in the treated seedlings themselves.

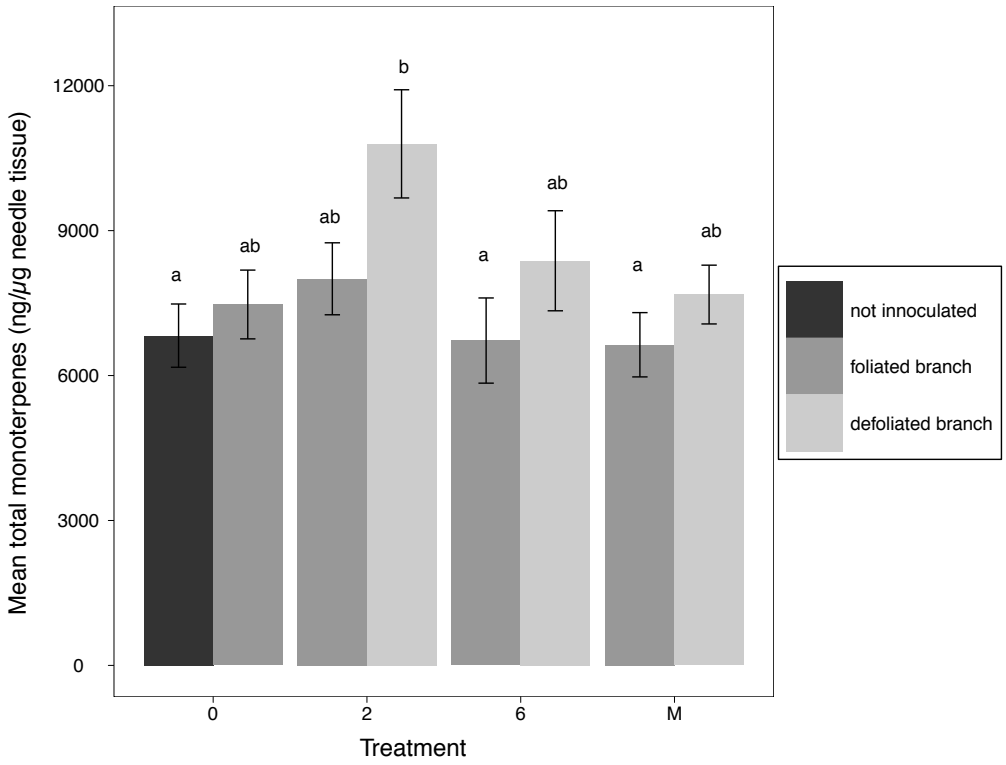


Figure 4.1: Total monoterpenes from needle tissue (ng/$\mu$g) of defoliated and foliated branches of jack pine (*Pinus banksiana*) seedlings, eight weeks post inoculation by *Grosmannia clavigera* (on stem), seedlings were subjected to different treatments prior to inoculation: 0= control, 2= two larvae defoliated, 6= six larvae defoliated, M= mechanically defoliated. Results from controls without inoculation are shown as well. Post-hoc comparisons were made between foliated and defoliated branches of each treatment type and compared among treatment types (using treatment, inoculation and foliated status to categorize each seedling), seedling enclosure was used as a random factor in the mixed model analysis. Different letters indicate statistical significance at $p<0.05$ between bars.

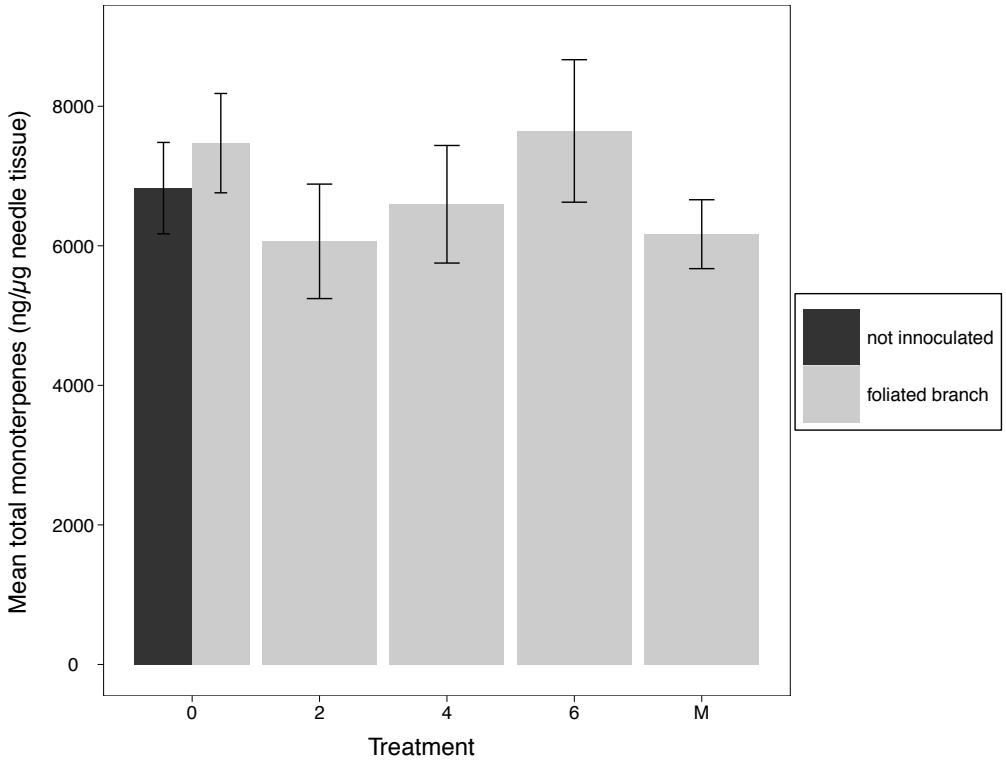


Figure 4.2: Total monoterpene concentration (ng/$\mu$g) from needle tissue of jack pine (*Pinus banksiana*) neighbouring seedlings, not previously defoliated yet exposed to volatiles from defoliated neighbours , eight weeks post inoculation by *Grosmannia clavigera* (on stem), grouped by treatment type of defoliated neighbour: 0=control, 2= two larvae, 6= six larvae, M= mechanical defoliation. Results from controls without inoculation are shown as well. Post-hoc comparisons are made among treatment types as well as between not inoculated controls and neighbours. Seedling enclosure was used as a random factor in the mixed model analysis. Different letters indicate statistical significance at $p<0.05$ between bars.

