The role of ectomycorrhizal fungi in induced defense chemistry of lodgepole pine

by

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Abstract

Ectomycorrhizal fungi are determinants of the success of pine regeneration in post-disturbance stands. These fungi promote resource acquisition and resistance in seedlings. They may alter plant chemistry in lodgepole pine (*Pinus contorta* var. *latifolia*), which may directly affect the success or failure of mountain pine beetle (*Dendroctonus ponderosae*) outbreaks in lodgepole pine stands of western Canada. It is unknown, whether the chemistry of pine seedlings differs on exposure to individual or a community of ectomycorrhizal fungi but could help to elucidate methods to promote healthy, post-disturbance regeneration. This project investigated such responses by examining induced monoterpene compounds as well as growth parameters of greenhouse-grown lodgepole pine seedlings whose roots were colonized by individual or a combination of ectomycorrhizal fungi. Ectomycorrhizal competition on artificial growth media was also assessed to support greenhouse findings. This research revealed that changes in ectomycorrhizal fungal species differently affect the induced chemistry of lodgepole pine depending on the fungal species, their interactions, and root colonization sequences. Considering the effects of these symbiotic fungi on plant growth and induced defenses, they can directly or indirectly affect the host tree susceptibility to their antagonistic biotic agents.

Preface

This thesis is an original work by Sanat S. Kanekar. No part of this thesis has been previously published. In this project two ectomycorrhizal fungal species; *Laccaria bicolor* and *Cenococcum geophillum* were used and their effect on lodgepole pine seedlings were quantified in terms of growth and defensive parameters like seedling biomass and secondary compounds.

To my parents

"फक्त उबो रावुन दर्याक पळयत जाल्यार आमी तो केन्नाच हुपुंक शकना."

– रबीन्द्रनाथ टेगोर

"You can't cross the sea merely by standing and staring at the water."

-Rabindranath Tagore

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Acronyms	*Full forms*
MPB	Mountain pine beetle
EM	Ectomycorrhizal
CEGE/ CE	*Cenococcum geophillum*
LABI/ LA	*Laccaria bicolor*
MJ	Methyl jasmonate
MS	Methyl salicylate
Tukey's HSD	Tukey's honest significant difference

Chapter 1- General Introduction

1.1 Fungal symbiont-host plant interactions

Plants are primary producers in ecosystems, and healthy plant development requires reproduction, growth, and defense to occur without significant perturbation. Environmental factors such as air, water, sunlight, and fertile soil are essential to these processes, but there are other factors which benefit plants such as above and below ground communities of microorganisms. One such group of microorganisms is the symbiotic mycorrhizal fungi which play an important role in promoting plant health (Lehto & Zwiazek, 2011). Mycorrhizae can be defined as a symbiotic association between plant roots and specific soil fungi (Janerette, 1991). This group of fungi differ in how they associate with plant roots (Janerette, 1991). In ectomycorrhizal (EM) fungi, the hyphae penetrate between the cell spaces of root hairs (Janerette, 1991).

Ectomycorrhizae form on many dominant groups of forest trees, such as pine (*Pinus*), spruce (*Picea*), hemlock (*Tsuga*), oak (*Quercus*), chestnut (*Castanea*), walnut (*Juglans*), beech (*Fagus selvatica*), birch (*Betula pendula*), and eucalyptus (*Eucalyptus*). During the formation of an ectomycorrhizal symbiosis, the fungus contacts a susceptible root, and on stimulation from the root, fungal hyphae extend outward and envelop the root and form a dense sheath called a "mantle" that physically separates the root from its surroundings. Enzymes secreted by the fungus enable hyphae from the mantle to penetrate the root and extend into the cortex. These hyphae are restricted to the spaces between root cells, where they form an interconnected network called a "Hartig net" which plays a role in the exchange of materials between the plant and the fungus. As the mycorrhiza develops, the fungus secretes growth regulating compounds which cause changes in root development (Janerette, 1991).

Ectomycorrhizal trees dominate many forest ecosystems from the subpolar to tropical zones (Horton, 2015). Fungal colonization is usually found in most of the fine roots of trees, sometimes in up to nearly all root tips of the host (Horton, 2015). Ectomycorrhizal fungal diversity usually exceeds the diversity of trees in temperate and boreal forests (Horton & Bruns, 2001). Host trees depend largely on their EM fungi for soil nutrient uptake (Smith & Read, 2008). Two major infection pathways which exist are spores (and sclerotia) and fungal networks. In developed forests, both types of inocula are ubiquitous and the lack of fungal inocula is not a limiting factor for seedling establishment in these forests. However, in severely disturbed areas, especially primary successional sites, the inoculum potential can decrease critically and affect seedling establishment (Horton, 2015).

1.2 Lodgepole pine and its ectomycorrhizal fungal association

Perennial conifer (Pinophyta) woody plants are dominant species in many parts of the world's forests, most notably in the Northern Hemisphere (Henry, 2005). One such conifer is lodgepole pine (*Pinus contorta* Dougl. Ex. Loud.) whose geographical range in North America ranges from the Pacific coast to the Rocky Mountain range and from Alaska to Baja California (Burns & Honkala, 1990; Ying & Liang, 1994; Pec, 2016). Lodgepole pine covers about 6 million hectares in the western United States and 20 million hectares of forest in Canada (Burns & Honkala, 1990). It may have to tolerate the widest range of environmental conditions of any conifer species in North America, and as such, grows in association with many plant species (Burns & Honkala, 1990). It can grow in a variety of soil conditions, but usually prefers moist soil. In Canada, however, extensive stands occur on calcareous glacial tills (Burns & Honkala, 1990). A balance of moisture and soil porosity is provided by glacial drifts and this helps the species

thrive. This can be clearly seen in Alberta, where it grows better on glacial tills than on alluvial soils or lacustrine deposits (Burns & Honkala, 1990). It is an early-successional, shade-intolerant species that colonizes areas following disturbance, such as stand-replacing wildfire (Pec, 2016).

Regeneration of lodgepole pine occurs naturally in response to wildfire, where cones release seeds in the presence of intense levels of heat (Teste et al., 2011). Seedling establishment is also linked to conditions following fire disturbance, particularly to exposed mineral soil, decayed wood and organic material for increased germination success (Nyland, 1998; McIntosh & Macdonald, 2013). In the absence of disturbance, lodgepole pine is eventually replaced by more shade-tolerant conifer species such as white spruce (*Picea glauca*) (Dhar & Hawkins, 2011). Similarly, successful infestation and mortality of lodgepole pine by outbreak insects can promote growth of shade-tolerant conifer species, which may lead to a non-pine dominated system (Cigan et al., 2015; Pec et al., 2017). In either scenario, tree loss may lead to complex effects on the structure, composition, and function of the forest system, both above- and belowground.

