Characterizing the changes in host tree chemistry after cutting and revisiting the nutritional role of fungi in the novel mountain pine beetle host jack pine

By

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A thesis submitted in partial fulfilment of the requirements for the degree of

Master of Science

In

Forest Biology and Management

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Abstract

Studies with conifer-infesting bark beetles commonly use tree bolts to evaluate the effects of host tree quality on various aspects of insect biology. Yet, whether host quality changes between live trees and bolts cut from these trees has not been assessed. Particularly, changes in concentrations of defense chemicals and nutrients have not been compared between live trees and their cut bolts. To determine whether monoterpene and nutrient concentrations differ after cutting, jack pine trees in Lac La Biche (Alberta) were selected and sampled for phloem tissue. Then, these trees were harvested into two bolts per tree and stored at 4°C. Phloem from one bolt was sampled after three months of storage and from the second bolt six months after cutting. I found that major monoterpenes of jack pine were higher in phloem from bolts than live trees. Storage time did not affect the results. Furthermore, some nutrients including nitrogen were also higher in bolts and varied between storage times. I conclude that researchers should be aware of the observed changes in the host quality which may have positive or negative effects on the development of bark beetles.

In addition, to determine how they change host quality in bolts, I evaluated the nutritional benefits that were provided by fungi associated with the mountain pine beetle. Mountain pine beetle is commonly associated with three symbiotic fungi: *Grosmannia clavigera, Ophiostoma montium*, and *Leptographium longiclavatum.* These fungi are an important source of nitrogen and ergosterol for beetles as plant tissues are low in nitrogen, and ergosterol cannot be synthesized by insects. In lodgepole pine, it has been reported that both nitrogen and ergosterol concentrations increase in the presence of these fungi; however, the relative contribution of each species is unknown. I investigated the nutritional benefits of the three fungi in terms of their ability to concentrate nitrogen and to produce ergosterol in the jack pine phloem. Eighty jack pine trees occurring in two

forest stands in Alberta were inoculated with live culture plugs of each of the three fungi or non-colonized media (mock inoculation, control) (n=20 trees per trt). Six weeks later, phloem samples were collected from within and outside of the lesions caused by fungal infections. A single tissue of mock inoculated trees was also collected. Nitrogen and ergosterol concentrations were determined, and results indicated that total nitrogen in lesions varied with fungal species, being higher in control trees and *O. montium* lesions. Furthermore, ergosterol incidence was significantly higher in lesions compared to the samples from outside lesions. I found a higher ergosterol concentration in lesion sampled from *O. montium* than from *G. clavigera* and *L. longiclavatum*. My results indicate that *O. montium* may provide an advantage in terms of ergosterol availability during the development of the mountain pine beetle larvae.

Preface

This thesis is an original work by Sydne Guevara Rozo. No part of this thesis has been previously published. In this project I aimed to evaluate how host tree quality changes between bolts, one of the *in vitro* techniques commonly used to study mountain pine beetle biology, and live standing trees. In addition, I evaluated and provided valuable information about the nutritional benefits, in terms of ergosterol and nitrogen, that fungal associates of the mountain pine beetle provide when they grow in jack pine tree phloem.

To my family

Acknowledgements

I would like to express my sincere and deep gratitude to my supervisor Nadir Erbilgin. Thank you for everything from the first day until today! Thank you for giving me the opportunity to be a part of your team without having met me in person when I was applying from Colombia. Thank you for your professional and personal support during these two years. Thank you for your open-mindedness, patience, willingness to help and teach, and for your way to motivate me to achieve my goals.

I deeply appreciate Altaf Hussain, Kayleen Sandrowski, Guncha Ishangulyyeva, Ethan Chen, Gail Classens, Melanie De Kappelle, Dave Kamelchuk, Marla Roth, Brosnon Peters, Christien Dykstra, and Ahmed Najar for their valuable help in the lab and field. I specially would like to thank Rahmat Rajabzadeh, Jennifer Klutsch, and Jonathan A. Cale for their advice during the development of the experiments, I learned a lot from you guys. Thank you to the entire Erbilgin's lab team for their help and support during my MSc project, thank you for your questions and advice.

I wish also to express my gratitude to the members of my committee, Carol Frost and Tod Ramsfield. Thank you for taking time to read and comment on my thesis.

Thank you to my lovely family and Nico for their understanding, patience, and endless support from afar. Thanks to Jean, Altaf, and Emma (Fuai) for being my colleagues and friends.

Thank you to the NSERC Strategic Network – TRIANet Turning Risk Into Action for the Mountain Pine Beetle Epidemic (tria-net.srv.ualberta.ca/) for the financial support to my project.

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Chapter 1- General Introduction

Bark beetles (Coleoptera: Curculionidae, Scolytinae) attack some of the most economically and ecologically important tree species around the world (Safranyik *et al.*, 2010). In North America, there are a few species of bark beetle in the genera *Dendroctonus* and *Ips* that periodically kill large numbers of living trees, mainly conifers. All tree killing bark beetle species rely on their ability to aggregate in host trees *via* aggregation pheromones, and to inoculate host trees with their associated fungi (Blomquist *et al.*, 2010; Six, 2012). Pheromones have multiple functions in bark beetle biology, including mate finding, overwhelming host defenses, and successful reproduction (Erbilgin *et al.*, 2017a). Fungal associates play a critical role in bark beetle biology as they are important for exhausting host tree defenses and providing essential nutrients such as nitrogen and ergosterol to beetle larvae (Bentz & Six, 2006; Bleiker & Six, 2007). Due to their importance, many studies have investigated bark beetle-host tree-fungal interactions either in the field or *in vitro*. In my thesis, I will focus on one of the most prominent members of *Dendroctonus* genus in North America, *Dendroctonus ponderosae* Hopkins, mountain pine beetle (MPB).

Mountain pine beetle has killed millions of hectares of pine (*Pinus*) forests over the past decades in western North America (Safranyik *et al.*, 2010). In Canada, MPB outbreaks were historically restricted to lodgepole pine (*P. contorta* Douglas) forests in British Columbia, but they have expanded to the eastern edge of lodgepole pine forests in western Alberta. More recently, MPB outbreaks have been found in jack pine (*P. banksiana* Lamb) forests in northern Alberta, and threatened to move towards the boreal forest, where jack pine is a dominant tree species (Cullingham *et al.*, 2011).

Biology of MPB is well characterized (Safranyik & Carroll, 2006; Safranyik *et al.*, 2010). Briefly, the host tree colonization process begins with the arrival of a female beetle at a new host. After successful entry into the host tissue, the female beetle releases an aggregation pheromone, *trans*-verbenol which is an oxidation product of host monoterpene α-pinene. This pheromone attracts beetles of both sexes and initiates a mass attack to overwhelm tree defenses. Following mating, female beetles construct maternal galleries along which they deposit eggs and inoculate host tissues with the symbiotic ophiostomatoid (Ascomycota: Ophiostomataceae) fungi. After hatching, larvae construct larval galleries while feeding on the host phloem and fungal hyphae. Once pupation occurs, beetles develop into teneral (sexually immature) adults which are pale colored and soft. Teneral adults feed on the phloem, harden, and become dark before emerging as adults from the natal host. Although the length of the MPB life cycle changes depending on the ambient temperature, most populations in western North America are univoltine. Hatching occurs within a week following deposition, and the third and early fourth instar are reached before winter. In spring, once temperatures are warm enough, larvae resume feeding, transforming to pupae by June. Teneral adults mature in one or two weeks, and mature beetles emerge during late June and early July in western Canada.

Much of the feeding occurs during larval stages, in which larvae exclusively rely on the nutritional component of host tissues and hyphae from the associated fungi inoculated during host colonization. Three ophiostomatoid fungi have been commonly associated with MPB: *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer, and Wingfield, *Ophiostoma montium* (Rumbold) von Arx, and *Leptographium longiclavatum* Lee, Kim, and Breuil (Lee *et al.*, 2005, 2006). These fungi are considered an important source of sterols and nitrogen for the developing larvae and can significantly improve beetle fitness (Six & Paine, 1998; Bentz & Six, 2006; Bleiker

& Six, 2007). Sterols affect beetle growth, metamorphosis, and reproduction and must be acquired through diet (Clayton, 1964; Behmer & Nes, 2003). Likewise, nitrogen plays a critical role in beetle development as it is a constituent of proteins, nucleic acids, and hormones (Mattson, 1980). Since nitrogen content in plants is lower than in insects, the fungi are considered an important factor to supplement the low nitrogen diet to which beetles are exposed by concentrating it in the phloem (Mattson, 1980; Ayres *et al.*, 2000; Cook *et al.*, 2010).

In order to study the nutritional benefits that the fungi provide to MPB and other aspects related to the beetle-host tree interactions such as the effects of host quality on beetle development, several *in vitro* techniques have been utilized. These techniques include the use of rearing tubes (e.g., (Myrholm & Langor, 2016), phloem sandwiches (Therrien *et al.*, 2015), and bolts (Erbilgin *et al.*, 2014). Of these techniques, bolts are commonly used to determine the impacts of host tree quality on beetle host preference, pheromone production, and brood production and development (Erbilgin *et al.*, 2014). Host tree quality is usually characterized by the concentrations of defense chemicals and nutrients. In addition, beetle performance can also be influenced by fungal growth and the nutrients that fungi accumulate in the phloem (Goodsman *et al.*, 2012; Arango-Velez *et al.*, 2016).

Despite the importance of fungi as a source of sterols and nitrogen in MPB biology, information regarding the benefits that each fungal species provides is limited, especially in the most northern expansion of MPB in western North America. Likewise, although bolts have been widely used in bark beetle research it is unknown how host quality changes after cutting live trees and hence, how well they emulate live conditions when they are used in the laboratory. In the present study, I focused on evaluating how host tree quality changes after cutting and determining the nutritional benefits that each of the three fungal associates provides to MPB in terms of nitrogen and sterols.

In Chapter 2, I compared monoterpene and nutrient concentrations before and after cutting live jack pine trees. I sampled phloem tissue from 30 healthy jack pine trees in Lac la Biche (Alberta) and their bolts and compared their macro and micro nutrient and monoterpene contents.

In Chapter 3, I further evaluated whether nitrogen and sterol concentrations vary among *G. clavigera*, *O. montium*, and *L. longiclavatum* when they grow in live standing jack pine trees. I selected 80 trees from two forest stands in Lac la Biche (Alberta) and I inoculated them with fungal culture or agar media plugs. I evaluated nitrogen and ergosterol concentrations in colonized and uncolonized fungal phloem six weeks after inoculations.