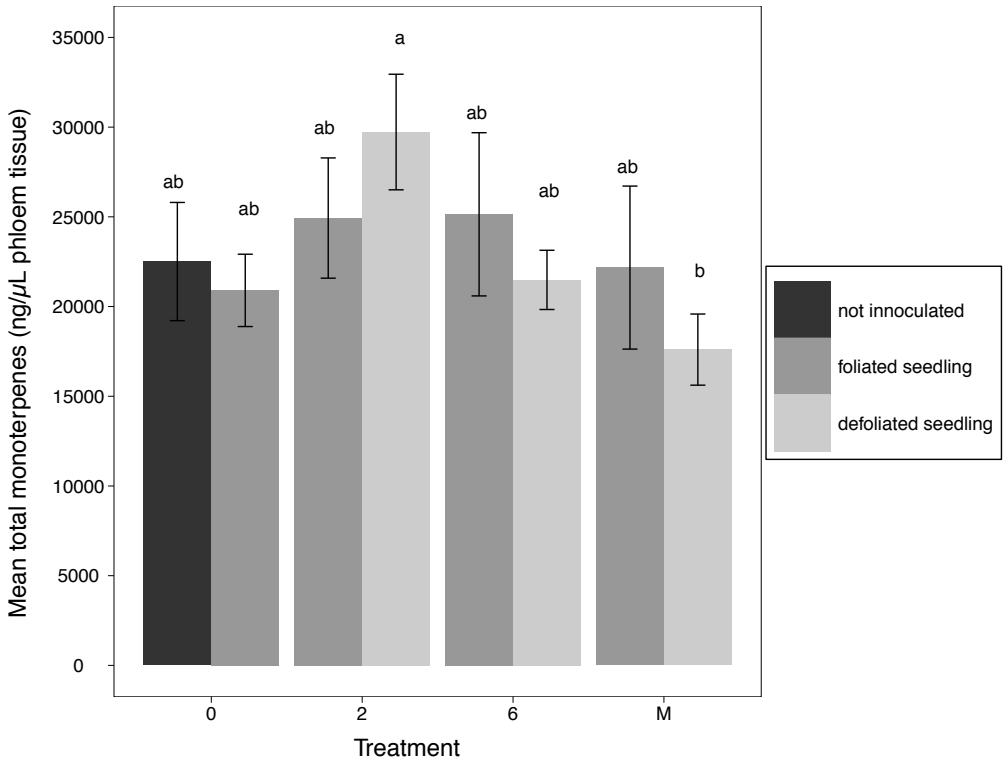


Figure 4.3: Total monoterpene concentration (ng/$\mu$g) in phloem of jack pine (*Pinus banksiana*) seedlings, eight weeks post inoculation by *Grosmannia clavigera* (on stem). Seedlings were subjected to different treatments prior to inoculation: 0= control, 2= two larvae defoliated, 6= six larvae defoliated, M= mechanically defoliated. Results from controls without inoculation are shown as well. Post-hoc comparisons were made between foliated and defoliated seedlings of each treatment type and compared among treatment types (using treatment, inoculation and foliated status to categorize each seedling), seedling enclosure was used as a random factor in the mixed model analysis. Different letters indicate statistical significance at $p<0.05$. Different letters indicate statistical significance at $p<0.05$ between bars.

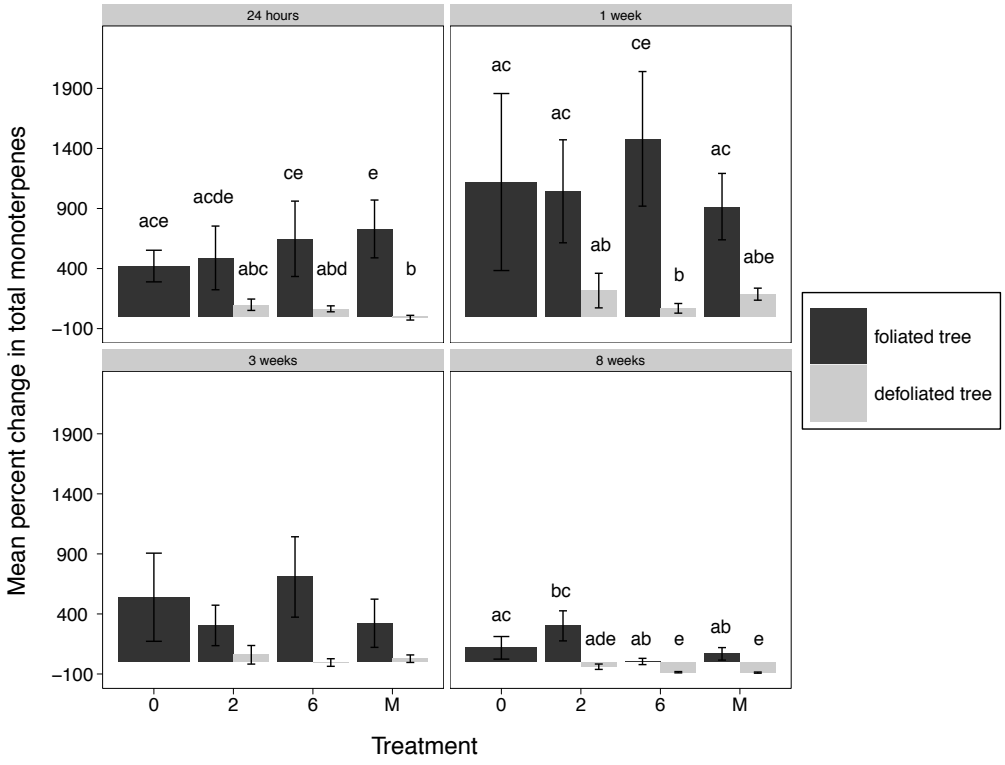


Figure 4.4: Relative mean percent change of volatile monoterpenes released from whole jack pine (*Pinus banksiana*) seedlings over time after initial inoculation by *Grosmannia clavigera* (on stem). Percent change is from before inoculation to indicated time (on each panel). Seedlings were subjected to different treatments prior to inoculation: 0= control, 2= two larvae defoliated, 6= six larvae defoliated, M= mechanically defoliated. Post-hoc comparisons were made between foliated and defoliated seedlings of each treatment type and compared among treatment types (using treatment and foliated status to categorize each seedling), seedling enclosure was used as a random factor in the mixed model analysis. Different letters indicate statistical significance at $p<0.05$ between bars within each panel.