Co-evolution with symbiotic organisms can mutually benefit and help sustain the organisms involved, and recent evidence such co-evolution likely occurred between lodgepole pine and its EM fungi (Arnold et al., 2003; Karst et al., 2015). Ectomycorrhizal fungi are critical determinants of successful pine regeneration after major disturbances and benefit plants in various ways such as increased seedling germination and growth (Marschner & Dell, 1994; Karst et al., 2014). Seedling germination and growth is directly dependent on the availability of nutrient supply during the early growing stage. Ectomycorrhizal fungi increase nutrient uptake by increasing the absorbing surface area and by excreting chelating compounds or ectoenzymes. Nutrient uptake is also increased by stimulating sparingly available nutrient sources (Marschner

& Dell, 1994). An ectomycorrhizal association helps plants in improving their defense systems and especially promotes the production of defense-related monoterpenes (Gershenzon, 1994). Ectomycorrhizal fungal association can also prevent entry of pathogen in a host plant (Marschner & Dell, 1994). The colonization of plant roots by EM fungi increases plant resistance to disease (Kernaghan et al., 2002), and improves plant growth in toxic soils contaminated with heavy metals (Kernaghan et al., 2002). This association can also increase plant tolerance to high salinity soils (Onwuchekwa et al., 2014). Ectomycorrhizal fungi are also responsible for soil mineral uptake in exchange for carbon (Kernaghan et al., 2002). Colonization by EM fungi enhances the nitrogen, phosphorus and carbon status of hosts, though specific effects may the species of colonizing fungus (Smith & Read, 2008). This association also helps plants to absorb water from the soil, helping plants survive in drought conditions (Lehto & Zwiazek, 2011).

Plants produce a range of secondary compounds, which mediate plant defensive interactions with insect herbivores and pathogens (Agrawal, 2011; Karst et al., 2015). A class of secondary compounds are monoterpenes—low molecular weight volatile compounds produced by terpenoid metabolism in gymnosperms (Phillips & Croteau, 1999). In lodgepole pine, investigation of compounds such as phenolics, fatty acids and monoterpenes has suggested that these compounds play roles as primary defense chemicals against bark beetles and their fungal symbionts (Phillips & Croteau, 1999; Keeling & Bohlmann, 2006; Boone et al., 2011; Erbilgin et al., 2016, 2017). Because differences in defensive chemical compounds are crucial for successful MPB attack, it is essential to simulate the attack conditions to study these differences. To simulate insect or pathogen attacks under controlled conditions, phytohormones such as methyl jasmonate and methyl salicylate are commonly used (Kozlowski et al., 1999; Davis et al., 2002; Erbilgin et al., 2006; Zhao et al., 2010; Erbilgin & Colgan, 2012; Derksen et al., 2013). Methyl

jasmonate and methyl salicylate are methyl esters of jasmonic acid and salicylic acid, respectively. They stimulate the biochemical pathways important in defense responses (Erbilgin & Colgan, 2012).

Despite the known benefits of the lodgepole pine-EM fungi association, little is known regarding how EM fungi affect the synthesis and production of secondary compounds (Gershenzon, 1994; Smith & Read, 2008: Karst et al., 2015). Karst et al. (2015) showed that EM fingi association can affect the composition of secondary compounds in lodgepole pine. However, how different species of EM fungi and their interactions affect induced defenses of conifer trees remain unclear (Bennett et al., 2006; Gehring & Bennett, 2009; Koricheva et al., 2009; Karst et al., 2015).

1.3 Thesis overview

In my thesis, I investigated the effect of EM fungal species on induced defense-related monoterpenes in lodgepole pine, mainly, how variation in the EM fungal species affects concentrations of individual monoterpenes in seedlings

I hypothesized that EM fungal species differentially affect monoterpene, concentrations in lodgepole pine seedlings. To test this hypothesis, I set the following objectives: (1) determine changes in monoterpenes and pine seedling growth in response to varying EM fungal associations; (2) determine how hormonal treatment affects the monoterpenes in lodgepole pine seedlings under different EM fungal associations; (3) determine the effects of inter-EM fungi competition between two species on growth and ability of each fungus to colonize host roots.

To fulfill these objectives, this project was carried out in two interlinked stages: greenhouse experiments and plates experiment. In the greenhouse experiment, I characterized the

responses of lodgepole pine seedlings whose roots were colonized by one EM fungal species or by two EM fungal species inoculated in different ways. Six different families of lodgepole pine seedlings were used. Mycorrhizal fungi *Cenococcum geophillum* and *Laccaria bicolor* were selected for study because they are commonly associated with pines in boreal forest (Bradbury et al., 1998; Onwuchekwa et al, 2014).

The seedlings were also treated with one of two phytohormones: methyl jasmonate or methyl salicylate. Then, I measured various responses that the plant had towards the fungal treatments as well as the phytohormone applications. The measured responses included final seedling biomass, root colonization by fungi, and concentration of plant monoterpenes. Further, to aid in understanding the interaction of the fungi with each other, a plates experiment was designed by growing *C. geophillum* and *L. bicolor* on artificial media. I quantified the growth of the EM fungi when they were alone or competing. This experiment helped provide potential explanation of how the fungi interacted with each other in the greenhouse experiments.

Chapter 2: Ectomycorrhizal fungal species differentially affect the induced defense chemistry of lodgepole pine (*Pinus contorta* Dougl. Ex. Loud.)

2.1 Introduction

Perennial conifer (Pinophyta) woody plants are often the dominant species in many parts of the global forests, especially in the Northern Hemisphere (Henry, 2005). They have achieved this dominance by providing a diversity of secondary compounds which facilitate plant interaction with biotic and abiotic factors (Arnold et al., 2003; Whitaker et al., 2017). For example, plant secondary compounds defend plants against insect pest and pathogens, attract natural enemies of herbivores, mediate interactions with pollinators, and protect them from adverse climatic conditions (Dixon & Paiva, 1995; Agrawal, 2011; Moore et al., 2013; Erbilgin et al., 2017). Secondary compounds can widely vary within- and between plant species, likely resulting from plant coevolution with insect herbivores as well as adaptive radiation in response to environmental selective pressures (Sequeira et al., 2000; Huber & Ralph, 2004; Howe & Jander, 2008; Moore et al., 2013; Erbilgin et al., 2014; Karst et al., 2015; Raffa et al., 2017). Although pines (*Pinus* spp.), for example, have established symbiotic relationships with ectomycorrhizal (EM) fungi , which are responsible for uptake of soil resources in exchange for carbon, and these fungi can modify the nitrogen, phosphorus and carbon status of hosts, their roles in the synthesis and production of secondary compounds have received little attention (Gershenzon, 1994; Smith & Read, 2008; Karst et al., 2015). In particular, while changes in EM Fungal species can alter constitutive defense-related chemicals in host trees, whether differences in EM fungal species affect the induced defenses of conifer trees remains unclear (Bennett et al., 2006; Gehring & Bennett, 2009; Koricheva et al., 2009; Karst et al., 2015).