Overall, I expect that the outcome of my thesis will lead to a better understanding about how *in vitro* experiments imitate live tree conditions, as well as allowing us to identify the relative nutritional benefit that each of the fungal associates is providing to the MPB.

Chapter 2 - Short and long-term cold storage of jack pine bolts are associated with higher concentrations of monoterpenes and nutrients

2.1 Introduction

Mountain pine beetle (MPB) (*Dendroctonus ponderosae*, Hopkins; Coleoptera: Curculionidae, Scolytinae) has killed millions of hectares of pine trees, mainly lodgepole pine (*Pinus contorta* Douglas) over the past decades in western North America (Safranyik *et al*. 2010). In Canada, MPB outbreaks have expanded to the novel host jack pine (*Pinus banksiana* Lamb) forests in northern Alberta (Erbilgin *et al*. 2014). Multiple approaches have been utilized to evaluate the effects of host tree quality on MPB in *in vitro* experiments including the use of rearing tubes (Myrholm & Langor, 2016), phloem sandwiches (Therrien *et al.*, 2015), and bolts (Erbilgin *et al.*, 2014). Of these, bolts, cut from trees to a designated length, usually 40-50 cm, have been commonly used to evaluate the impact of host quality on different aspects of MPB biology (Erbilgin *et al.*, 2014) because they can be easily transported and maintained in the laboratory.

Host tree quality is critical for both adult and immature stages of bark beetles. Host monoterpenes, for example, can act as a precursor for MPB pheromones, as anti-attractants, or anti-feedants (Erbilgin *et al.*, 2017a). Beetle development also depends on the available host phloem nutrients. Nitrogen, for instance, can be a major limiting factor for the growth and reproduction of bark beetles due to its low content in plant tissues (Goodsman *et al.*, 2012). Likewise, metal ions obtained from plant tissues play an important role as cofactors of several enzymes and are involved in the osmoregulation, cold tolerance, and purine metabolism in insects (Dow, 2017).

Approaches comparing the quality of beetles' hosts using bolts may not truly account for host quality effects on MPB as this may be substantially altered when trees are cut. To date, how cutting

trees affects host quality has not be evaluated. Thus, my goal was to determine the changes in monoterpene and macro and micro nutrient concentrations before and after cutting live jack pine trees. I expect that the results obtained through this study should lead us to a better understanding of the potential consequences of cutting on bark beetle biology and ecology.

2.2 Materials and Methods

2.2.1 Experimental Design and Sampling

I selected 30 healthy jack pine trees (i.e. no signs of biotic or abiotic stress) in two forests stands in Lac la Biche, Alberta, Canada, in June of 2016. Fifteen trees (diameter at breast height = 27.7 cm ± 0.84 SE) were selected per stand (55°04'13.8" N, 111°59'48.2" W; 54°55'10.2" N, 111°28'05.6" W). Two phloem samples (3cm x 3cm) from the north and south aspect of each tree were taken at 1.3 m height. Samples were kept in liquid nitrogen in the field and then stored at -40°C in the laboratory until extraction. Two bolts (each 35 cm long) were cut from each tree above and below the 1.3 m height (total of 60 bolts). Bolts were sealed with melted wax in both ends, transported to the laboratory, and stored at 4°C. After three months of storage, half the bolts representing the 30 trees were sampled for phloem tissue as it was described above, and the remaining bolts were similarly sampled six months after cutting. All samples were stored at -40°C until chemical analysis. Phloem moisture in the bolt was calculated only after six months of storage using the equation (wet weight-dry weight)/ dry weight.

2.2.2 Defense Chemical Analysis

To extract host defense compounds, mainly monoterpenes, the two samples taken from each tree or bolt in the different time periods were pooled (trees and bolts by separated) and ground in liquid nitrogen. Samples were prepared and analyzed using similar procedures as those described by Erbilgin *et al*. (2017b). Briefly, ground tissue (100 mg) was extracted twice with 0.5 mL of pentane and 0.004% tridecane as an internal standard in 1.5 mL microcentrifuge tubes. Extracts were vortexed for 30s at 3,000 rpm, sonicated for 10 min, centrifuged at 13,000 rpm for 15 min at 4°C, and placed in a freezer for at least 2 hrs. Extracts were then transferred into gas chromatograph (GC) vials and 1 µL were injected at a split ratio of 10:1 in a coupled GC-mass spectrometer (GC-MS) (7890A-5975C, Agilent Tech., Santa Clara, CA, USA) equipped with an HP Innowax column (ID 0.25 mm; length 30 m; Product ID: 19091N-233, Agilent Tech) with a helium carrier gas flow of 1.1 mL min^{-1}, temperature of 55°C for 1 min, then 40°C min^{-1} to 65°C (held for 1 min), then 40°C min^{-1} to 75°C (held for 0.5 min), then 7 °C min^{-1} to 130°C, and then 20°C min^{-1} to 250°C (held for 0.5 min). Compounds were identified by comparing retention times and mass spectra with the commercial standards and quantified through calibration curves using the standards. Monoterpenes were reported as ng mg^{-1} of fresh weight tissue.

2.2.3 Nutrient Analysis

Ground tissue pooled (40 mg) from each tree and bolt was dried at 40°C during 24 hrs in the oven and transported to Natural Resources Analytical Laboratory at the University of Alberta (http://nral.ualberta.ca) for macro and micronutrient analyses. Samples were analyzed in Thermo FLASH 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Bremen, Germany). Thirty samples were run twice to corroborate the results. Nutrients are reported as apercentage of dry weight.

2.2.4 Statistical Analyses

To compare monoterpene and nutrient results between live-trees and bolts, linear mixed model were performed for each monoterpene and nutrient using the function lme from the nlme package in R (Pinheiro *et al.*, 2018). I used time as a fixed effect (time 0: tissue taken from live trees, and time 3 and 6: tissues taken from bolts after three and six months of storage, respectively) and trees and sites as random effects. Data were natural log transformed to meet model assumptions of normality and homoscedasticity. Significance of the fixed effect in the model was determined using the *Wald chi-square* test from the car package in R (Fox & Weisberg, 2011). *Post-hoc* Tukey's honest significance difference test was carried out with the general linear hypothesis function (glht) in the package multcomp (Hothorn *et al.*, 2008) using an $\alpha=0.05$ to indicate significant differences between times.

2.3 Results

2.3.1 Defense Chemicals

Both three and six month-stored bolts had higher concentrations of monoterpenes than live trees (Table 2.3; Fig. 2.3.1a-c). Specifically, α-pinene (3.73 X after 3 months and 2.93 X after 6 months) and β-pinene (2.69 X after 3 months and 2.17 X after 6 months), camphene (3.06 X after 3 months and 1.98 X after 6 months), limonene (2.63 X after 3 months and 2.41 X after 6 months), and myrcene (2.63 X after 3 months and 2.42 X after 6 months) were significantly higher in bolts and were present in almost all trees and bolts sampled. β-phellandrene, terpinolene, and 3-carene were mostly detected in bolts than in trees being significantly higher in bolts (β-phellandrene 21.9 X after 3 months and 27.48 X after 6 months; terpinolene 1.28 X after 3 months and 1.29 X after 6

months; 3-carene 2.57 X after 3 months and 2.46 X after 6 months). No statistical differences were found between storage times. I did not conduct any statistical analysis for the remaining monoterpenes as they were either below detection limit or present in very few samples collected.

2.3.2 Macro and Micro Nutrients

Regarding nutrients, higher concentrations were generally found in bolts than in trees and varied with storage times (Table 2.3; Fig. 2.3.2a, b). Concentrations of calcium (1.13 X after 3 months and 1.29 X after 6 months), nitrogen (1.21 X after 3 months and 1.46 after 6 months), potassium (1.08 X after 3 months and 1.24 X after 6 months), phosphorus (1.18 X after 3 and 1.15 X after 6 months), magnesium (1.05 X after 3 and 1.21 X after 6 months), sulfur (1.12 X after 3 and 1.17 X after 6 months), manganese (1.03 X after 3 months and 1.17 X after 6 months), and zinc (1.11 X after 3 months and 1.72 X after 6 months) were higher in bolts than in trees. Concentration of cooper was lower after 3 months of storage (0.01 X) but higher after 6 months of storage (1.89 X). Carbon and iron concentrations did not vary significantly during the storage times (Carbon: 1.02 X after 3 months and 0.98 X after 6 months; Iron 1.13 X after 3 months and 1.08 X after 6 months), although carbon-to-nitrogen ratio (C/N) was lower after six months of storage (C/N 176:1 and 118:1 for time 0 and 6, respectively).

2.4 Discussion

The higher concentration of monoterpenes in bolts is partially explained by a progressive loss of water from bolts. Although the reduction of moisture was not quantified in the present study, Redmer *et al.* (2001) stored red pine (*P. resinosa*) bolts at 10°C and reported that bolts lost about 20% of phloem water after three months of storage and about 50% after six months of storage. In

the current study, after six-months of storage, phloem moisture content in bolts was about 64%. Since my quantification methods for monoterpenes were based on the fresh weight of phloem tissues, loss of moisture likely reduced the overall fresh weight of tissues quantified thus increasing their concentration. However, since monoterpene concentrations did not differ between three-month and six-month storage periods and that a larger portion of moisture remained in the phloem, we suspect that water loss cannot fully explain the changes observed in the bolts. One likely explanation is that most of the changes observed in the bolts occurred during the first three months and storage beyond this point did not influence monoterpenes. In this study, monoterpenes were quantified based on fresh tissues as they represent a more realistic proxy to what beetle larvae are facing under wood, than dry tissues.

Increase in nutrient levels in bolts, on the other hand, has been reported in other studies (Holub *et al.*, 2002). Although the mechanism is not clear, it is likely that during the storage process, nutrients were redistributed into bolt phloem, leading to increased nutrient concentrations (Boddy & Watkinson, 1995). For nitrogen, fixation by bacteria in bolts may also be a potential mechanism during storage (Graham & Cromack, 1982). Decrease in carbon levels with storage time may be caused by microbial activity during bolt decomposition (Boddy & Watkinson, 1995). Since I used dried phloem for nutrient analysis, water loss likely did not influence the nutrient concentrations observed in the current study.