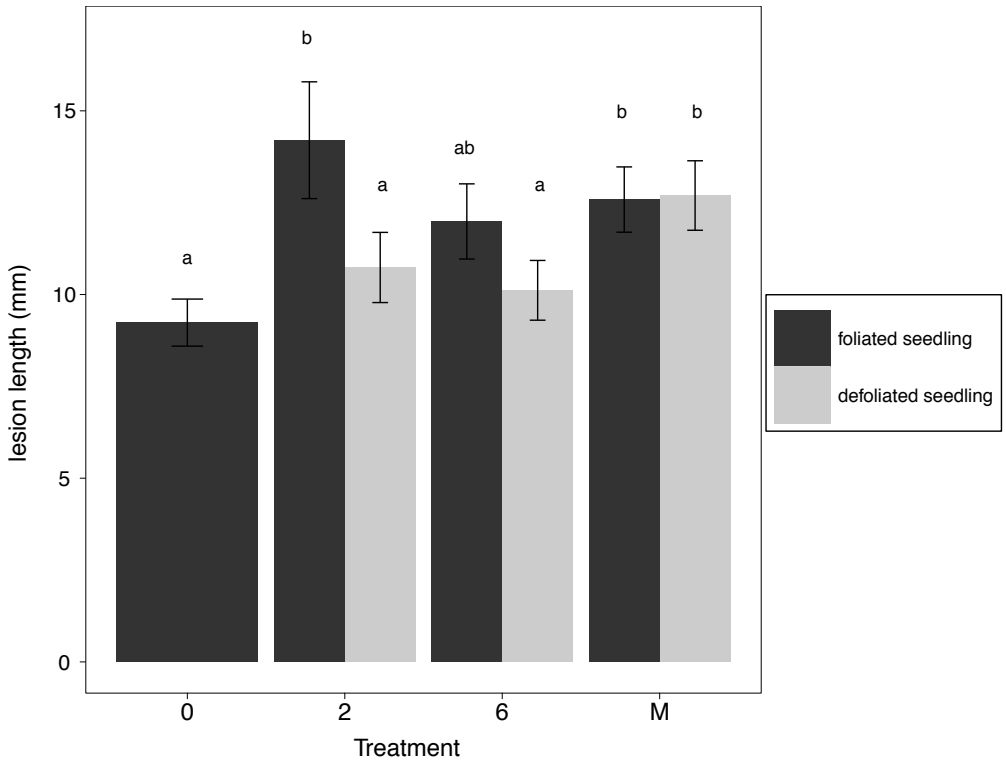


Figure 4.5: Average lesion length (mm) per jack pine (*Pinus banksiana*) seedling from *Grosmannia clavigera* inoculation after eight weeks. Seedlings were subjected to different treatments prior to inoculation: 0= control, 2= two larvae defoliated, 6= six larvae defoliated, M= mechanically defoliated. Post-hoc comparisons were made between foliated and defoliated seedlings of each treatment type and compared among treatment types (using treatment and foliated status to categorize each seedling), seedling enclosure was used as a random factor in the mixed model analysis. Different letters indicate statistical significance at $p<0.05$ between bars.

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# Chapter 5

# Jack pine Budworm Infestation in Forest Stands Affects Monoterpene Chemistry in Mature Jack Pine

## 5.1 Introduction

Damage by herbivory or pathogenic infection is known to induce physiological changes in conifers (Franceschi et al., 2005; Miller et al., 2005; Keeling and Bohlmann, 2006). An important induced response to damage in pines is resin accumulation in existing resin ducts, and production of new resin ducts (Franceschi et al., 2005; Miller et al., 2005). These kinds of induced responses become evident a 2-3 weeks after attack (Miller et al., 2005), and help the tree defend against future attack. Resin (or oleoresin) is an essential part of a conifer's primary defences, and is present in the needles, stems and roots (Bohlmann, 2008). Oleoresin primarily consists of terpenoids, of which monoterpenes are the most abundant (Keeling and Bohlmann, 2006).

Monoterpenes and the less abundant sesquiterpenes are volatile, and their evaporation upon wounding causes the non-volatile diterpenes to harden around a

wound and seal it (Langenheim, 2006). These monoterpenes can also deter insects and prevent fungal growth (Raffa and Smalley, 1995; Wallin and Raffa, 1999; Miller et al., 2005; Bonello et al., 2006; Thoss and Byers, 2006). Some induced responses may even persist to defend against another attack on the same plant at later time (Agrawal, 1998; Krokene et al., 2003, 2008; Eyles et al., 2010). Induced responses may be offset spatially or temporally from the initial place and time of damage, and systemically transfer their effects within the tree (Franceschi et al., 2005; Bonello et al., 2006; Howe and Schaller, 2008).

Evaporation of volatile terpenes also mediates an array of ecological interactions, such as the attraction of herbivores, and their parasitoids or predators (Hulcr et al., 2006; Raffa et al., 2007). Insect sequestration of plant terpenes for use as pheromones can also occur during herbivory which may attract the herbivore's conspecifics, or act as kairomones to attract their predators (Erbilgin and Raffa, 2001; Seybold et al., 2006; Raffa et al., 2007; Borden et al., 2008). $\alpha$-pinene is a good example of a monoterpene which is converted to use as *trans*-verbenol, an aggregtion pheromone of the mountain pine beetle (*Dendroctonus ponderosae*, Coleoptera: Curculionidae) (Pitman, 1971; Gries et al., 1990). In this study, I sought to understand the monoterpene changes in jack pine (*Pinus banksiana* Lambert) tissues of trees of different defoliation intensities. The questions of interest in this chapter include: 1) how do jack pine trees respond to different intensities of budworm defoliation in stands? 2) What is the immediate effect of herbivory on needles of jack pine? In a manipulative experiment, I further compared foliar monoterpenes to better understand the pattern of allocation of the defensive compounds in jack pine before and after herbivory by jack pine budworm (*Choristoneura pinus* Freeman, Lepidoptera: Tortricidae). The jack pine budworm is the most notorious defoliator of jack pine in the eastern and central boreal forest of Canada. When populations are high, feeding can result in top-kill, reduction in pollen-cone production, and continuous defoliation may cause tree death (McCullough, 2000). Periodic high populations occur in Ontario roughly every 6-12 years, with outbreaks lasting

approximately 2-4 years (Nealis, 1995).

Defoliation by jack pine budworm exponentially increases the likelihood of colonization by bark beetles (*Ips grandicollis* Coleoptera: Scolytidae) and their associated fungal symbionts (*Ophiostoma ips*) as well as wood boring beetles (*Monochamus carolinensis* Coleoptera: Cerambycidae) (Wallin and Raffa, 1999). This response was attributed to the higher monoterpene levels in the phloem of jack pine of highly defoliated trees, causing susceptibility to *I. grandicollis* (Wallin and Raffa, 1999).

Investigation of the natural mechanisms of plant defence may help researchers to better understand how stands respond to future invaders, or potentially forecast risk of infestation. Defoliation by jack pine budworm could be a significant biotic factor in the behaviour of other invasive tree pests such as the mountain pine beetle (*D. ponderosae*). This research can be useful for future investigators seeking to understand mechanisms behind the ecological interactions of the jack pine and jack pine budworm system.