I investigated the induced defense responses of lodgepole pine (*Pinus contorta*), one of the most abundant conifer tree species in western North America. Within its range, lodgepole pine can grow in many different environments, from bogs to dry sandy soils, and from cold and wet winters to warm and dry summers, and show different chemotypic (chemically distinct groups) variations (Forrest, 1981; Clark et al., 2014; Erbilgin et al., 2017a). Evolutionary explanations for variation in secondary compounds of lodgepole pine are not clear, but have been primarily attributed to selective pressures imposed by natural enemies, such as mountain pine beetle (MPB; *Dendroctonus ponderosae*, Hopkins, Coleoptera: Curculioniade, Scolytinae) (Erbilgin et al., 2017; Raffa et al., 2017). Recent evidence indicates that EM fungal association can also affect the composition of constitutive secondary compounds, particularly monoterpenes, of lodgepole pine (Karst et al., 2015). In this earlier study, pine seedlings grown with EM fungi collected from MPB-killed stands had lower concentrations of monoterpenes and lower monoterpene richness, compared with seedlings grown with fungi collected from healthy lodgepole pine stands (Karst et al., 2015). However, the mechanism underlying such differences in monoterpene concentrations in pine seedlings is not clear. Likewise, whether fungal symbionts can also influence induced secondary compounds of pines remains to be investigated.

Many EM fungi have an obligate association with pine species (Simard et al., 1997; Karst et al., 2014). For example, *Cenococcum geophillum* and *Lacarria bicolor* are commonly associated with pines in boreal forests (Bradbury et al., 1998; Onwuchekwa et al, 2014). Ectomycorrhizal fungi benefit plants in many ways. Association with these fungi can improve plant growth (Bennett et al., 2006), and seedlings grown along with EM fungi have a higher germination rate as compared to others without such association (Marschner & Dell, 1994). Mycorrhizal associations can help plants improve their defense systems, especially the synthesis

of defense-related monoterpenes (Gershenzon, 1994). Such associations can also prevent the entry of pathogens in host plant roots (Marschner & Dell, 1994). The colonization of EM fungi in plant roots increases plant resistance to diseases and improves plant growth in toxic soil with heavy metals (Kernaghan et al., 2002). Likewise, colonization by ectomycorrhizal fungi enhances host nitrogen, phosphorus and carbon status, the effect of which may vary with EM fungal species (Smith & Read, 2008). Lastly, it also helps plants to absorb water from soil, especially in drought conditions (Lehto & Zwiazek, 2011).

I focused on *C. geophillum* and *L. bicolor* commonly associated with pines in boreal forests (Bradbury et al., 1998; Onwuchekwa et al, 2014). The phytohormones such as methyl jasmonate (MJ) or methyl salicylate (MS) are commonly used to simulate insect or pathogen attacks on trees, respectively (Kozlowski et al., 1999; Davis et al., 2002; Erbilgin et al., 2006; Zhao et al., 2010; Erbilgin & Colgan, 2012; Derksen et al., 2013). These compounds elicit cascading effects that induce plant secondary compounds (Erbilgin & Colgan, 2012). The advantage of hormone application over other induction methods such as wounding is that a mechanical wounding is not required to observe plant induced defenses. In lodgepole pine, toxic monoterpenes have been suggested the primary defense chemicals against bark beetles and their fungal symbionts (Phillips & Croteau, 1999; Keeling & Bohlmann, 2006; Boone et al., 2011; Erbilgin et al., 2017a,b).

Monoterpenes are constitutively (existing prior to attack) present in pine tissues, providing immediate resistance to insect attacks (Franceschi et al., 2005). If the insect attack persists and is not deterred, induction responses are triggered to further protect the plant (Franceschi et al., 2005; Raffa et al., 2005). For example, within a few days of bark beetle attacks, induced monoterpene levels in pines can overcome the physiological tolerance thresholds of beetles,

inhibit or repel later-arriving beetles, and alter the growth of their associated fungi (Raffa et al., 2005). Generally, induced defenses qualitatively and quantitatively differ from constitutive defenses (Raffa et al., 2005). Both constitutive and inducible responses can form the basis of conifer defenses to bark beetles (Franceschi et al., 2005; Erbilgin et al., 2006, 2017a; Keeling & Bohlmann, 2006; Eyles et al., 2010).

I hypothesised that EM fungal species differentially affect production of monoterpenes relative to single EM fungus. This project incorporated the outcome of two separate but complimentary experiments including a greenhouse experiment and plates experiment. In the greenhouse experiment, I characterized the responses of lodgepole pine seedlings to *C. geophillum* and *L. bicolor* individually or in their various combinations. The same seedlings were also treated either with MJ or MS to induce monoterpenes. Then, I measured various plant responses to the fungal and phytohormone treatments including biomass, fungal root colonization, and monoterpene concentrations. The goal of plates experiment was to investigate interactions between *C. geophillum* and *L. bicolor* as a potential mechanism underlying treatment responses observed in the greenhouse experiment.

2.2 Materials and Methods

2.2.1 Greenhouse Experiment

2.2.1.1 Experimental set up and mycorrhizal treatment application

A greenhouse experiment was conducted in order to investigate the effect of EM fungi, individually or in various combinations, on the growth and defense-related chemistry of

lodgepole pine seedlings. Seedlings were grown from seeds representing six families. The seeds were provided by Tree Improvement Branch, Kalamalka Forestry Centre (Vernon, BC, Canada).

Prior to sowing, the seeds were stratified for 28 days. This process included a surface sterilization by soaking seeds in 5% bleach for 15 min followed by rinsing with distilled water and soaking the seeds for 24 h in distilled water. Excess water was drained, and seeds were surface dried by patting with Kim wipes. Seeds were then stored in the dark for 28 days at 4°C.