2.5 Conclusions

The results reported here are relevant for studies determining the potential effect of host pine quality on bark beetles. First, relatively higher concentrations of monoterpenes along with reduced

phloem moisture content may differentially alter larval development and pheromone production by mature bark beetles. The presence of pheromone precursors, such as α-pinene may have a positive effect on pheromone production as there is a positive linear relationship between α-pinene concentrations and *trans*-verbenol production in MPB (Erbilgin *et al.*, 2014). In contrast, the presence of toxic compounds such as limonene may inhibit larval development as limonene is one of the most toxic monoterpenes present in the phloem of MPB host trees (Chiu *et al.*, 2017). Second, higher nutrient concentrations, particularly nitrogen in bolts may have a positive impact on larval development because trees tend to be limited in nitrogen, and thus increased concentrations of nitrogen can improve larval development (Goodsman *et al.*, 2012). Third, even though we took many steps to minimize moisture loss from our bolts (i.e., waxed both ends of bolts in the field, stored bolts at 4°C a few hours after cutting), apparently significant changes in monoterpene and nutrient concentrations are unavoidable. Further studies are needed to determine if storage temperatures below 4°C could further minimize the changes observed in this study and whether the observed changes can also occur in other pine species that are used to rear bark beetles. Nevertheless, researchers should consider the consequences of changes in tree defense chemicals and nutrients on bark beetles when they conduct experiments with bolts or any other *in vitro* experiments in the laboratory.

Table 2.3. Results from linear mixed model analyses comparing monoterpene and nutrient concentration of live jack pine (*Pinus banksiana*) trees with stored bolts. Bolts were stored at 4°c for three and six months after cutting and half of the bolts were sampled at each storage time.

	Wald X^2 (2)	***P- value***
Monoterpenes (ng mg^{-1} of fresh weight phloem tissue)		
α-Pinene	232.67	<0.001
β-Pinene	123.45	<0.001
Camphene	119.60	<0.001
β-Phellandrene	82.27	<0.001
Limonene	73.14	<0.001
Myrcene	68.63	<0.001
Terpinolene	18.54	<0.008
3-Carene	7.11	<0.028
Nutrients *(*percentage in dry weight phloem tissue*)*		
Nitrogen	128.16	<0.001
Zinc	90.62	<0.001
Calcium	69.73	<0.001
Copper	53.62	<0.001
Magnesium	33.80	<0.001
Potassium	37.88	<0.001
Sulfur	31.68	<0.001
Manganese	23.03	<0.001
Phosphorus	20.31	<0.001
Carbon	4.06	0.2624
Iron	0.43	0.804

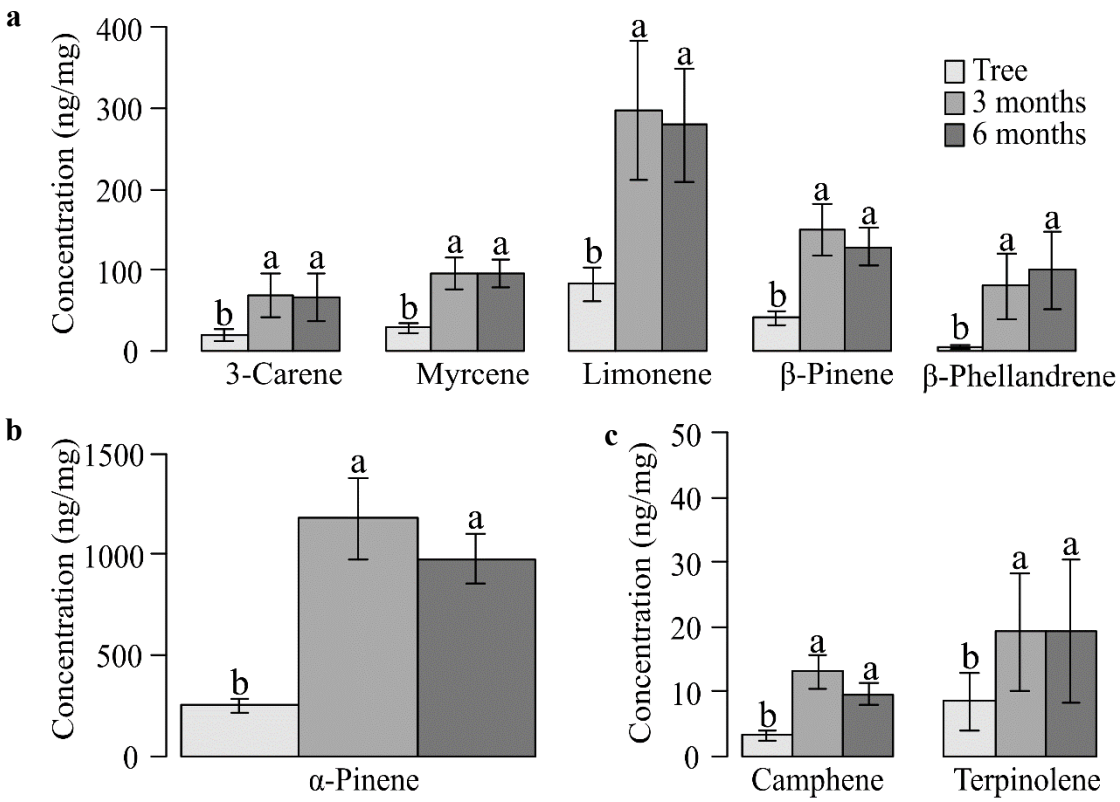


Figure 2.3.1. Mean (± SE) Monoterpene concentrations in phloem tissues sampled from live jack pine (*Pinus banksiana*) trees and bolts after three and six months of storage. Time 0 indicates tissue taken from live trees, and times three and six indicate tissues taken from bolts after three and six months of storage, respectively. Bars with different letters are statistically different from each other.

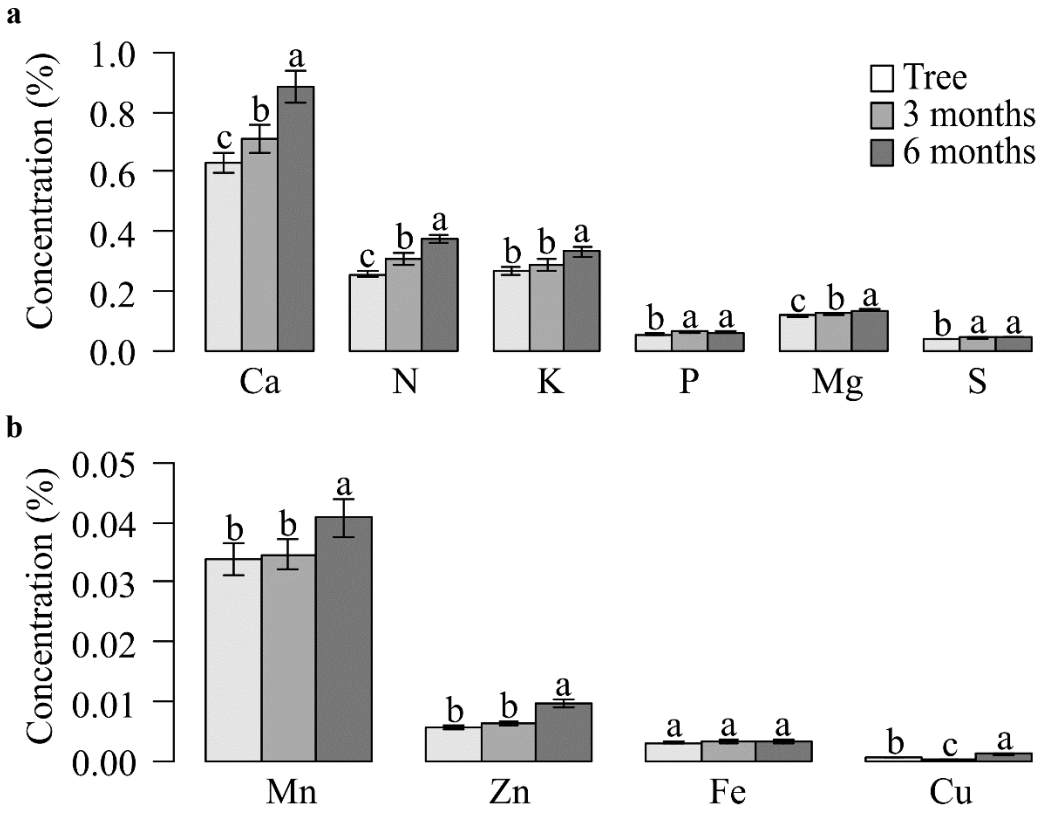


Figure 2.3.2. Mean (± SE) Nutrient concentrations in phloem tissue sampled from live jack pine (*Pinus banksiana*) trees and bolts after three and six months of storage. Ca: calcium, N: nitrogen, K: potassium, P: phosphorus, Mg: magnesium, S: sulfur, Mn: manganese, Zn: zinc, Cu: copper, Fe: iron. Time 0 indicates tissue taken from live trees, and times three and six indicate tissues taken from bolts after three and six months of storage, respectively. Bars with different letters are statistically different from each other.

Chapter 3 – Symbiotic fungi of mountain pine beetle provide different sources of nitrogen and ergosterol

3.1 Introduction

Mountain pine beetle (MPB) outbreaks (*Dendroctonus ponderosae*, Hopkins; Coleoptera: Curculionidae, Scolytinae) are historically restricted to lodgepole pine (*Pinus contorta* Douglas) forests in North America including British Columbia; however they have recently expanded to jack pine (*Pinus banksiana* Lamb) forests in Alberta and threaten to move toward eastern boreal forests (Safranyik *et al.*, 2010). The beetle is associated with three symbiotic fungal species: *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel, de Beer, and Wingfield, *Ophiostoma montium* (Rumbold) von Arx, and *Leptographium longiclavatum* Lee, Kim, and Breuil (Lee *et al.*, 2005, 2006), which can affect beetle fitness by providing nutrients during the larval development. These fungi can increase nitrogen and sterol concentrations in attacked lodgepole pine trees (Bentz & Six, 2006; Bleiker & Six, 2007); however, it is unknown the relative contribution of each fungus on the novel host jack pine trees.

The mountain pine beetle life cycle is well described (Safranyik *et al.*, 2010). The host colonization process begins with the arrival of a female beetle at a new host. Female beetles release an aggregation pheromone that attract beetles of both sexes and causes a mass attack on the host tree. After a successful host colonization, female beetles excavate galleries along which they oviposit and inoculate fungal associates into the host. Following hatching, larvae feed on the phloem and fungal hyphae through their four instars of development. Once pupation occurs, immature adults (called tenerals) primarily feed on the fungal spores prior to emergence from hosts.