## 5.2 Methodology

### 5.2.1 Plot descriptions and quantification of defoliation levels

In late June and early July 2011, two jack pine budworm infested locations were sampled in Ontario. Trees were sampled around Britt and Sturgeon Bay Provincial park, Ontario (45° 37 20.22, 80° 24 34.2 and 45° 46 10.5, 80° 37 14.69). We also sampled in the north-eastern corner of Algonquin Provincial Park, Ontario near Lake Travers off of Achray road (45 56 45.48, 78 0 38.16).

To characterize the *C. pinus* populations on each tree: 1) a visual and physical examination of jack pine trees prior to experimentation, coarsely dividing high

and low groups in terms of previous year defoliation and current year populations of early instar ($2^{nd}$ and $3^{rd}$) jack pine budworm, and 2) a detailed assessment of defoliation was made at the end of the growing season, after our experiments had been completed. Groups were categorized as low defoliation if previous year's defoliation was (on average) less than 40% or if fewer than 5 larvae were found per 1 m long branch sampled from the lower crown of the tree in the spring. If trees had higher levels of defoliation (>40% on average) in the previous year or more than 15 larvae were found on a branch in spring, it was categorized as high defoliation. I also selected trees within each location as a controls >10km from infested trees; controls had no visual signs of budworm feeding. In Britt, I sampled 15 control trees, 24 trees from the low category and 20 trees from the high category. In Algonquin, 10 control trees were sampled with no signs of low-crown defoliation, 19 trees from the low category, and 15 in the high category.

Needles from each sample tree were collected with garden clippers from low crown branches, and phloem was collected from each tree at around breast height. Cores from each tree were taken as well for growth rate analyses, and later processed using the WinDendro program (Gagnon and Morin, 1996). Sample tissues were stored in aluminium foil on dry ice in the field, in freezer (-18°C) until expedited shipment to Edmonton.

### 5.2.2 Manipulative experiment

Budworms were collected from trees in other highly infested trees in the Britt area. 10 larvae ranging from fourth to sixth instar, were placed on single branches on 15 uninfested (control) trees after initial needle sampling. Budworms were contained in mesh bags (20x50cm) with the open ends secured to a branch, and left to defoliate needles for three days. Following defoliation needles were resampled from budworm-defoliated branches, and from an adjacent foliated branch. All samples were stored at -40°C upon arrival to the laboratory.

### 5.2.3 Chemical analysis

Tissues were ground with mortar and pestle and extracted with 1ml of dichloromethane (Sigma-Aldrich, St. Louis, Missouri, USA) spiked with 0.01% (v/v) tridecane (Sigma-Aldrich, St. Louis, Missouri, USA) as surrogate standard and subsequently stored at -40°C before Gas Chromatograph/ Mass Spectrometer (GC/MS) analysis. Samples (1$\mu$l) were injected in an Agilent 7890A/5062C (GC/MS) (Agilent Technologies, Santa Clara, California, USA) equipped with an HP Innowax (Agilent Technologies) column (I.D. 0.25 mm, length 30m). The helium carrier gas flow was set at 1.0ml/min and the following temperature programme was applied: 50°C for 2 min, increased to 60°C by 1°C per min and then ramped up to 250°C by 20°C.

The following standards were used to determine sample concentrations: Borneol, pulegone, $\alpha$-terpinene, $\gamma$-terpinene, $\alpha$-terpineol (Sigma-Aldrich, St. Louis, Missouri, USA), camphor, 3-carene, $\alpha$-humulene, terpinolene, $\alpha$-thujone and $\alpha$-thujone, (-)-$\alpha$-pinene, (-)-$\beta$-pinene, ($S$)-(-)-limonene, sabinene hydrate, myrcene, (-)-camphene, p-cymene (Fluka, Sigma-Aldrich, Buchs, Switzerland), bornyl acetate and cis-ocimene (SAFC Supply Solutions, St. Louis, Missouri, USA), $\beta$-phellandrene (Glidco Inc., Jacksonville, Florida, USA).

### 5.2.4 Statistical analyses

All tests were performed using R statistical software (R, 2010). For all comparisons, Shapiro-Wilk tests for normality and Levene's test for homogeneity of variances were performed to meet the assumption of a mixed model anova. Log transformations were made to the data to achieve normality. Nlme package was used in order to incorporate location (Britt or Algonquin) as a random factor in the mixed model analysis. The dependent variable was monoterpene concentration with replicates being individual tree tissues (needles or phloem) monoterpene

concentration, with the category of budworm infestation level as the independent factor.

For the feeding experiment in Britt, individual trees were considered as a random factor in the mixed model, using total needle monoterpenes as the dependent variables with independent factor being needle category (either needles without budworm defoliation, larvae treated needles, or untreated needles on nearby branches, in proximity to budworm treated branch).

## 5.3 Results

### 5.3.1 Needle and phloem monoterpenes by infestation level

Needle monoterpene concentrations did not differ among infestation categories ($F_{(2,99)}$=2.244, p= 0.1114, Fig. 5.1), though there was a trend suggesting that high and low categories of infestation have higher concentrations of total monoterpenes compared to no infestation.

Phloem monoterpene concentration was suggestively higher in stands with high budworm defoliation ($F_{(2,82)}$=3.85, p= 0.0532, Fig. 5.2). Total phloem monoterpenes from highly infested trees were higher than in non-infested trees (p=0.0221), though there was no difference between high or low levels of infestation (p=0.1598).

The concentration of individual terpenes in the phloem and needles followed a similar trend to the total concentration (Table 5.1); with exceptions for a couple of minor compounds. In needle monoterpenes, only concentration of bornyl acetate differed between infestation categories ($F_{(2,99)}$=7.31, p= 0.0011); high infestation having higher concentrations than low (p=0.0085) and controls with no signs of infestation (p=0.0005). There were significant differences between

$\alpha$-pinene concentrations in different infestation levels ($F_{(2,81)}$=5.09, p= 0.0083); highly infested trees having higher concentrations than low (p=0.007) and controls (p=0.0033). Other monoterpenes generally showed an increasing trend with defoliation intensity, though this was not statistically different.

### 5.3.2 Feeding assay

Monoterpene concentrations significantly decreased in needles after three days of herbivory ($F_{(2,28)}$=9.29, p<0.0001, Fig. 5.3). Needles from branches nearby to those affected by herbivory had lower monoterpene concentrations compared to controls not affected by herbivory, before larvae were introduced (p=0.0002, p= 0.0267 respectively).

## 5.4 Discussion

The results of the current study indicate that phloem monoterpene concentration is significantly higher in highly infested jack pine budworm stands, than in stands with no infestation. Although not statistically different, needle monoterpene concentrations tended to be higher in highly infested stands compared to uninfested stands. However, in the feeding experiment which specifically focused on needles after herbivory, defoliated branches and the adjacent foliated branches had lower monoterpene concentrations than prior to herbivory. Some possible explanations for these findings are proposed here, and I propose possible consequences for other biotic agents in this system, with directions for future research.