Seeds were sown into 400 ml pots filled with sterilized potting material (70:30 sterile sand: top soil). Four seeds from one of the six families were sown into each pot, which was at the same time inoculated with 10 ml of a mycelial slurry representing one of six mycorrhizal treatments (Fig. 2.2): (1) *Cenococcum geophillum* (isolate #UAMH 5512; CEGE) (n=41 seedlings) alone, (2) *Laccaria bicolor* (isolate #UAMH 8232; LABI) alone (n=36), (3) *C. geophillum* plus *L.* bicolor combined (n=40), (4) *C. geophillum* followed by *L. bicolor* (LA Interaction) (n=31), (5) *L. bicolor* followed by *C. geophillum* (CE Interaction) (n=44), and (6) non-inoculated as a control (n=36).

Both fungi were isolated from lodgepole pine forests in Alberta and provided by the University of Alberta Microfungus Collection and Herbarium. Fungal inoculum was prepared by growing fungal cultures in modified Melin Norkrans liquid media started from 30 plugs (8 mm dia.) taken from the margins of actively growing cultures on potato dextrose agar. The cell density of two-week old liquid cultures was quantified and standardized among culture bottles by dilution, as needed. For the combined treatment, 5 ml of each fungus were mixed at the time of inoculation. For the treatments 4 (LA Interaction) and 5 (CE Interaction), pots were inoculated with the second fungus two weeks after sowing and initial inoculation with the first fungus. Treatment 6 was served a control where in no fungal treatment nor culture solution was

inoculated. Inoculation treatments were reapplied six times with each reapplication 15 days apart.

2.2.1.2 Greenhouse conditions

Pots were thinned to one seedling one week after germination began; the most vigorous in growing seedling was retained in each pot. Seedlings were grown for a total of 12 months at 21 ºC under a natural light-dark regime. Seedlings were placed in dormancy conditions of 4 ºC and a 12:12 hrs light-dark regime for six weeks (after an acclimation period of a graduate temperature decline from 21 ºC to 4 ºCover a two-week period). Seedlings were reconditioned to warmer temperatures reflecting growing season conditions over one week when temperatures were gradually returned to 21 ºC. Pots were fertilized with a 8:20:30 (N:P:K) formulation prior to (50 ppm) and twice (125 ppm) during dormancy to alleviate a developing phosphorus deficiency which was identified by reddening of seedling needles. A 10:52:10 fertilizer (400 ppm) was applied immediately following dormancy, whereas a 10:20:10 fertilizer (100 ppm) was otherwise applied three times a week until three weeks prior to defense-related hormone application (described below). Iron chelate (17.5 ppm) was periodically added to the latter fertilizations. Pots were rotated to a new location in the greenhouse once a week to ensure equal sunlight exposure.

2.2.1.3 Hormone treatment application and root morphotyping

To investigate how the mycorrhizal treatments influence defense-related induction in lodgepole pine, seedlings were treated with MJ or MS. After 12 months of growth, half of the seedlings received the MJ treatment while the other half received MS. Fifty µl solutions of MJ or MS in

0.1% (v/v) Tween 20 were applied to the seedling stems using a foam brush. Seedlings were not watered for 24 hrs to ensure that solutions were absorbed and kept in separate greenhouse rooms for 24 hrs in order to avoid cross-contamination among treatments. No fertilizers were applied after the hormone application to avoid potential nutrient-defense feedbacks. Seedlings were harvested 10 days post-treatment by carefully uprooting them from the pots without disturbing the roots. The roots were cleaned of potting mixture using a brush and then wrapped in aluminium foil and stored at 4 °C. Seedlings roots and above ground parts were separated and weighed to measure their biomass. The parts were put in the same bag and kept at -40 °C. Furthermore, stem and foliage tissues were combined for later chemical analysis.

The success of mycorrhizal treatment applications was assessed by measuring percent colonization of each fungus as determined by morphotyping a subset of 100 randomly selected root tips per seedling. Root tips were cut into lengths of ca. 1-2 cm and put into a Petri dish containing water and evaluated for morphotypes and other characteristics indicative of *C. geophillum*, *L. bicolor*, or other/non-colonized (Goodman, 1996; Martin & Selosse, 2008).

2.2.1.4 Monoterpene extraction and chromatographic analysis

Defense-related monoterpenes were extracted from the aboveground tissues of each seedling. Needle and stem tissues were ground together in liquid nitrogen using a mortar and pestle. Ground samples were stored at -40 °C prior to extraction. Terpenes were extracted using methods described by Erbilgin et al. (2017a). Briefly, 100 mg of ground tissue was extracted twice with 0.5 ml of dichloromethane and 0.019% tridecane as an internal standard. Extractions were vortexed for 30 s, sonicated for 10 min, and centrifuged at 13,200 rpm at 0 °C for 15 min.

Centrifuged extractions were stored at -40 °C for an hour to facilitate separation between supernatant and ground sample. The supernatant was collected and transferred to a glass vial through a glass-wool filter. Extractions were then pushed through a glass wool filter inside a glass pipette using a rubber plunger in order to remove fine sample particulate from extracts. Filtered extracts were collected in 2 mL glass gas chromatography vials and stored at -40 °C until chromatographic separation.

Extractions (1 μL) were injected into a gas chromatograph/mass spectrometer (Agilent 7890A/5062C, Agilent Tech., Santa Clara, CA, USA) equipped with a HP-Chiral-20B column (I.D. 0.25 mm, length 30 m) (Agilent Tech.) with helium carrier gas flowing at 1.1 ml min^{-1}, and a temperature program of 50°C for 1 min, increased to 65°C for 2 mins by 40°C min^{-1}, then to 85°C for 2 mins by 40°C min^{-1} and then to 240°Cfor 1 min by 10°C min^{-1}. To identify and quantify individual and total compounds (mainly monoterpenes), the following standards were used: (−)-α-pinene, (+)-α-pinene, (−)-β-pinene, (+)-β-pinene, (−)-camphene, (+)-camphene, myrcene, (−)-limonene, (+)-limonene, 3-carene, terpineol (chem purity >90%), (+)-cymene, sabinene, terpinolene, p-cymene, β-thujone(enantiomeric ratio 92.5/7.5), pulegone, terpinolene (>90%), borneol, 4-allyanisole (Fluka, Sigma-Aldrich, Buchs, Switzerland), γ-terpinene, α-terpinene (Sigma-Aldrich, St. Louis, MO, USA), *cis*-ocimene (>90%), bornyl acetate (SAFC Supply Solutions, St. Louis, MO, USA), and β-phellandrene (Erbilgin lab). Where chemical purity was not noted, the purity was 97%. Compounds were identified by comparing retention times and mass spectra to those of the standard chemicals. Quantity of chemicals was calculated using calibrated curves generated from analyses of a serial of dilution of known quantities of standards, and calculated as μg of compound per mg of wet tissue.