Fungi associated with the MPB are transported by the beetle in a specialized structure called the mycangium or phoretically on the exoskeleton, and their presence and relative abundance is highly

affected by temperature conditions (Six & Bentz, 2007; Bleiker *et al.*, 2009). *Grosmannia clavigera* and *L. longiclavatum* appear to be more tolerant of cold conditions than *O. montium* and the relative abundance of the fungi is strongly influenced by latitude (Kenkel *et al.*, 1997; Six & Bentz, 2007; Roe *et al.*, 2011). *Leptographium longiclavatum* is more abundant in northern sites and replaced by *G. clavigera* as latitude decreases (Roe *et al.*, 2011). In contrast, optimal growth of *O. montium* occurs in warmer climates and its abundance is less dependent on latitude (Six & Bentz, 2007; Roe *et al.*, 2011). In general, it has been shown that the three fungi commonly coexist, the most common association being between *O. montium* and *G. clavigera* or *O. montium* and *L. longiclavatum*, and *G. clavigera – L. longiclavatum* rarely occur together (Rice & Langor, 2009; Roe *et al*., 2011).

Grosmannia clavigera, *L. longiclavatum* and *O. montium* significantly improve MPB fitness and are considered an important source of nitrogen and sterols (Bentz & Six, 2006; Bleiker & Six, 2007; Cook *et al.*, 2010; Goodsman *et al.*, 2012). Nitrogen content and requirement in beetles is higher than in plants (Mattson, 1980) and it is thought that fungi supplement the beetle's low nitrogen diet by concentrating nitrogen within the hyphae and conidia that surround larvae developing in the phloem (Ayres *et al.*, 2000; Bleiker & Six, 2007; Cook *et al.*, 2010; Goodsman *et al.*, 2012). Likewise, sterols cannot be synthesized by beetles and must be acquired through the diet. Sterols are required in lipid biostructures and also play an important role as a precursor of insect steroid hormones which regulate different developmental processes (Clayton, 1964; Behmer & Nes, 2003). The dominant sterol in most Ascomycota fungi is ergosterol ($C_{28}H_{44}O$) (Behmer & Nes, 2003). Ergosterol is an important element of fungal cell membranes, and in several insect-fungus symbioses it has been shown that it is required for the pupation and oviposition of viable

eggs in the associated insect (Behmer & Nes, 2003; Six, 2003). In MPB, ergosterol can play critical roles in beetle development (Bentz & Six, 2006).

Studies in MPB-fungal interactions have primarily focused on nitrogen (Bleiker & Six, 2007; Cook *et al.*, 2010; Goodsman *et al.*, 2012). Experiments *in vitro* have shown that fungi associated with MPB can concentrate nitrogen in their hyphae, with *G. clavigera* apparently being more efficient than *O. montium* (Bleiker & Six, 2007; Cook *et al*., 2010). However, to date, it has not been evaluated whether the fungi can concentrate higher nitrogen levels in colonized phloem than uncolonized, as occurs in other bark beetle species (Ayres *et al.*, 2000). On the other hand, although ergosterol concentrations increase following bark beetle attacks, relative to those in non-attacked trees, to date, the relative contribution of each fungal associated is unknown (Bentz & Six, 2006). Furthermore, all available literature has focused on the historical host of MPB, and similar information is lacking in the jack pine.

The objective of this study was to determine whether nitrogen and ergosterol concentrations in live jack pine trees varied among *G. clavigera*, *O. montium*, and *L. longiclavatum* when trees were artificially inoculated with these fungi. I compared the concentrations of nitrogen and ergosterol between fungal inoculations and mock inoculations (no living fungal cultures in the inoculations) and measured the concentrations of nitrogen and ergosterol in the lesions (reaction zones on phloem in response to fungal growth) and uncolonized phloem. In addition, I evaluated whether the growth and performance of the fungi and their overall nitrogen and ergosterol contributions vary with tree heights, as it has been suggested that tree height can influence fungal growth (Goodsman *et al*. 2013). I hypothesized that nutritional benefits provided by all three associated fungi vary depending on their phylogenetic relationship (Six & Paine, 1999; Rice & Langor, 2009).

3.2 Materials and Methods

3.2.1 Sampling collection

I selected 40 healthy jack pine trees (i.e. no signs of biotic or abiotic stress) from each of two forest stands located near Lac la Biche (55°03'27.9"N 112°01'36.4"W; 55°04'31.7"N 111°59'42.7"W), Alberta, in early June of 2017. In each stand, trees (diameter at breast height= 21.58 cm ± 0.23 SE) were randomly assigned to one of the four inoculation treatments, with ten trees per treatment. Treatments consisted of inoculations with culture plugs (1 cm dia.) of three fungi, *G. clavigera*, *L. longiclavatum*, *O. montium*, or agar growth media as a mock control. All trees were inoculated at three heights along the tree bole (1m, 3m, 5m) in the four cardinal directions (north, south, east, west). A single isolate of each of *O. montium* (EL005), *G. clavigera* (EL033), *L. longiclavatum* (EL0380) was used for the inoculations. Fungi were isolated from blue stain sapwood between *D. ponderosa* galleries in *P. contorta* (for *O. montium*), *P. banksiana* (for *L. longiclavatum*) and *P. contorta – P. banksiana* hybrid (for *G. clavigera*)(Supplementary Table 3.2.1a). Additionally, during inoculations, phloem samples from each height and direction were collected to determine constitutive nitrogen level. Inoculation points were covered with plastic wrap to avoid moisture loss and further fungal infections.

Six weeks later, phloem samples from lesions, which were formed as a result of fungal growth in host tissues, and phloem samples from outside-lesions, were collected to determine ergosterol and nitrogen concentrations (Suppl. Fig. 3.2.1b). From each height on all trees, I collected the whole lesion area in the north aspect of trees inoculated with *G. clavigera* and *L. longiclavatum*, and the whole lesion area in north and east aspects of trees inoculated with *O. montium*. I collected additional lesion tissues for *O. montium* because the size of lesions formed was too small to provide the amount of tissues required for chemical analyses (see details below). On each tree, the lesion in the north aspect was also photographed to estimate the fungal growth using ImageJ. The phloem tissue adjacent the lesions (about 4 cm right of the lesions, Suppl. Fig. 3.2.1c) was also collected

(5cm x 4cm) from all fungal inoculated trees. From control trees, one 5cm x 4cm phloem sample was collected above the wound inoculation point in the north aspect of trees.

All tissue samples were kept in liquid nitrogen in the field and transferred to -40°C in the laboratory. Tissue samples collected from the four cardinal directions were pooled by height to determine constitutive nitrogen level for each individual tree and then ground in Tissue Lyser II (QIAGEN Corp., Germany). Lesion samples, samples collected adjacent to lesions (outside-lesion hereafter), and phloem from control trees were ground in liquid nitrogen using mortar and pestle. These samples were used to determine ergosterol and nitrogen concentrations per treatment. In the case of trees inoculated with *O. montium*, the two lesions collected were pooled together prior to grinding.

3.2.2 Nitrogen analysis

Ground tissue (40 g) from samples collected before and after inoculations was dried at 40°C for 24h in the oven and transported to NRAL Laboratory at the University of Alberta (http://nral.ualberta.ca) for total nitrogen determination. Samples were analyzed in a FLASH 2000 HT Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Bremen, Germany). Nitrogen is reported as a percentage of dry weight.

3.2.3 Ergosterol Analysis

Ground tissues from lesions and outside-lesion samples were freeze dried for four days (Labconco Corp., USA). Ergosterol in 300 mg of the dried tissue was extracted with 5 mL 10% KOH in methanol following the protocol described by Sterkenburg *et al.* (2015) with slight modifications. Briefly, after 15 min of sonication and 90 min of incubation in a water bath (70°C), 1 mL of water was added to increase the polarity of the methanol phase. After adding 3 mL of pentane, samples

were vortexed for 1 min at 3,000 rpm, followed by 5 min centrifugation at 1,000 rpm. The nonpolar phase (upper layer) was transferred into a new glass tube of 20 mL. The same process was repeated after adding 2 mL of pentane to the original tubes. I then pooled both resulting extracts. Pentane from each tube was evaporated on a 40°C heating block under N_2 gas flow and the samples were resuspended in 300 µL of hexane containing 0.001 mg/mL of methyl tricosanoate (internal standard). Samples were heated in a water bath (40° C) for 5 min and sonicated 3 min before filtering. Extracts were transferred into glass chromatography vials and 2 µL were injected in pulsed splitless mode into a coupled Gas Chromatograph-Mass Spectrometer (7890A-5975C, Agilent Tech., Santa Clara, CA, USA), equipped with an HP-5 column (ID 0.320 mm; Film 0.25 µm; length 30 m; Agilent Tech.) with a helium carrier gas flow of 1.5 mL min^{-1}, temperature of 50°C for 2 min, then 25°C min^{-1} to 240°C, and then 15°C min^{-1} to 300°C (held for 7 min). The ergosterol and internal standard were detected and quantified using scan and selective ion monitoring mode (SIM), respective and simultaneously.

Peak identification was based on the comparison with a MS-library (NIST) and the retention time of a commercial standard (Ergosterol >95%, Sigma-Aldrich, ON, CA; Methyl Tricosanoate > 99%, Nu-Chek Prep Inc., MIN, USA). The fragment ions of m/z 363, 396 were used for ergosterol quantification and 368 for internal standard quantification. A calibration curve was constructed with commercial ergosterol standard solutions in hexane, ranging from 0.002 to 0.2 mg/mL at five points, plotting the peak height (ion signal) of the analyte divided by the ion signal of the internal standard versus the concentration of the analyte over the concentration of the internal standard. The equation generated by the curve was used for ergosterol quantification (Suppl. Fig. 3.2.3). The Limit of Detection (LOD) and the Limit of Quantification (LOQ) were determined from the background response of ten repetitions of an extracted control sample. They were calculated as

three and ten times (for LOD and LOQ, respectively) the standard deviation of the signal response plus their average. Ergosterol is reported as μg g^{-1} dry weight phloem.