First, it is important to note the difference between monoterpene concentrations in continuously defoliated trees in naturally infested areas, and concentrations of needle monoterpenes after manipulative larval defoliation assay. The former represents a more long term response and general condition whereas the latter

demonstrates an immediate consequence of herbivory on the plant tissues. Generally, herbivory and wounding tissues causes the increase of monoterpenes cyclases, enzymes which catalyze the synthesis of monoterpenes; however, this process of increased monoterpene production is not instantaneous, and may take several days after herbivory to be detected in the tissues (Lewinsohn et al., 1991; Litvak and Monson, 1998). This increase in monoterpene cyclase production is counteracted by the high rate of volatilization of monoterpenes from tissues defoliated by tiger moth larvae *Halisdota ingens* (Litvak and Monson, 1998; Martin et al., 2003). The aforementioned study by Litvak and Monson (1998) also showed an immediate decrease in monoterpene concentration in the needles in response to herbivory in white fir (*Abies concolor*), lodgepole (*Pinus contorta*) and ponderosa pines (*P. ponderosa*). This decrease was only returned to initial concentrations 12 days after feeding.

In contrast to the needle monoterpenes in naturally infested stands, the phloem monoterpenes increased in stands of high infestation. This may be due to an increase in monoterpene cyclases, which are likely induced in phloem resin ducts. The finding of higher phloem monoterpenes in highly defoliated trees relative to control trees suggests that a stress on one tissue type (foliage) may affect resource allocation to another type (stem phloem). Erbilgin and Colgan (In Press) similarly showed that phloem tissues had higher monoterpene concentrations than needles. The direct loss of photosynthetic tissue, depletion of starch reserves upon feeding, and volatile losses from foliage, decreases storage of valuable plant resources (Litvak and Monson, 1998). This may reduce the potential for induction of carbon-based secondary compounds in foliage (Bryant et al., 1988). Monoterpene storage in the the foliage may be short lived since herbivory causes rapid evaporation (Litvak and Monson, 1998), thus storing these defensive compounds in better protected tissues such as phloem in the stem would be less metabolically costly.

Differences between monoterpene levels in different tissue types may also be attributed to the importance of biological organisms associated with specific tissue

types. Although jack pine has evolved with the patterns of jack pine budworm outbreaks (Nealis, 1995) they rarely kill trees (McCullough, 2000). The most significant cause of mortality in jack pine is not the budworm infestation, but the subsequent attacks by bark beetles (Wallin and Raffa, 1999). For example, colonization of *I. grandicollis* and *Massarina carolinensis* rose exponentially with degrees of jack pine budworm infestation (Wallin and Raffa, 1999). That study is confirmed by our findings of induced phloem monoterpenes of jack pine stands after herbivory, and more specifically increased $\alpha$-pinene concentrations in highly infested stands. Since the most recent host and range expansion of the mountain pine beetle in western Canada's jack pine forests (Cullingham et al., 2011), it would be paramount to further explore the effects jack pine budworm defoliation may have on this potential forest threat. This is particularly interesting since $\alpha$-pinene in particular is required for the production of *trans*-verbenol, an important aggregation pheromone of the mountain pine beetle (Pitman, 1971; Gries et al., 1990).

The rapid decrease in monoterpenes in needle tissue of both defoliated and nearby branches of the same tree after defoliation indicates systemic communication. In a recent study on jack pine seedlings, systemic communication was similarly detected by volatile emissions from foliated branches of defoliated jack pine seedlings (Chapter 4, this thesis). If high volatilization occurs from wounded branches as well as from unwounded ones, as shown in the aforementioned study, the loss in monoterpene concentration in the tissues themselves is expected after only 3 days of herbivory. Though I did not test the mechanism of this response, the rapid changes observed in neighbouring branches might be best attributed to volatile signalling (from the defoliated branch), rather than through vascular means. Airborne molecules have recently been demonstrated to trigger communication among hybrid poplar leaves (*Populus deltoides x nigra*), showing that volatile communication was essential for priming the recipient leaves against herbivory (Frost et al., 2007). In that study, primed leaves received little or no vascular signalling.

Overall, jack pine trees with higher levels of of infestation tended to have

higher concentrations of specific monoterpenes. In needle monoterpenes, bornyl acetate was significantly more concentrated in needles of trees with higher budworm infestation . This increased response of bornyl-acetate was also noted in drought stressed jack pines (Lusebrink et al., 2011), as well as methyl jasmonate treated Douglas-fir *(Pseudotsuga menziesii)* seedlings (Huber et al., 2005), suggesting that bornyl-acetate plays an important role for tree responses to biotic or abiotic stress. The impacts of this particular compound on jack pine budworm may be interesting to investigate.

In phloem tissues, the main driver for the total monoterpene increases at high defoliation was $\alpha$-pinene, the most abundant terpene in jack pine trees. These results indicate that defoliation triggers responses in individual compounds as much as they do total monoterpenes, and it has been argued that fluctuations of individual monoterpenes may be especially important for biological implications (Mumm and Hilker, 2005; Colgan and Erbilgin, 2011). $\alpha$-pinene in particular may be especially relevant to the mountain pine beetle system (*D. ponderosae*) as it is converted for use as *trans*-verbenol, an aggregtion pheromone of the beetle (Pitman, 1971; Gries et al., 1990).

In conclusion, our findings show that herbivory by jack pine budworm may initially cause rapid decreases of monoterpenes in tissues, which confirm research on the rapid volatilization on monoterpenes on wounded tissues. Systemic communication is also demonstrated in these trees by a response in the branches nearby. Testing the mechanisms of monoterpene movement after herbivory would be a worthwhile topic for future research. Understanding how these changes in tree chemistry affect interactions with other organisms would also be essential for future ecological research and improvements in forest management.