2.2.2 Plates experiment

Plate experiment was conducted to compare the potential interaction between *C. geophillum* and *L. bicolor* on artificial medium in order to elucidate how the fungi may be interacting on the roots of seedlings in the above greenhouse experiment. The short coming of this experiment was that there was ample amount of nutrient supply in the form of potato dextrose agar for the fungal growth. Growth measurements were made using fresh cultures of the same fungal strains used in the greenhouse experiment. Fungi were grown in six culture treatments reflecting the mycorrhizal treatments used in the greenhouse experiment (Fig. 2.3): (1) *C. geophillum* alone as (CEGE control), (2) *L. bicolor* alone as control (LABI control), (3) both the fungi on separate halves of a partitioned plate (Partitioned), (4) both the fungi on opposite ends of a non-partitioned plate (combined), (5) *C. geophillum* was grown on an established *L. bicolor* culture (CE Interaction), and (6) *L. bicolor* grown on an established *C. geophillum* culture (LA Interaction). Each treatment was replicated 15 times, for a total of 90 plates.

Fungal cultures were prepared by first growing master cultures on potato dextrose agar. After 15 days, master cultures were sub-cultured from the margins of master cultures by placing a culture plug (8 mm dia.) onto either the center (Interaction and each fungus alone treatments) or equidistant locations (Partitioned and Combined treatments) of 100 mm dia. Petri dishes of potato dextrose agar. Fungi were grown in total darkness at room temperature (22 ºC) for a length of time dependent on treatment. This variable growth period was used in order to allow growth measurements to be made before cultures covered the entire plate surface.

Culture growth (as area in mm^2) measurements were made using image analysis techniques. Images were taken using a Nikon D7100 camera mounted on a stand 50 cm from the surface. Camera was set to ISO: Auto, F: 5.3, A:40. Images were taken with a ruler in frame in

order to later scale images and measure image elements. Images were taken at several times (0, 9, 15, and 30 (except *L. bicolor* cultures) days post-inoculation) according to the treatment growth period (Fig. 2.3). Images were loaded into and quantified using the software program Image J (http://imagej.net/ImageJ). The final growth as well as the per day rate of growth were calculated and used for data analysis.

2.2.3 Data Analysis

For the greenhouse experiment, the development of mycorrhizal roots in each treatment, and thus treatment application success, was determined by assessing the percent colonization of each fungus on seedling roots. Differences in percent colonization among treatments for each fungus were separately tested for statistical significance by ANOVA. To determine whether mycorrhizal treatments affected the biomass of lodgepole pine seedlings, I used one-way ANOVA to test the statistical significance of differences among treatment. Separate models were run for aboveground, belowground, and total biomass (g) response variables. Tukey's Honest Significant Difference (HSD) tests were performed following all significant one-way ANOVA models. Main effects of mycorrhizal treatments and the interaction between the composition of seedling monoterpenes (proportion- calculated by concentration of total monoterpenes divided by concertation of a particular monoterpene compound) or total monoterpene concentration (ng/mg fresh weight) were assessed by two-way ANOVA for each hormone.

For the plates experiment, the competitive effect of *C. geophillum-L. bicolor* interactions on the culture area (mm^2) and growth rate (mm^2 day^{-1}, calculated from the final culture area) of each fungus was examined by one-way ANOVA followed by Tukey HSD tests, as needed. The

Partitioned treatment was not considered in the *L. bicolor* analysis because this fungus covered the entire media before the experiment concluded, and thus I was unable to accurately measure the growth behavior.

All statistical analyses were performed in the R software environment version 3.3.2 (R Core Team, 2016). Data was log transformed to satisfy model assumptions of normality and homogeneity of variance, as needed. Figures were generated using non-transformed data.

2.3 Results

2.3.1 Greenhouse experiment

2.3.1.1 Fungal root colonization

The ectomycorrhizal treatments were successful as morphotyping indicated that each of the inoculated fungi had colonized roots of pine seedlings in their respective treatments (Fig. 2.1), ranging from 50% to 80% colonization rate (Fig. 2.4). Percent colonization of seedling roots colonized only by *C. geophillum* ($F_{3,152} = 13.52$, $P<0.001$) or *L. bicolor* ($F_{3,147} = 53.76$, $P<0.001$) significantly varied among treatments (Fig. 2.4a, b). The other species was found only in control treatment with mean of 14.62% and these were not found in any other treatment. For both fungi, the highest root colonization occurred when each fungus was inoculated on the roots by itself and percent colonization declined when they were inoculated together. For *C. geophillum*, percent colonization was 38.7% lower when both *C. geophillium* and *L. bicolor* were inoculated at the same time (combined treatment), and 72.4% lower when *L. bicolor* was inoculated prior to *C. geophillium* (CE Interaction treatment), relative to the *C. geophillum* control treatment (Fig. 2.4a). Similarly, the percent colonization of seedling roots by *L. bicolor* was 32.5% lower for the

LA Interaction treatment where *C. geophillum* was inoculated prior to *L. bicolor* and 60.3% lower for the combined treatment, relative to the *L. bicolor* control treatment (Fig. 2.4b). These fungi were not observed colonizing roots of the control seedlings. Mean hyphal cell count of the both the species were quantified before each inoculation form the fungal slurry separately. Further, 95 percent confidence interval was applied to calculate the difference. There was no difference found in the hyphal cell count of *C. geophillum* ($5.46\times10^6 \pm 3.33\times10^6$) and *L. bicolor* ($7.93\times10^6 \pm 5.17\times10^6$) and due to this overall effect of both the species was same.

2.3.1.2 Response of lodgepole pine seedling biomass to individual or a combination of fungal treatments

Total seedling biomass did not vary among treatments (Fig. 2.5; $F_{5,221}$=1.63, P=0.152). Seedling biomass responses were relatively higher for single-fungus inoculations for both *C. geophillum* and *L. bicolor* but there was no statistical difference in treatments. The total biomass of treated seedlings ranged from 2.0% to 30.1% greater than that of control seedlings without any fungal inocula.

2.3.1.3 Differences in seedling monoterpenes after hormone applications

Induced monoterpenes varied among mycorrhizal treatments for both hormone applications. For MJ-treated seedlings, I detected a significant effect of mycorrhizal treatment on the proportions of (−)-α-pinene ($F_{5,116}$ =5.19, P<0.001), (+) α-pinene ($F_{5,116}$=2.68, P=0.025), and myrcene ($F_{5,116}$=3.13, P=0.011) (Fig. 2.6). For (−)-α-pinene, seedlings treated with both fungi together had the highest and seedlings treated with *L. bicolor* had the lowest proportion. For (+)-α-pinene,

seedlings treated with both fungi together or with LA Interaction had the highest and seedlings treated with *C. geophillum* had the lowest proportion. For myrcene, seedlings treated with *L. bicolor* had highest and seedlings treated with both fungi together had the lowest proportion.