3.2.4 Statistical Analyses

Fungal growth and total nitrogen concentrations before and after fungal inoculations were analysed through a linear mixed model using the function lme from the nlme package in R (Pinheiro *et al.*, 2018). I used site and tree ID as random factors, and height and fungal treatments as fixed factors. Data was log transformed to meet model assumptions of normality and homoscedasticity. These were visually assessed using Q–Q (quantile–quantile) normality and residual plots. Significance of the fixed effects in the model was determined using Wald chi-square tests from the car package in R (Fox & Weisberg, 2011). *Post-hoc* Tukey's honestly significance difference (HSD) test was carried out with the general linear hypothesis function (glht) in the package multcomp (Hothorn *et al.*, 2008) using α=0.05 to indicate significant differences between samples before and after inoculations.

To analyze ergosterol results from lesion and outside-lesion tissues and control trees, we used the function prop.test in R to carry out a proportional test. Ergosterol concentrations in lesions among fungal species were compared through a linear mixed model as was described above. Data was reciprocal transformed and zero values were eliminated in order to meet the normality and homoscedasticity assumptions of the model.

3.3 Results

3.3.1 Constitutive total nitrogen concentration along tree bole

Percent total nitrogen concentrations (0.272 ± 0.051 SE) did not vary among tree heights (1m, 3m, 5 m) (X^2= 0.556, df= 2, P= 0.757). Thus, I included only 1m and 5 m heights for the remaining chemical and statistical analyses.

3.3.2 Lesion area and length in response to inoculations

I did not find any differences in lesion area (X^2= 0.870, df= 2, P= 0.647) and length (X^2= 0.035, df= 2, P= 0.982) by tree height or an interaction effect between fungal treatments and tree heights on lesion area (X^2= 11.731, df= 6, P= 0.068) or length (X^2= 2.855, df= 6, P= 0.826). All fungal inoculated trees had larger lesion areas and lengths than control trees (Fig. 3.3.2ab; P< 0.001). Furthermore, fungal species had significant effects on lesion area (X^2= 250.064, df= 3, P< 0.001) and length (X^2= 153.904, df= 3, P< 0.001). Trees inoculated with *G. clavigera* and *L. longiclavatum* had larger lesion than those inoculated with *O. montium* (P< 0.001). There was no difference in lesion area between *G. clavigera* and *L. longiclavatum,* but their lesion lengths varied (P>0.05; P=0.044).

3.3.3 Total nitrogen before and after inoculations

Variations in percent nitrogen were found among samples collected before and after inoculations (Fig. 3.3.3; X^2= 49.455 df= 3, P< 0.001). Total percent nitrogen was higher in samples from control and outside-lesions compared to the lesion samples and samples collected before inoculations (constitutive). I did not find any significant differences among sampling heights (X^2= 0.722, df= 1, P= 0.396) or sampling height-treatment interactions (X^2= 4.4934, df= 3, P= 0.212).

3.3.4 Total nitrogen concentrations among treatments

Total percent nitrogen was not significantly different among treatments when control and outside-lesion samples were compared (Fig. 3.3.4a; X^2= 4.789, df= 3, P< 0.001). Neither sampling heights (X^2= 0.067, df= 1, P= 0.795) or sampling height-treatment interactions (X^2= 3.001, df= 3, P= 0.391) were significant. For tissues collected from lesions, variations in total percent nitrogen concentrations were more evident among the four treatments (Fig. 3.3.4b; X^2= 42.263, df= 3, P< 0.001). Percent nitrogen was higher in control samples and *O. montium*, compared to *G. clavigera* or *L. longiclavatum*. Trees inoculated with *L. longiclavatum* had the lowest nitrogen concentrations among the three fungi. Sampling heights (X^2= 0.025, df= 1, P= 0.874) or sampling height-treatment interactions were not significant (X^2= 1.762, df= 3, P= 0.623).

3.3.5 Ergosterol incidence

I found significantly higher ergosterol incidence in-lesion than in outside-lesion samples (Fig. 3.3.5a; X^2= 77.941, df= 1, P< 0.001). I identified that over 77% of lesion samples and 17% of outside-lesion tissue samples had ergosterol. Ergosterol incidence in-lesion samples was significantly higher for each fungal treatment than their outside-lesion samples. When I compared the ergosterol incidence between control samples and lesions, I found a significant higher ergosterol incidence in lesion tissues (Fig. 3.3.5b; X^2= 27.637, df= 1, P< 0.001). Ergosterol incidence did not significantly differ between control and outside-lesion samples (X^2= 1.505, df= 1, P= 0.219).

3.3.6 Ergosterol concentration in lesion samples

There was not a significant difference in ergosterol concentrations by tree heights (X^2= 0.339, df= 1, P= 0.560) or any interaction effect between treatments and tree heights (X^2= 0.604, df= 2, P= 0.739). However, fungal inoculations significantly altered ergosterol concentrations (Fig. 3.3.6;

X^2= 57.106, df= 2, P< 0.001). Ergosterol was higher in lesions collected from trees inoculated with *O. montium*, compared to those collected from trees inoculated with *G. clavigera* and *L. longiclavatum*. Ergosterol concentration did not differ between *G. clavigera* and *L. longiclavatum* (P> 0.05).

3.4 Discussion

3.4.1 Ergosterol availability

This is the first study reporting ergosterol concentration in each of the three most common fungi associated with MPB in jack pine or in any other host trees of MPB. These results demonstrate that fungal species differ in their ergosterol concentrations. *Ophiostoma montium* had the highest ergosterol concentration among all three fungi including *G. clavigera* and *L. longiclavatum*. In contrast to the fungi, samples taken outside lesions contained none or only low amounts of ergosterol. A higher ergosterol concentration in *O. montium* relative to the other two fungi indicates that slow-growing *O. montium* has a greater fungal biomass than *G. clavigera* or *L. longiclavatum* in the phloem (Xue *et al.*, 2006; Porep *et al.*, 2014). Slow but denser growth of *O. montium* was also reported in other studies (e.g., Myrholm & Langor, 2016).

In general, ophiostomatoid fungi require tolerance to high moisture and low-oxygen conditions to grow in the sapwood (Solheim & Krokene, 1998; Bleiker & Six, 2009). Apparently, *G. clavigera* and *L. longiclavatum* can handle such conditions better than *O. montium* because growth of the former two fungi were more than the latter. Likewise, earlier studies in other insect-plant systems demonstrated that ergosterol production varies with fungal species and is highly dependent on the availability of oxygen and nutrients (Bills *et al.*, 1930; Appleton *et al.*, 1955; Charcosset &

Chauvet, 2001). Thus, in the current study, slow and denser growth of *O. montium* in the phloem may be explained in part by its lower tolerance to the oxygen-limited conditions in the sapwood (Solheim & Krokene, 1998).

Differences among fungi species in terms of ergosterol contributions support their differential roles in MPB biology and are linked to their temporal and spatial prevalence in and around beetle galleries (Adams & Six, 2007; Six & Bentz, 2007; Bleiker & Six, 2009; Roe *et al.*, 2011). *Ophiostoma montium* is the most prevalent in the phloem, associated with eggs, early larvae, and prepupa, and pupa stages of MPB when conditions are warm (usually early fall or spring) and suitable for growth and development of beetle larvae, while *G. clavigera* is more likely to be isolated from third and fourth instar larvae when temperatures are relatively cool (usually in later fall and winter) (Adams & Six, 2007; Bleiker & Six, 2009). During the teneral stage, both *O. montium* and *G. clavigera* fungi could be equally found (Adams & Six, 2007; Bleiker & Six, 2009). Prevalence of *O. montium* in the phloem during early stages of larval development may provide much needed sterols for critical physiological processes, such as formation of cellular membranes (Clayton, 1964). Also, during prepupa, pupa and teneral stages a high availability of ergosterol in the diet may be needed for molting hormone production, full ovarian development, hardening, and tanning processes (Clayton, 1964; Behmer & Nes, 2003; Six, 2003). In fact, ergosterol acquired during the pupal and later stages may influence beetle reproduction, particularly egg viability, and the amount of ecdysteroids in eggs (Clayton, 1964; Behmer & Nes, 2003). Low ecdysteroid content in eggs, for example, interferes with meiotic reinitiation, dropping of the serosal cuticle, and can cause complex malformations in the embryos (Behmer & Nes, 2003).

3.4.2 Nitrogen availability

This is the first study that has investigated nitrogen concentrations of fungi associated with MPB in jack pine. In general, bark beetle fungi are considered to be an important source of nitrogen for MPB larvae (Bleiker & Six, 2007; Cook *et al.*, 2010; Goodsman *et al.*, 2012). I observed that while nitrogen concentrations increased in the phloem of control and outside-lesions, nitrogen levels in lesions remained similar to those found before inoculations, and in fact, they were slightly lower. Although I did not observe a significant difference in percent nitrogen among control and outside-lesion samples from the three fungal treatments, the opposite was observed in tissues collected from the lesions. *Leptographium longiclavatum* and *G. clavigera* had lower nitrogen concentration than *O. montium*, which had a similar nitrogen concentration compared to the control samples.

While knowledge about how nitrogen metabolism in trees changes after fungal infection is limited, two mechanisms may explain the higher nitrogen concentrations found in control and outside-lesion samples. First, plants may have actively remobilized nitrogen away from sites of fungal infection as a defense strategy to deprive pathogens of nitrogen needed for their growth and to safeguard this valuable resource to support plant defense and growth processes (Pageau *et al.*, 2006; Schultz *et al.*, 2013). In parallel to this suggested mechanism, pathogens and mechanical wounding can induce expression of genes associated with senescence that limit pathogen growth by promoting organic nitrogen remobilization which leads to the death of the infected tissues (Snoeijers *et al.*, 2000; Pageau *et al.*, 2006).

Second, an increase in nitrogen concentration around damaged tissues has been observed in pine trees and is considered an important boost for *de novo* synthesis of defense compounds (Moreira *et al.*, 2012). In conifers, nitrogen is required for the differentiation of new xylem resin canals and for the production of terpenoid synthases and other biosynthetic enzymes that catalyze the synthesis of defense compounds (Moreira *et al.*, 2012; Schultz *et al.*, 2013). In fact, when damage occurs,

defense responses are not only confined to the damaged zone (Arango-Velez *et al.*, 2016), but usually result in increased secondary chemical synthesis in tissues surrounding the damaged area as well (Arango-Velez *et al.*, 2014; Erbilgin *et al.*, 2017b). This also may explain the higher nitrogen levels in mechanically wounded tissues and outside-lesion samples in the current study as trees likely accumulated nitrogen around the wounded and infected tissues to support *de novo* synthesis of defense products. Regardless of the mechanisms, tissues surrounding the infection/wounding zone contained more nitrogen than the phloem without such induction.