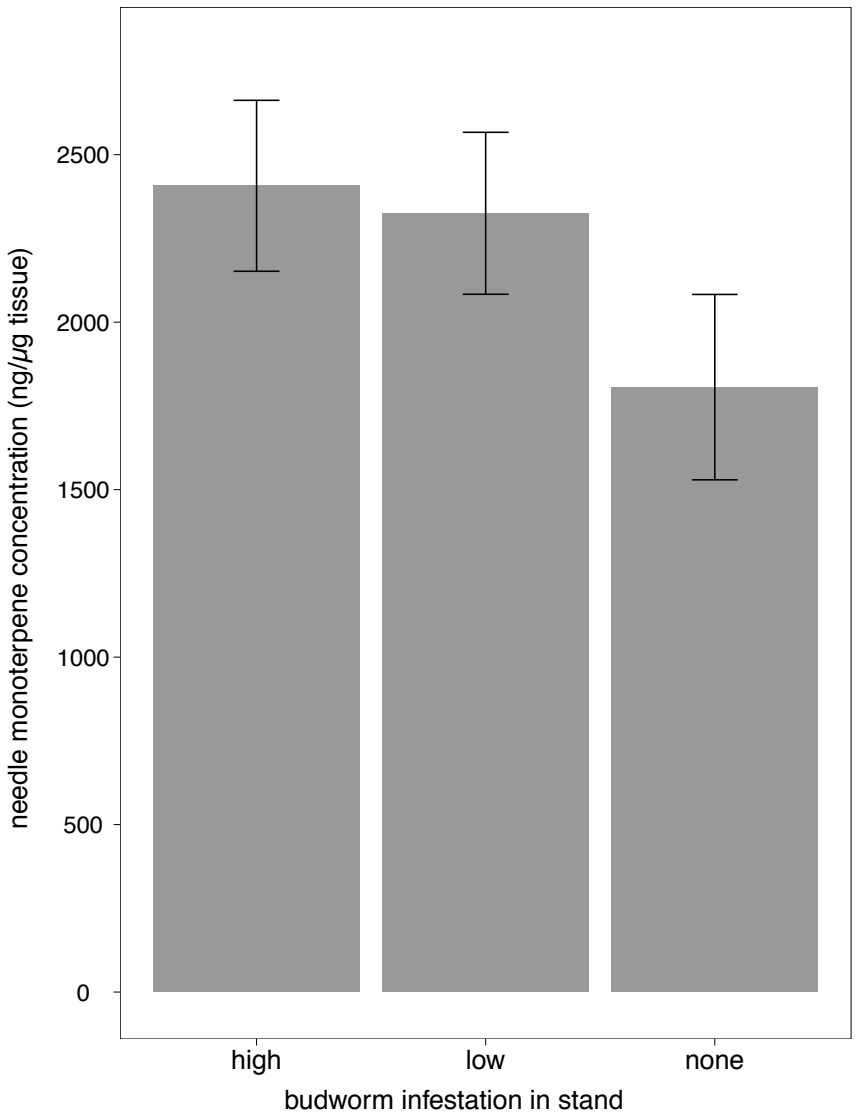


Figure 5.1: Total monoterpene concentration (ng/$\mu$g) in needles from mature jack pine (*Pinus banksiana*) trees by level of infestation by jack pine budworm (*Choristoneura pinus*) in Britt and Algonquin Park, Ontario.

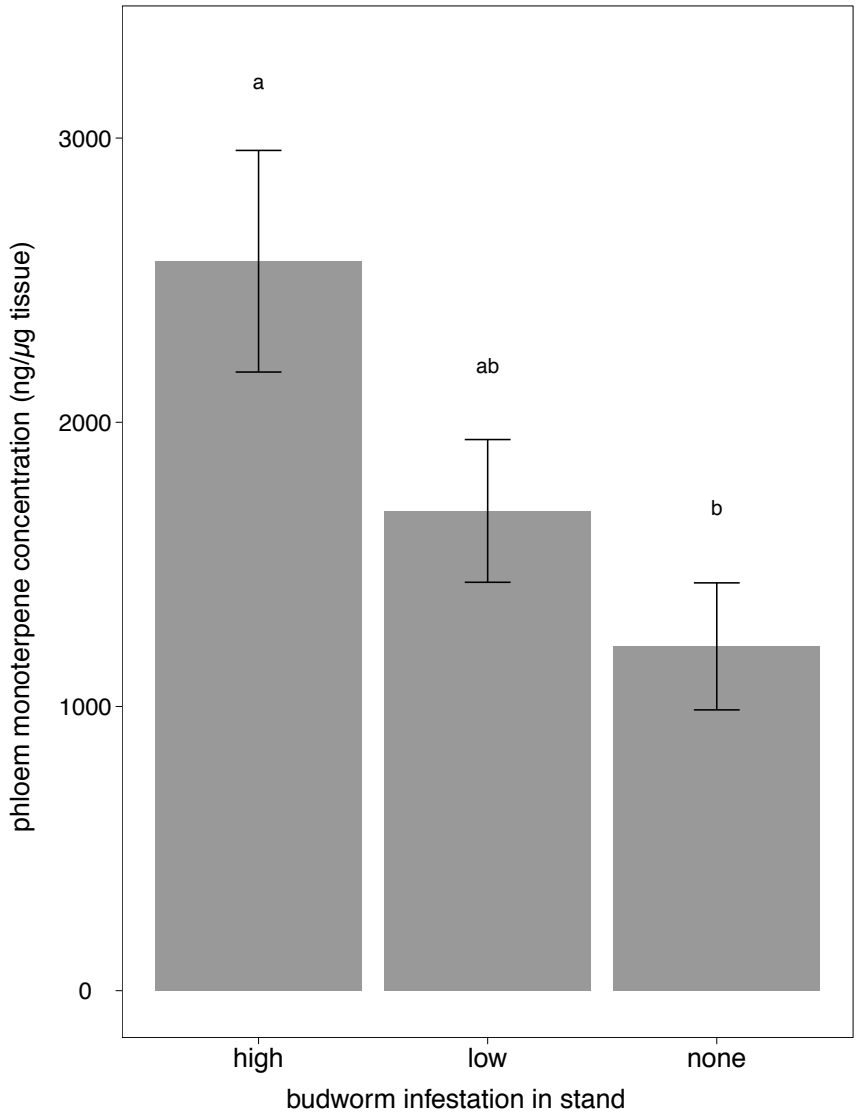


Figure 5.2: Total monoterpene concentration (ng/$\mu$g) in phloem from mature jack pine (*Pinus banksiana*) trees by level of infestation by jack pine budworm (*Choristoneura pinus*) in Britt and Algonquin Park, Ontario. A mixed model analysis was conducted with location as random factor, and intensity of defoliation categories as the independent factor. Different letters indicate significant differences in phloem monoterpenes at $p<0.05$.