For MS-treated seedlings, I detected significant effects of mycorrhizal treatments for myrcene ($F_{5,86}$=2.63, P=0.029) and (+)-limonene ($F_{5,86}$=2.48, P=0.038) (Fig. 2.7). For myrcene, seedlings treated with *C. geophillum* had the highest and control seedlings had the lowest proportions. For (+)-limonene, seedlings treated with both fungi together had the highest and the control seedlings had the lowest proportions.

2.3.1.4 Overall comparisons of monoterpenes among fungal treatments between MJ and MS treated seedlings

Heatmaps (Figs. 2.8 and 2.9) were generated by taking the mean of the concentration of all compounds in the five treatments and individually comparing them to the mean of the same compounds in the control treatment. Based on the comparison, each compound in a treatment was given a score of either +1, 0 or -1, any compound which had its mean concentration higher than in the compound was scored +1, those with a lower concentration -1, and those which had no changes as compared to the control as 0.

In CEGE and LABI treatments (individual treatments), in MJ treated seedlings, pulegone was found in higher proportion as compared to all other treatments. Whereas, in MS treated seedling, (−)-camphene was found in higher proportion in CEGE treatment as compared to all other treatments. In combination treatment, in MJ treated seedlings, β-phellandrene was lower in proportion than all other treatments. In MS treated seedlings, no such trend was observed. In

competition treatment, in MJ treated seedlings, ocemine was found higher in LA Interaction as compared to all other treatments and no trend could be seen in MS treated seedlings.

2.3.2 Plates experiment

The total mycelial growth of *C. geophillum* (30-day growth) and *L. bicolor* (15-day growth) differed among treatments. For *C. geophillum*, total mean culture area of the partitioned, combined, and CE Interaction treatments were 33.0%, 54.1%, and 98.0%, respectively, lower than the *C. geophillum* control ($F_{2,42}$=93.41, P<0.001) (Fig. 2.10). Similarly, for *L. bicolor*, total mean culture area of the combined and LA Interaction treatments were 22.7% and 94.3 lower than the *L. bicolor* control %, respectively ($F_{2,42}$=282.71, P<0.001) (Fig. 2.11).

Growth rates of the fungi were separately compared among interaction treatments and controls. For *C. geophillum*, growth rate was significantly different across all the treatments ($F_{2,42}$=103.66, P=<0.001) (Fig. 2.12). These cultures had slower growth rates in the interaction treatments compared to the control; growth was 38.2% slower in the partitioned treatment, 63.4% in the combined, and 100% less in the CE Interaction treatment. Similarly, for *L. bicolor*, mean culture growth rate differed significantly among treatments ($F_{2,42}$=120.83, P<0.001) (Fig. 2.13). The mean growth rate of cultures in the interaction treatments was slower than the control, with the cultures in the combined treatment being 31.4% slower, and those in the LA Interaction treatment being 93.5% slower.

2.4 Discussion

Changes in belowground EM fungal species can affect the induced chemistry of conifer trees. Through my greenhouse experiment, I showed that induced monoterpene production in pine seedlings varied depending on the EM fungal species (*C. geophillium* or *L. bicolor*), their interactions, and the root colonization sequence by the two fungi. These results are in agreement with the changes in constitutive monoterpenes of lodgepole pine seedlings grown with fungi collected from MPB-killed or healthy pine stands (Karst et al., 2015). However, not all monoterpenes were affected by EM fungi, supporting the general idea that some of these monoterpenes are genetically controlled while others are sensitive to the changes in growing conditions (Forrest, 1981; Ott et al., 2011; Clark et al., 2014; Erbilgin et al., 2017).

In boreal forests, lodgepole pine trees are often found in symbiotic relationship with EM fungal species that help them to tolerate environmental stress (Bradbury et al., 1998; Arnold et al., 2003; Onwuchekwa et al., 2014; Karst et al., 2015). In my experiments, I selected two common EM fungi of pine forests in Alberta, *C. geophillium* or *L. bicolor* (Bradbury et al., 1998;). It is clear from my investigations that their effects on seedling induced monoterpene responses were different. For example, one of the most abundant monoterpenes in lodgepole pine is (−)-α-pinene and its proportion changes depending on the fungal species colonizing pine roots (Fig. 2.6). After induction treatments, seedlings grown with *C. geophillum* alone had proportionally more (−)-α-pinene that seedlings grown with *L. bicolor* alone. We can see similar differences in other monoterpenes such as (+)-α-pinene, myrcene, and (+)-limonene between these two fungi (Figs. 2.6, 2.7). Changes in monoterpene composition can likely be explained by the relative contributions from each fungus to the nutrient pool of host plants as monoterpene production is affected by both carbohydrate (Goodsman et al., 2013) and non-carbohydrate

(Gershenzon, 1994; Bennett et al., 2006; Smith & Read, 2008; Karst et al., 2015; Pec et al., 2017). To date , we do not know how the relative contribution by each fungus affects host plant nutritional composition (Bennett et al., 2006; 2009; Bennett & Bever, 2007). However, the current study provides the first evidence that pine induced monoterpenes are sensitive to the changes in the ectomycorrhizal fungal species.

I also found that plants do not necessarily benefit from having associations with multiple species of fungi, supporting the earlier work (Parladé & Alvarez, 1993; Baxter & Dighton, 2001; Bennett & Bever, 2007; Kennedy et al., 2007). I found both positive and negative effects of single vs. multiple species of fungi colonized the roots of pine seedlings on induced monoterpene responses. For example, after induction treatments, myrcene was higher in pine seedlings grown with either of the fungus alone, but was lower in seedlings grown with both fungi together (Figs. 2.6, 2.7). In contrast, in some cases having both fungi benefitted the monoterpene responses of pine seedlings more when they were present together. For example, both enantiomers of α-pinene were higher in seedlings grown with both fungi combined whereas lower when seedlings growth with either of the fungus alone (Fig. 2.6). Similar results were shown with arbuscular fungal associates of other plant species (Bennett & Bever, 2007; Bennett et al., 2009).