Nitrogen percentage in lesions, on the other hand, apparently seems to have an inverse relationship between the lesions as *O. montium* had the shortest/smallest lesions compared with the other two fungi. Considering that nitrogen can be 100 times higher in the phloem than in the xylem (Mattson, 1980), fungi likely remobilize nitrogen from the phloem to support their growth in the phloem and xylem. Thus, a faster growth may result in a higher rate of remobilization to the xylem and lower nitrogen level in the phloem.

Differences in nitrogen concentrations found in this study and others (e.g. Goodsman *et al.*, 2012) could be explained by differences in host chemistry, physiology and morphology of jack pine and lodgepole pine (Lusebrink *et al.*, 2016; Arango-Velez *et al.*, 2016; Erbilgin *et al.*, 2017a). For example, differences in host chemistry and morphology have been related to the larger lesions in lodgepole pine (13-18 cm) than in jack pine (4-9 cm) after fungal inoculation with *G. clavigera* (Arango-Velez *et al.*, 2016; Lusebrink *et al.,*2016). Further studies are needed to determine if nitrogen concentration would be different beyond the six-week duration of this study, especially when tree defenses are exhausted and active remobilization of nitrogen by plants is stopped.

3.5 Conclusions

Apparently MPB associated fungi may be more critical as a source of sterols during the beetle's development and may be less important as a source of nitrogen, at least during the initial stages of infection, in jack pine. Furthermore, these fungi likely provide different but complementary benefits to MPB. I propose that in the early stages of host colonization, *G. clavigera* and *L. longiclavatum* likely be involved in helping beetles to weaken host tree defenses due to their faster growth (cf. *O. montium*). In fact, I provided initial evidence that both *G. clavigera* and *L. longiclavatum* likely use more nitrogen to support their own growth within woody tissues (sap wood). *Ophiostoma montium*, on the other hand, grows slowly and its slow growth likely coincides when the early stages of larvae (1^{st} and 2^{nd} instar larvae) do not move much (a total of a couple mm) and need high ergosterol containing diet in the phloem. As larvae grow, they move more and are able to access larger areas infected by *O. montium* and other fungi species. Additional studies are needed to determine whether nitrogen and ergosterol concentrations in phloem vary with time, as environmental and host tree conditions change and may differentially affect fungal growth.

Chapter 4- General Discussion

Host tree quality is highly important for MPB development (Erbilgin *et al.*, 2017a). Furthermore, fungal associates of the MPB are considered to be an important source of nitrogen and sterols (Bentz & Six, 2006; Bleiker & Six, 2007). I evaluated how laboratory conditions affect host tree quality using bolts compared to live standing trees as well how nitrogen and ergosterol concentrations vary among the three fungal associates, *G. clavigera*, *L. longiclavatum*, and *O. montium*.

I observed that jack pine bolts have higher concentrations of monoterpenes, such as limonene and α-pinene, than live jack pine trees and their concentrations are not affected by storage time, at least between three and six months. Also, I observed that several nutrients, including nitrogen, are in higher concentrations in bolts but in contrast with monoterpenes, their concentrations are affected by storage time. Changes in host tree quality when bolts are used likely affect beetle performance. Toxic monoterpenes such as limonene may negatively affect beetle fitness while increasing nutrients such as nitrogen may positively affect beetle development (Goodsman *et al.*, 2012; Chiu *et al.*, 2017). Although water loss may partially explain the results that were obtained, it seems that the main changes observed related to chemical defenses occured before three months of storage. My results suggest that host quality differs between bolts and live trees and appears to be affected by the storage time of the bolts in the case of nutrients.

While evaluating the fungal nutritional benefits I found that the uncolonized phloem (outside-lesions) contained higher nitrogen concentrations than the colonized phloem. In addition, nitrogen concentration in lesions varied among fungal species, being relatively lower in *G. clavigera* and *L. longiclavatum* lesions, compared to *O. montium* lesions. Although the mechanism behind the lower

nitrogen concentration observed in the lesions is not clear, in general, my results suggest that the fungi, contrary to what is believed, may not increase nitrogen levels in lesions compared to the outside-lesions, at least at initial stages of infection. In fact, this would suggest that the fungi are probably using the nitrogen from the phloem to support their own growth.

Ergosterol, on the other hand, was higher in *O. montium* than in the other two fungi. Therefore, *O. montium* may provide an important advantage in terms of the availability of sterols for the developing MPB larvae. I propose that the higher ergosterol concentration provided by *O. montium* may boost larval development in combination with the nutritional benefits provided by *G. clavigera* and *L. longiclavatum*. This assertion is compatible with the report by Bleiker and Six (2007) where they observed that MPB larvae preferentially feed on phloem colonized by *G. clavigera* and *O. montium* more than on phloem colonized by either fungus alone or uncolonized, which may suggest a nutritional benefit to feeding on both fungi.

In summary, my results provide evidence that emulation of live trees with bolts is limited and that conclusions based on experiments that use only bolts as well as other *in vitro* techniques must be done cautiously. In addition, I showed that nutritional benefits provided by fungi associated with MPB change depending on the fungal species. *Ophiostoma montium* seems to be more beneficial in that it produces higher levels of ergosterol and keeps nitrogen levels stable in the phloem relative to *G. clavigera* and *L. longiclavatum*. This fact along with the less restricted growth of *O. montium* in different latitudes may likely positively affect MPB success across different environments in the boreal forest.

Future studies could evaluate changes in monoterpenes, nutrients, as well as water loss in bolts in shorter periods of storage time (i.e., the first 8 weeks of beetle inoculations). This evaluation may give us valuable information about the recommended time that bolts should be kept in the

laboratory in order to better emulate live tree conditions. Likewise, determining how these changes affect beetle fitness through a bioassay may give us a better idea about their practical importance.

Regarding the nutritional benefits from MPB-fungal interactions there are several points that could be of interest for future studies. Beetle development is enhanced by the presence of symbiotic fungi. The key nutrients provided by these fungi, however, are unknown. In several insect-fungus symbioses the insects rely on a specific concentration of ergosterol provided by the associated fungi that allows a successful populational growth. To date, the degree of dependence of MPB on the ergosterol provided by its fungal symbionts is unknown. Future studies may evaluate the range of doses of ergosterol that different developmental stages of MPB require. Furthermore, although they were not reported in this study, phytosterols such as campesterol, sitosterol, and stigmasterol were found in higher concentrations than ergosterol in the phloem. Future studies may evaluate the role that phytosterols play in the MPB sterols diet.

Second, ergosterol and nitrogen concentrations should be determined in older stages of fungal infection as changes in environmental and host tree conditions may differently alter fungal performance. Third, paired inoculations in tree phloem (i.e., *G. clavigera* and *O. montium*, etc.) may provide valuable information about how fungal competition affects ergosterol production or nitrogen concentration when two fungi coexist together. Finally, it may be highly interesting to compare fungal performance in terms of production of ergosterol and concentration of nitrogen between the novel (jack pine) and the historical (i.e., lodgepole pine) hosts.

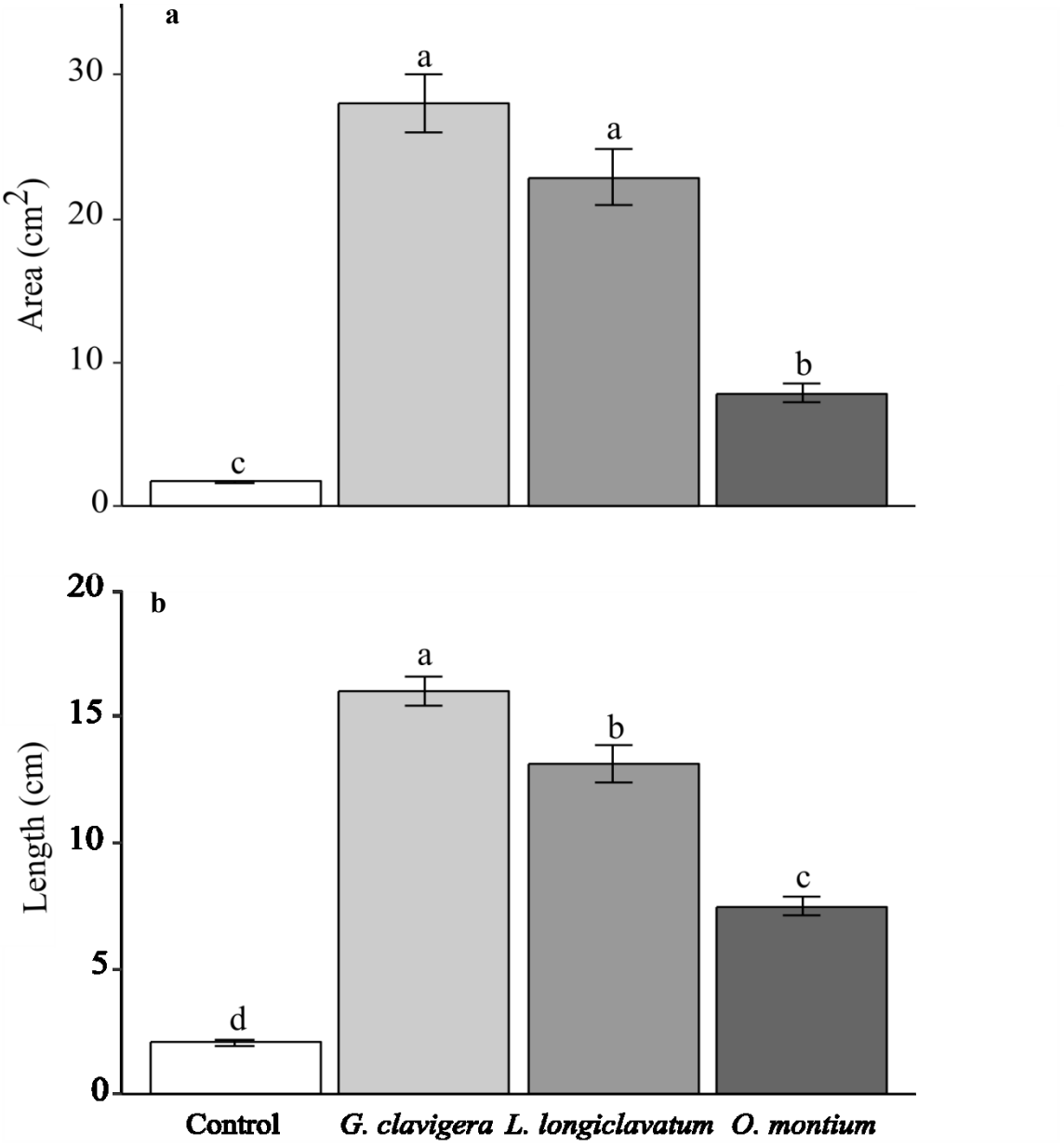


Figure 3.3.2. Mean (± SD) lesion area and length in response to inoculations of jack pine (*Pinus banksiana*) with three fungi associated with mountain pine beetle (*Dendroctonus ponderosae*). (a) Area of lesions from jack pine (*Pinus banksiana*) trees inoculated with the fungi *Ophiostoma montium*, *Grosmannia clavigera*, *Leptographium longiclavatum*, and agar media as a mock control. (b) Lesion lengths from the same four treatments as in (a) above.