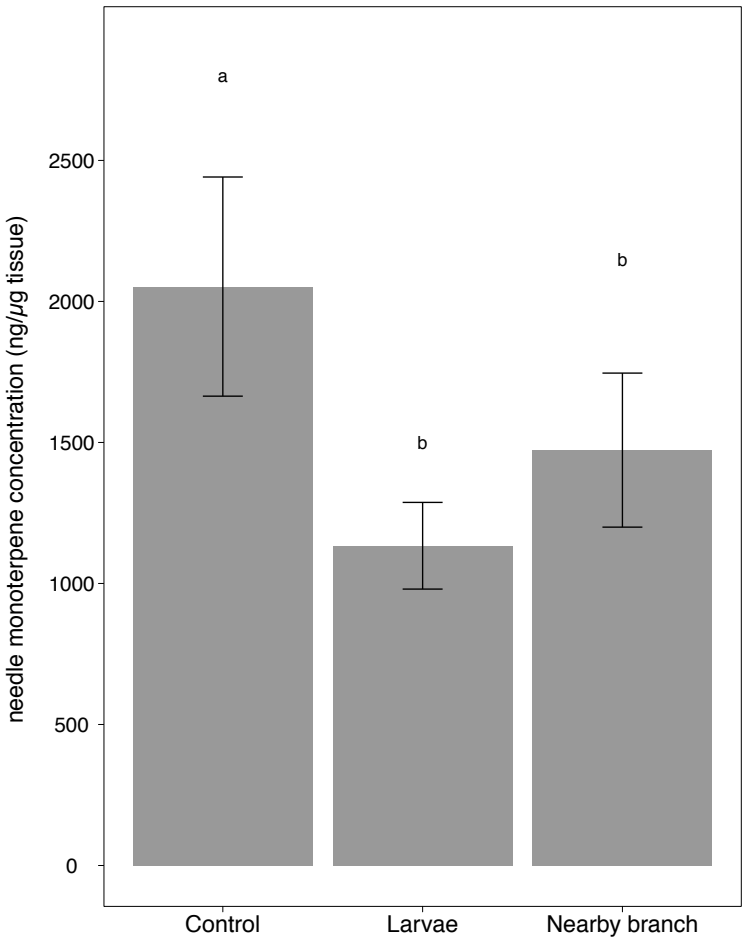


Figure 5.3: Total monoterpene concentration (ng/$\mu$g) in needle tissue of uninfested mature jack pine (*Pinus banksiana*) from Britt before jack pine budworm (*Choristoneura pinus*) larval onset (Control), 3 days after larval onset (Larvae) and on nearby branches also 3 days after larval onset (Nearby branch) in Britt, Ontario. A mixed model analysis was conducted with individual tree as random factor. Different letters indicate significant differences at $p<0.05$.

| Terpenes | Plot level defoliation | Total terpene concentration(ng/µg) Needles (±SE) | | Phloem (±SE) | |
|---|---|---|---|---|---|
| α-Pinene | high | 608.2 | (72.5) | 1129.9**(a)** | (199.3) |
| | low | 590.2 | (65.2) | 746.9**(b)** | (96.3) |
| | none | 473.1 | (78.2) | 517.8**(bc)** | (121.1) |
| β-Pinene | high | 316.6 | (48.2) | 380.0 | (69.0) |
| | low | 263.8 | (54.5) | 244.8 | (37.2) |
| | none | 199.2 | (36.0) | 201.8 | (39.2) |
| Myrcene | high | 276.8 | (50.9) | 916.3 | (189.2) |
| | low | 256.5 | (37.9) | 508.4 | (122.1) |
| | none | 194.8 | (30.4) | 423.3 | (170.1) |
| β-Phellandrene | high | 183.5 | (35.5) | 43.1 | (7.8) |
| | low | 154.0 | (45.4) | 21.9 | (4.4) |
| | none | 133.4 | (39.5) | 12.4 | (3.3) |
| Camphene | high | 449.5 | (61.6) | 50.1 | (9.4) |
| | low | 487.8 | (60.9) | 29.6 | (5.6) |
| | none | 357.7 | (73.5) | 27.2 | (13.2) |
| Carene | high | 35.4 | (12.3) | 0.0 | (0.0) |
| | low | 33.1 | (9.2) | 5.0 | (4.8) |
| | none | 34.9 | (13.0) | 0.0 | (0.0) |
| Limonene | high | 40.3 | (11.6) | 28.2 | (10.7) |
| | low | 30.4 | (6.5) | 95.0 | (50.0) |
| | none | 18.2 | (3.4) | 15.0 | (9.4) |
| cis-Ocimene | high | 44.7 | (19.7) | 0.0 | (0.0) |
| | low | 25.4 | (12.5) | 0.0 | (0.0) |
| | none | 41.6 | (32.7) | 0.0 | (0.0) |
| α-Terpineol | high | 5.1 | (0.9) | 4.0 | (1.3) |
| | low | 5.0 | (0.8) | 1.8 | (0.5) |
| | none | 7.7 | (2.5) | 0.9 | (0.5) |
| Bornyl-acetate | high | 23.9**(a)** | (3.9) | 3.6 | (0.9) |
| | low | 13.0**(b)** | (3.5) | 1.4 | (0.4) |
| | none | 8.1**(bc)** | (1.4) | 2.4 | (1.7) |
| Geraniol | high | 2.2 | (0.3) | 1.1 | (0.3) |
| | low | 2.4 | (0.2) | 0.8 | (0.2) |
| | none | 1.2 | (0.2) | 0.0 | (0.0) |
| ρ-Cymene | high | 0.2 | (0.1) | 0.1 | (0.1) |
| | low | 0.1 | (0.1) | 0.1 | (0.0) |
| | none | 0.1 | (0.1) | 0.0 | (0.0) |
| Terpinolene | high | 6.1 | (1.8) | 0.3 | (0.3) |
| | low | 18.7 | (6.9) | 25.4 | (11.7) |
| | none | 9.2 | (3.1) | 0.0 | (0.0) |
| Linalool | high | 2.8 | (0.8) | 0.1 | (0.0) |
| | low | 2.8 | (0.9) | 1.2 | (0.7) |
| | none | 1.3 | (0.6) | 0.0 | (0.0) |
| Pulgeone | high | 0.7 | (0.2) | 0.0 | (0.0) |
| | low | 0.3 | (0.1) | 0.0 | (0.0) |
| | none | 0.7 | (0.3) | 0.0 | (0.0) |

Table 5.1: Average concentrations of specific terpenes present in the needles and phloem of mature jack pine (*Pinus banksiana*) from different defoliation intensities of jack pine budworm (*Choristoneura pinus*), shown with standard error. Different letters in brackets indicate significant differences ($p<0.05$) within each monoterpene compound.

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# Chapter 6

## Thesis discussion

My research on jack pine (*Pinus banksiana*), and jack pine budworm (*Choristoneura pinus pinus*), encompassed experiments which tested systemic induction within a seedling upon herbivory, as well as the response of neighbouring seedlings receiving volatile exposure (Chapter 3). After induction by herbivory or volatile exposure, I tested the resistance of these seedlings to a subsequent challenge by a fungal pathogen (Chapter 4). Furthermore, I investigated how these systems affect the tissues of mature jack pine in the field, assessing phloem and needle tissues of trees defoliated at different intensities. In a manipulative assay, larvae were placed on non infested trees to assess immediate results of defoliation at the needle level (Chapter 5). The general findings provide evidence of systemic communication in both jack pine juveniles. First, exhibited by elevated emission of volatiles from foliated branches of defoliated seedlings (Chapter 3), and then in mature trees, demonstrated by the decrease in monoterpene concentrations of needles in defoliated and in nearby branches (Chapter 5). I also tested induction of susceptibility to a pathogenic fungus *Grosmannia clavigera* from seedlings previously mechanically defoliated, and exposed to volatiles from neighbouring seedlings (Chapter 4). Seedlings previously defoliated by larvae, or those receiving volatiles from highly defoliated seedlings were similarly defended against *G. clavigera* as those not previously defoliated or exposed to volatiles from defoliated neighbours. In

general, this research has given rise to many interesting ideas and further questions regarding conifer inducible responses.