In addition, I demonstrated that the effects of EM fungi on monoterpenes can occur when early-colonizing species inhibit the colonization or development of later-arriving species. I showed that *L. bicolor* can have such an inhibitory effect on *C. geophillum*, which had reduced colonization of seedling roots and slowed growth rates on artificial media. However, *C. geophillium* did not have such inhibitory effect on *L. bicolor* when both species were growing together. In addition, *L. bicolor* had colonized a higher percentage of seedling roots and had a higher growth rate on artificial medium compared to *C. geophillum*. Such interactions between

these two fungi had a cascading effect on the production of monoterpenes in pine seedlings. For example, seedlings after induction treatments had lower (−)-*β*-pinene relative to the untreated control when *C. geophillium* was inoculated seedlings prior to *L. bicolor*, relative to any other fungal treatments. These results suggest that there is an associated cost on plant induced chemicals when EM fungal species compete with one another for the same host plants. I suspect that the competition between fungal symbionts apparently diverts some of their resources to supress or inhibit the growth of the competing fungal species. My experiments indicate that the growth rate of *C. geophillum* or *L. bicolor* was reduced when the other fungus was present. Negative competition is a fairly common among co-occurring EM fungi (Wu et al., 1999; Kennedy & Bruns, 2005; Kennedy et al., 2009; Kennedy, 2010).

Although my study provided evidence that *C. geophillium* and *L. bicolor* are likely competing for root colonization, their growth on the artificial media demonstrated that the mycelium of *L. bicolor* did not overlap that of *C. geophillum* (i.e., there was no uncolonized media and there was a distinct boundary between the interacting cultures). I currently do not know the mechanism underlying the observed non-overlapping competitive growth between the two fungi. Ectomycorrhizal fungi are known to secrete various fungal volatile organic compounds (Müller et al., 2013). Indeed, *C. geophillum* and *L. bicolor* have been shown to emit such organic compounds with each fungus releasing different profiles of chemicals (Müller et al., 2013). Cale et al. (2016) have demonstrated that organic fungal volatiles from a given fungal species can selectively or broadly inhibit growth and/or spore production of other fungal species. Thus, it is possible that the volatiles emitted from *L. bicolor* may adversely affect *C. geophillum*, resulting in *L. bicolor* being the dominant competitor when these fungi co-occur. Another potential antagonistic factor is secreted enzymes. Ectomycorrhizal fungi secrete enzymes

necessary to breakdown and assimilate nitrogen- and phosphorous-containing compounds (Pritsch & Garbaye, 2011). However, these compounds can also inhibit the growth of other fungi (Cale et al., 2016).

Although the mixed effects of fungi on plant induced defenses, their effects on plant biomass is all beneficial. All fungal treated seedlings tents to have a higher total biomass compared to non-treated control seedlings but there was no significant difference. Among treated seedlings, total biomass was higher in the single-fungus treatments compared to two-fungal treatments for both *L. bicolor* and *C. geophillum*. Similarly, jack pine seedlings colonized by a single EM fungal species had higher biomass than seedlings colonized by more than more fungus (Onwuchekwa et al., 2014). When considering seedling root colonization by more than one EM fungi, Kennedy et al. (2007) observed that colonization by the dominant competitive species lead to higher plant performance in terms of total seedling weight and leaf nitrogen as compared to the other competing species. In our study system, *L. bicolor* appears to be the superior competitor as it showed greater root colonization and culture area when growing in proximity to *C. geophillum*. Seedlings grown only with *L. bicolor* tended to have greater total biomass than those grown only with *C. geophillum*. Co-colonization may not be as beneficial to host plants as colonization by a single species, which can provide greater seedling growth and nitrogen uptake. This may result from inter-fungal competition, which may negatively affect plant performance and biomass as nitrogen is used by competing fungi rather than being supplied to the host plant. Thus, the benefits that the plant receives from EM fungi may be limited by the dominant EM fungal species and the presence of other competitive fungi.

In conclusion, the outcome of this study provided evidence that ectomycorrhizal fungal symbionts can affect the induced defense chemistry of conifers depending on the fungal species,

their interactions, and the root colonization sequence. Considering the effects of these symbiotic fungi on plant growth and induced defenses, they can directly or indirectly affect the host tree susceptibility to insect herbivores. They can directly contribute to the nutritional pool of host trees, especially nitrogen, by providing nutrients or accelerating plant growth, which in turn accelerates the photosynthetic carbon uptake. In directly, they can contribute the production of secondary compounds as even carbon-based defense compounds need nitrogen and other minerals in their production. These results suggest that ectomycorrhizal fungal communities should also be considered a part of host plant co-evolution against insect herbivores (Karst et al., 2015) as they can influence the induced secondary chemistry of pines and likely the host plant-insect herbivory interactions.

Figure 2.1. Scanning electron microscope images of ectomycorrhizal fungal association with lodgepole pine (*Pinus contorta*) seedling root hairs. (a) *Cenococcum geophillum* and (b) *Laccaria bicolor.*