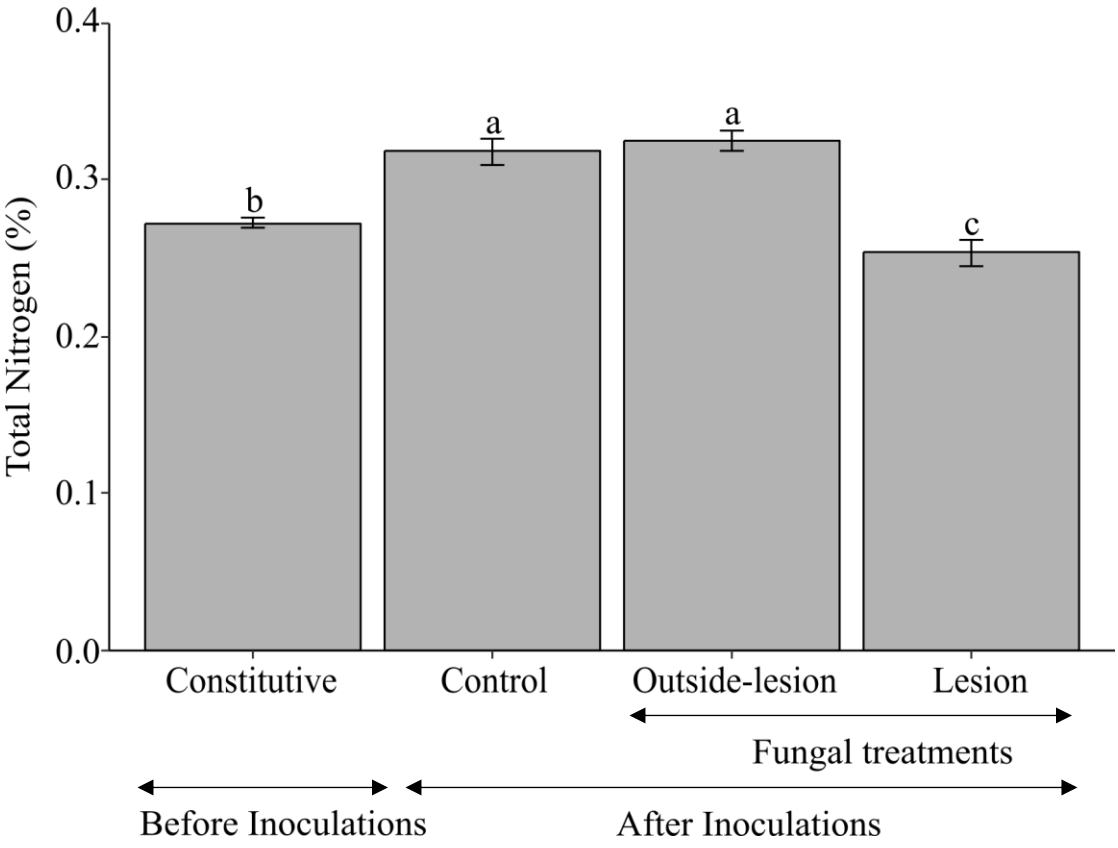


Figure 3.3.3. Mean (± SE) total nitrogen in samples collected before and after inoculations of jack pine (*Pinus banksiana*) with three fungi associated with mountain pine beetle (*Dendroctonus ponderosae*). Jack pine (*Pinus banksiana*) trees were inoculated with *Ophiostoma montium*, *Grosmannia clavigera*, *Leptographium longiclavatum* or mock inoculated at two heights (1 and 5 m). Samples are pooled together by height.

a

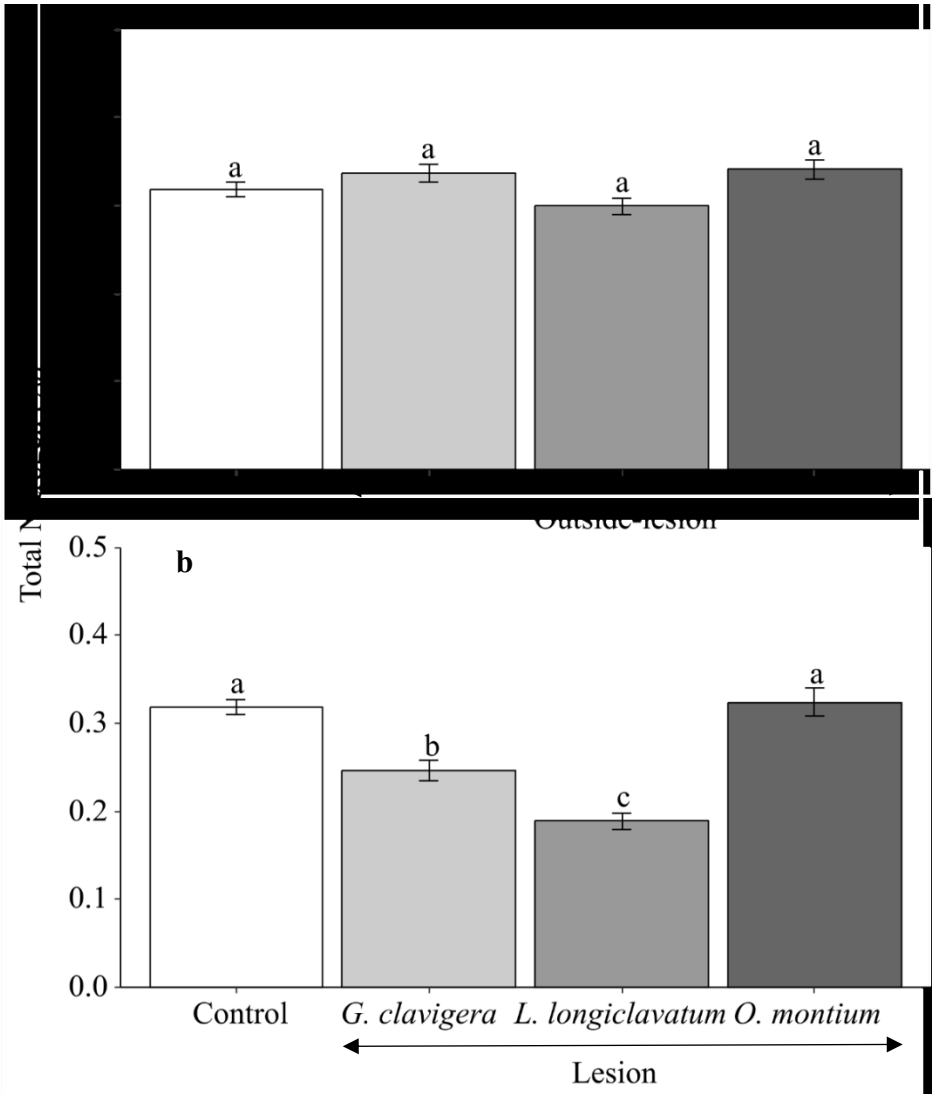


Figure 3.3.4. Mean (± SE) total nitrogen from outside-lesion and lesion tissues and control jack pine (*Pinus banksiana*) trees. (a) Trees were inoculated with *Ophiostoma montium*, *Grosmannia clavigera, Leptographium longiclavatum*, or agar media as a control. Percent nitrogen was not different in outside-lesion samples. In contrast, it varied from fungal treatments compared to control trees. (b) Percent nitrogen in lesions varied in tissues from lesions, being lower in *G. clavigera* and *L. longiclavatum* compared to control and *O. montium*. No significant difference was found in total nitrogen among 1m and 5m in either outside-lesion or lesions.