In terms of systemic communication, this research supports several findings of induced responses in conifers (Lewinsohn et al., 1991; Litvak and Monson, 1998; Bonello et al., 2001, 2006), yet is the first to show these responses as induced by needle herbivory and expressed by emissions of volatile monoterpenes from nearby needles. A recent study on Scots pine, *Pinus sylvestris*, also showed evidence of systemic communication within tissues, as the large pine weevil *Hylobius abietis* increased volatile monoterpene emission in needle tissues (Heijaria et al., 2011). An interesting question remains about what mediates this systemic response within the seeding: vascular or volatile signalling? My results further complicate this question and suggest that: a) systemic inductions are dose dependent, and have an effect on the length of systemic response, and b) systemic inductions are also type-dependent, demonstrated by the lack of systemic response with mechanical wounding. These results are in accord with several studies that confirm mechanical wounding does not induce the same responses in trees that real herbivory can (Haukioja, 1990; Litvak and Monson, 1998; Erbilgin and Colgan, In Press).

This thesis further elaborated on conifer induction responses by testing the hypothesis from the review in Chapter 2, that signalling between trees was possible in conifer systems. Though we found induction of volatile monoterpenes from the emissions in a different part of the same plant, the same could not be said for neighbouring plants. Monoterpenes were chosen as the best candidate for signalling molecules for their broad ecological significance in conifer systems (Keeling and Bohlmann, 2006); though it was not clear from these experiments whether they were responsible for the response observed in terms of longer lesions on neighbouring seedlings. This could be directly tested, by systematically exposing seedlings to blends of volatiles and observing their responses to future challenge treatments. As mentioned in the review, ethylene is another likely candidate for signalling molecule which could be tested in the future. In Douglas-fir (*Pseudotsuga menziesii*) and

Giant Redwood (*Sequoiadendron giganteum*), ethylene induces similar responses to methyl jasmonate (Hudgins and Franceschi, 2004), a plant hormone often used as a proxy for herbivory. This molecule is small, volatile and likely active within a very short time frame (Tscharntke et al., 2001).

The signalling mechanism remains elusive, though the findings from Chapter 4 suggest that there was a volatile-mediated response from seedlings. While induction of resistance was my initial prediction, I observed the induction of susceptibility in volatile exposed seedlings, as well as seedlings previously treated by mechanical defoliation. These findings suggest that there is cross-talk occurring between the pathways responsible for responses to herbivory and pathogen attack. These findings are supported by recent findings in Austrian pine, *Pinus nigra*, seedlings showing that resistance or susceptibility to a fungal inoculum is dependent on the initial organ of induction (Blodgett et al., 2007). Researching the metabolic pathways following herbivory as compared to fungal inoculation would be a very interesting next step for understanding response mechanisms in conifers. Comparing the metabolic processes triggered by the plant hormones methyl jasmonate and methyl salicylate, would be good start for confirming hypotheses about cross-talk in conifers.

The results from mature jack pine trees suggest that herbivory induces chemical changes within a tree, confirming the study by Wallin & Raffa (1999). In that study, phloem monoterpene concentration also increased after defoliation by jack pine budworm; and furthermore increased jack pine susceptibility to attacks by bark beetles, *Ips grandicollis*, and woodborers, *Monochamus carolinensis*. These findings, as well as those from the current research, imply that budworm infestation may be a very important factor for determining the movement of mountain pine beetle, *Dendroctonus ponderosae*, on jack pine. The recent range expansion of this pest into Canada's western jack pine forests (Cullingham et al., 2011) makes this a very relevant question for future research.

Reduced monoterpene concentration in needles after herbivory in mature trees would definitely merit deeper examination. The phloem of these trees was not tested

after the manipulation of larvae. However, this could be useful to confirm whether systemic communication is possible from needles to phloem, in direct response to herbivory. These results would be ideal to compare with the current findings of plot-level phloem responses.

Another point of interest regarding these studies is the large differences in magnitude between concentrations of monoterpenes found in tissues of seedlings versus mature trees. This pattern was also noted in a recent study by Erbilgin & Colgan (In press). In the aforementioned study, possible reasons for their findings, are discussed. One important factor may be that mature trees have better physical defences, thus need not allocate as much to chemical defences as the more vulnerable seedlings. Further investigation is needed to better understand the mechanisms of these observations.

The implications of this first exploratory study on interplant communication in conifers has lead to many interesting new questions and discoveries. There is however, always room to improve, and design future experiments with fewer constraints, and more directly aimed towards answering more pointed hypotheses. For example, for more accurate answers to questions of induction in seedlings, the use of clones would be ideal in order to reduce the huge variation in monoterpene responses. Another element of variation in these greenhouse studies were larvae used to defoliate seedlings- these were taken from the field and reared to older instars in the lab, but following eclosion, some larvae were found to be parasitized. This may be a factor in the specifics of volatile responses from feeding. In terms of responses of needles and phloem after inoculation by pathogenic fungus, my experimental design did not allow for tissue collections earlier than 8 weeks after onset of fungus because otherwise volatile collection would be compromised. Testing the responses of these tissues closer to the inoculation period would be a more accurate measure of their response to the challenge. In the field, defoliation from prior years as well as more accurate assessments of infestation levels and stages of outbreak would have been ideal, especially for studying the cyclical jack pine budworm outbreak. Another

important factor mediating budworm populations is Armillaria root rot (*Armillaria ostoyae*) (Mallett, 1995). This factor was overlooked in these assays and merits a closer look, as it is very likely these pathogens could alter jack pine chemical ecology. The effects of the manipulative larval feeding on the phloem of experimental mature jack pines remains unknown. Such constraints and suggestions could be useful for future research of chemical ecology in conifers. Future research could incorporate monoterpene ratios and chirality analyses in order to better predict ecological consequences, as well as directly testing possible volatile chemicals which may trigger responses in plants.

Research on insects and conifers will continue to be important in the future, by improving our understanding of forest ecosystem processes and eventually contributing to better, more sustainable forest management practices. Range expansions like that of the mountain pine beetle will be easier to predict and manage with a better understanding of the interactions between trees, and their multiple pests and pathogens.

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