a

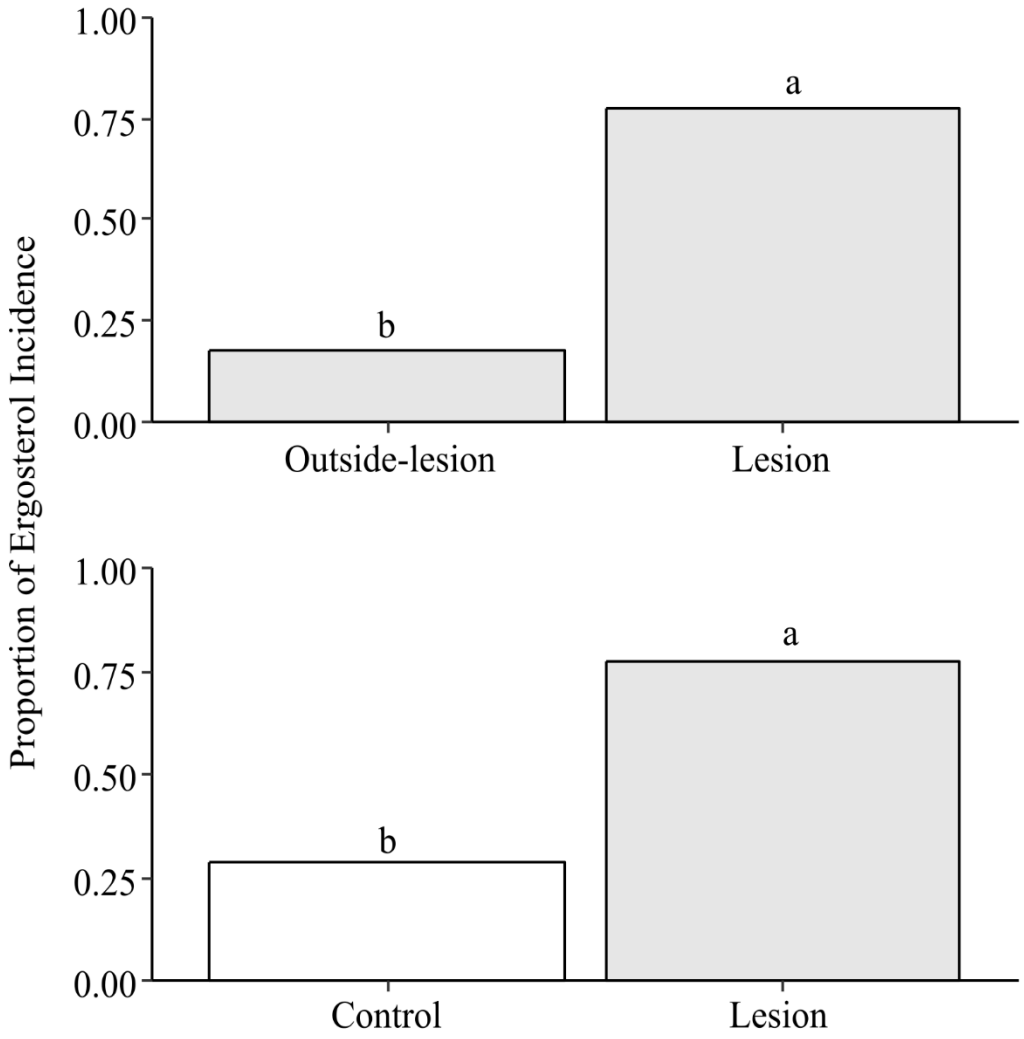


Figure 3.3.5. Ergosterol incidence in lesion, outside-lesion, and control samples of jack pine (*Pinus banksiana*) trees. (a) Ergosterol incidence in lesions from trees inoculated with *Grosmannia clavigera*, *Leptographium longiclavatum* and *Ophiostoma montium* was significantly higher than in their outside-lesions. (b) Ergosterol incidence in lesion from trees inoculated with the fungi was significantly higher than ergosterol incidence in samples from control trees.

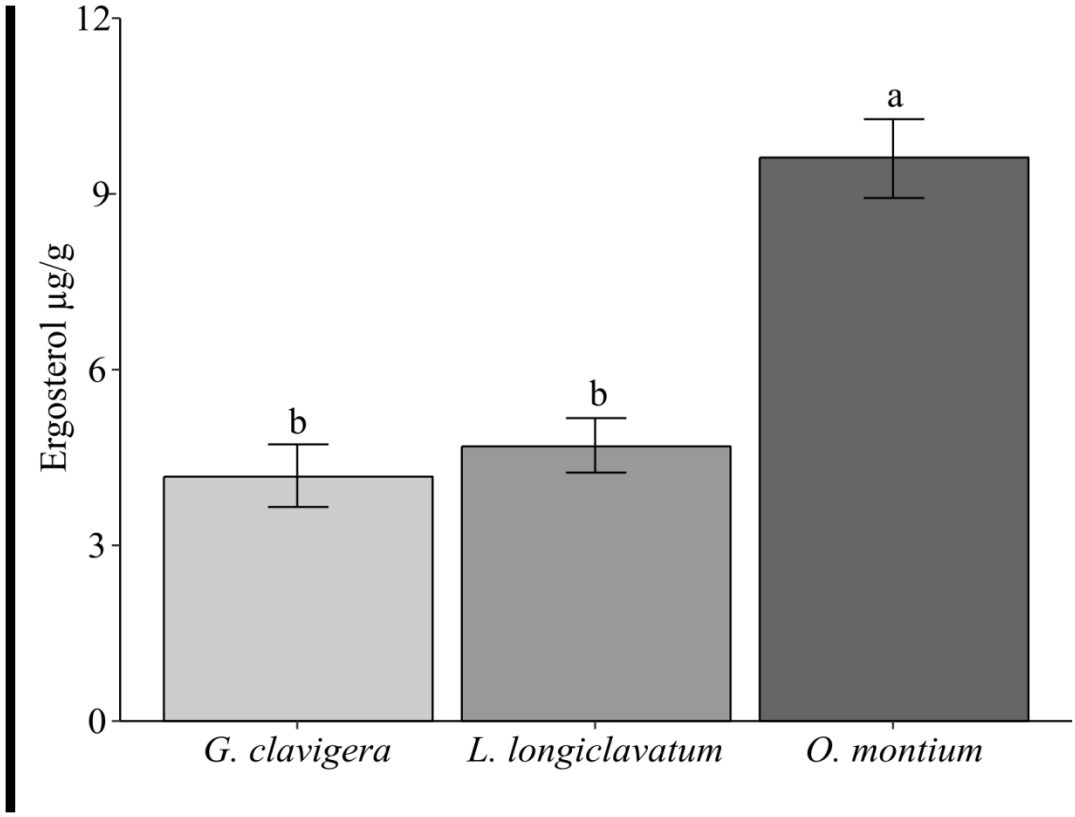
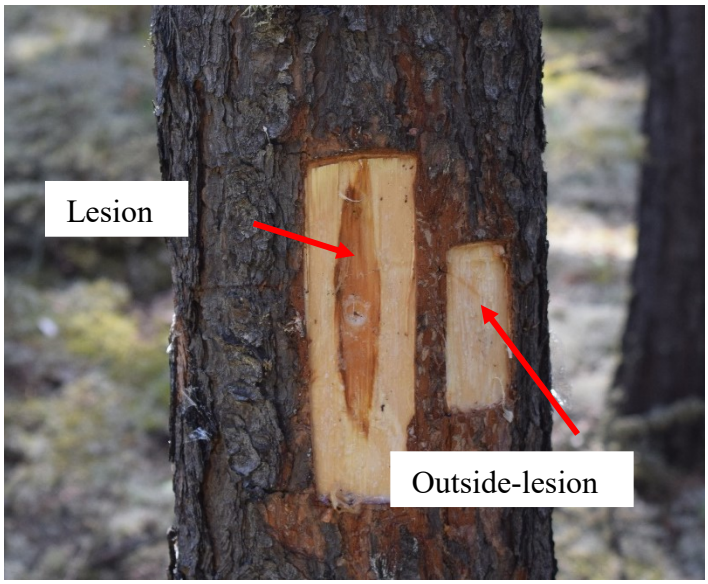


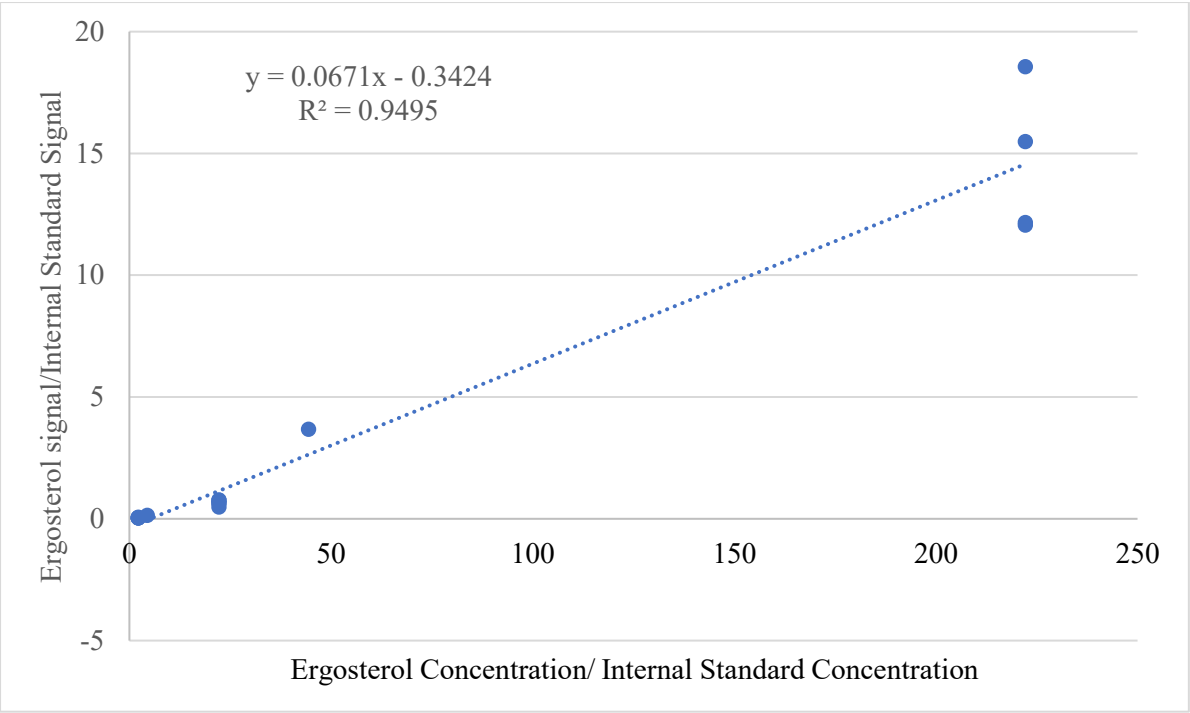
Figure 3.3.6. Ergosterol concentration in lesion samples from fungal treatments in jack pine (*Pinus banksiana*) trees. Ergosterol concentration was significantly higher in lesion samples from trees inoculated with *Ophiostoma montium* than from trees inoculated with *Grosmannia clavigera* or *Leptographium longiclavatum*. Ergosterol concentration was not significantly different in terms of height (between 1m and 5m).

Supplementary Table 3.2.1. Detailed source information about isolates of ophiostomatoid fungi used during tree inoculations

Accession number	Location collection	Date collected	Species	Notes
EL033	Banff Alberta, Canada	May 2016	*Grosmannia clavigera*	Isolated from a MPB gallery in *P. contorta*
EL038	Graham fire base Alberta, Canada	May 2016	*Leptographium longiclavatum*	Isolated from a MPB gallery in *P. banksiana* x *P. contorta* hybrid
EL005	Westcastle Alberta, Canada	January 2015	*Ophiostoma montium*	Isolated from a MPB gallery in *P. contorta*



Supplementary Figure 3.2.1a. Lesion and outside-lesion phloem samples collected from jack pine (*Pinus banksiana*) trees. Lesions corresponded to the dark reaction zones on phloem, where typically terpenoid and phenolic compounds are accumulated, in response to the fungal growth. Outside-lesion corresponded to the non-reaction zones on phloem where fungal growth was not expected.



Supplementary Figure 3.2.1b. Standard curve used to calculate ergosterol concentration in the phloem samples of jack pine (*Pinus banksiana*). The calibration curve was constructed with commercial ergosterol standard solutions in hexane ranging from 0.002 to 0.2 mg/mL at five points. The equation generated by the curve was used for ergosterol quantification.

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