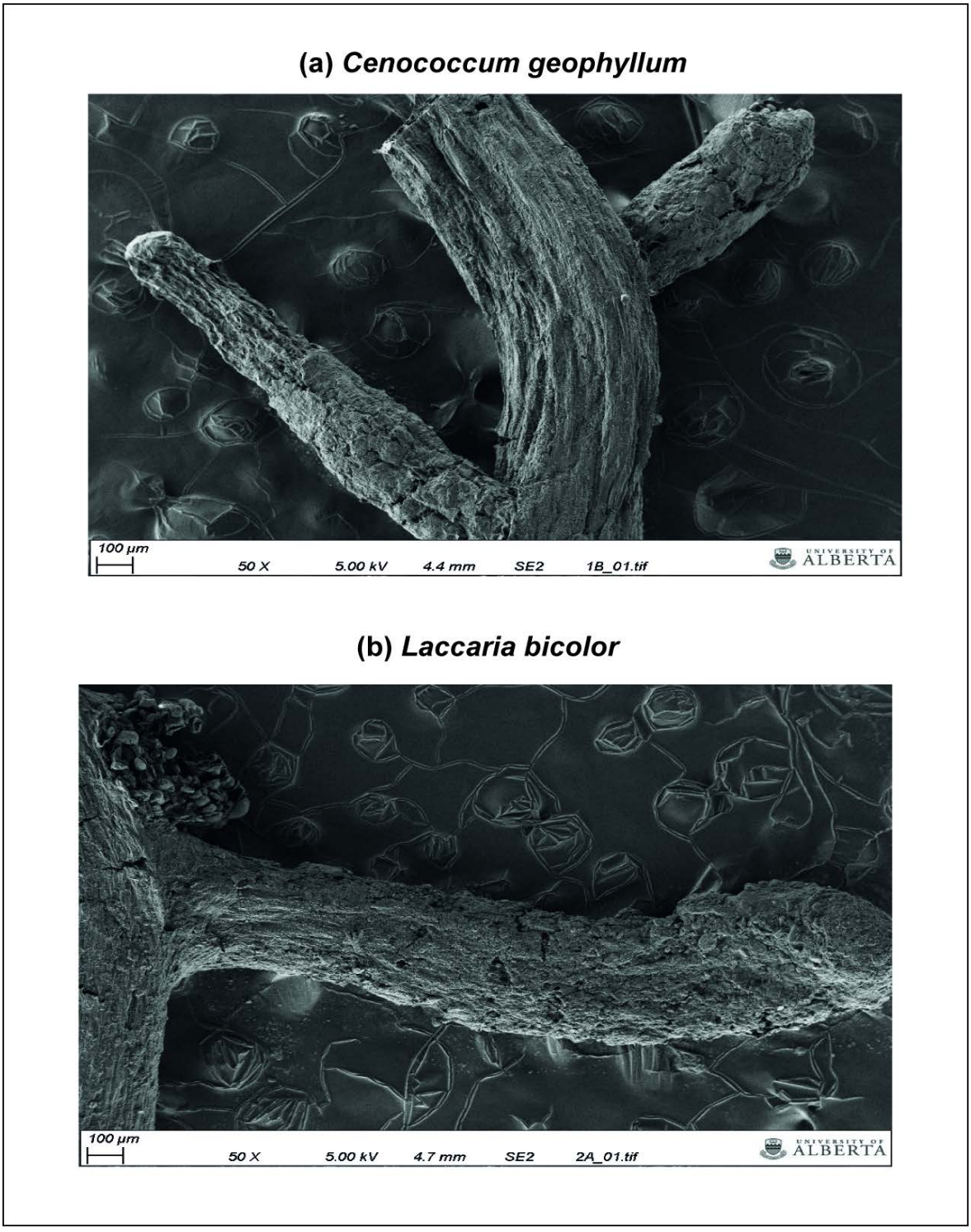


Figure 2.2. Experimental design showing the fungal inoculation in various treatments. Where in, CEGE: *Cenococcum geophillum* was inoculated alone; LABI: *Laccaria bicolor* was inoculated alone; Combined: Both the fungi were inoculated together; LA Interaction: *C. geophillum* was inoculated first and then *L. bicolor;* CE Interaction: *L. bicolor* was inoculated first and then *C. geophillum;* Control: No fungi were inoculated and were served as control.

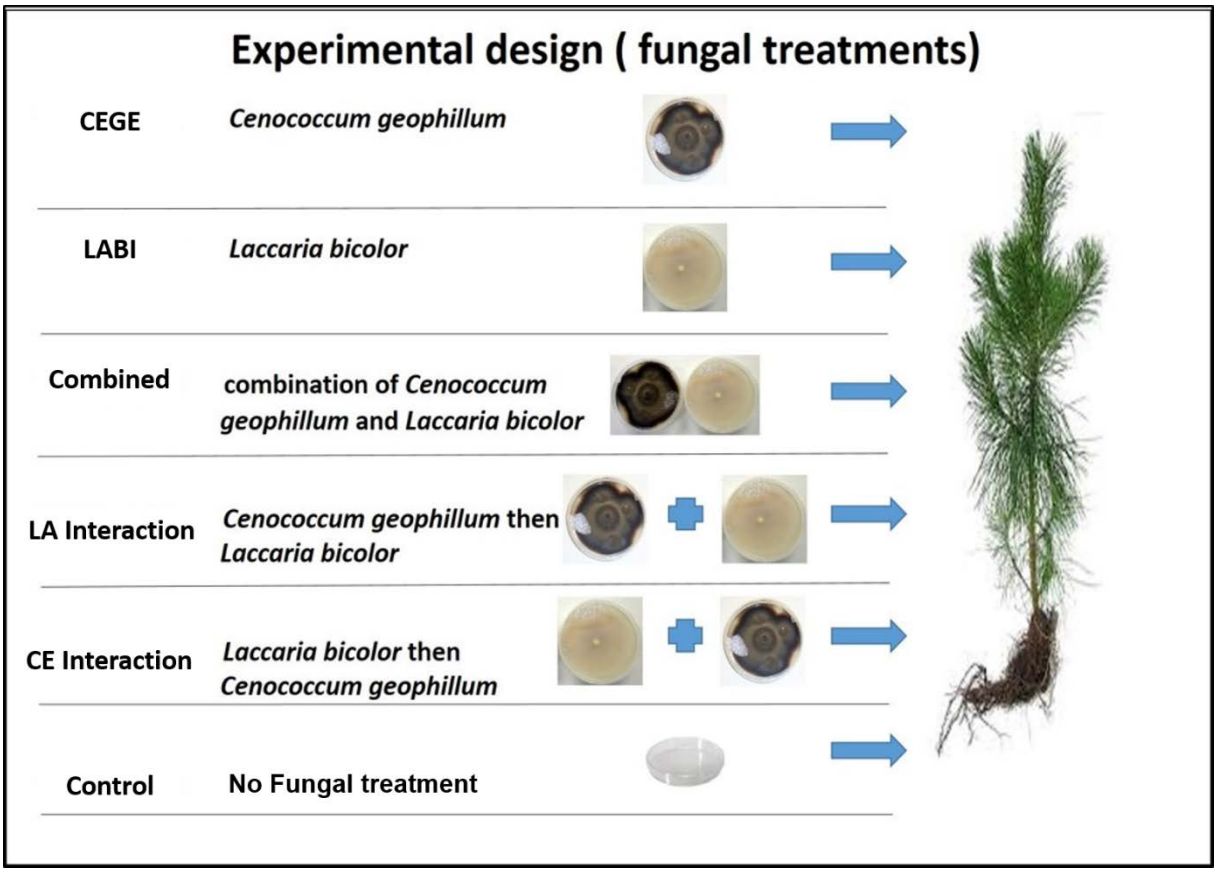


Figure 2.3. Experimental design showing the inoculation point of each fungus in various treatments considering time as a main component. Where in, CEGE control: *Cenococcum geophillum* was grown alone; LABI control: *Laccaria bicolor* was grown alone; Partitioned: Both the fungi were grown together in partitioned Petri dish; Combined: Both the fungi were grown together in non- partitioned (regular) Petri dish; CE Interaction: *C. geophillum* was grown on established *L. bicolor*; LA Interaction: *L. bicolor* was grown on established *C. geophillum.*

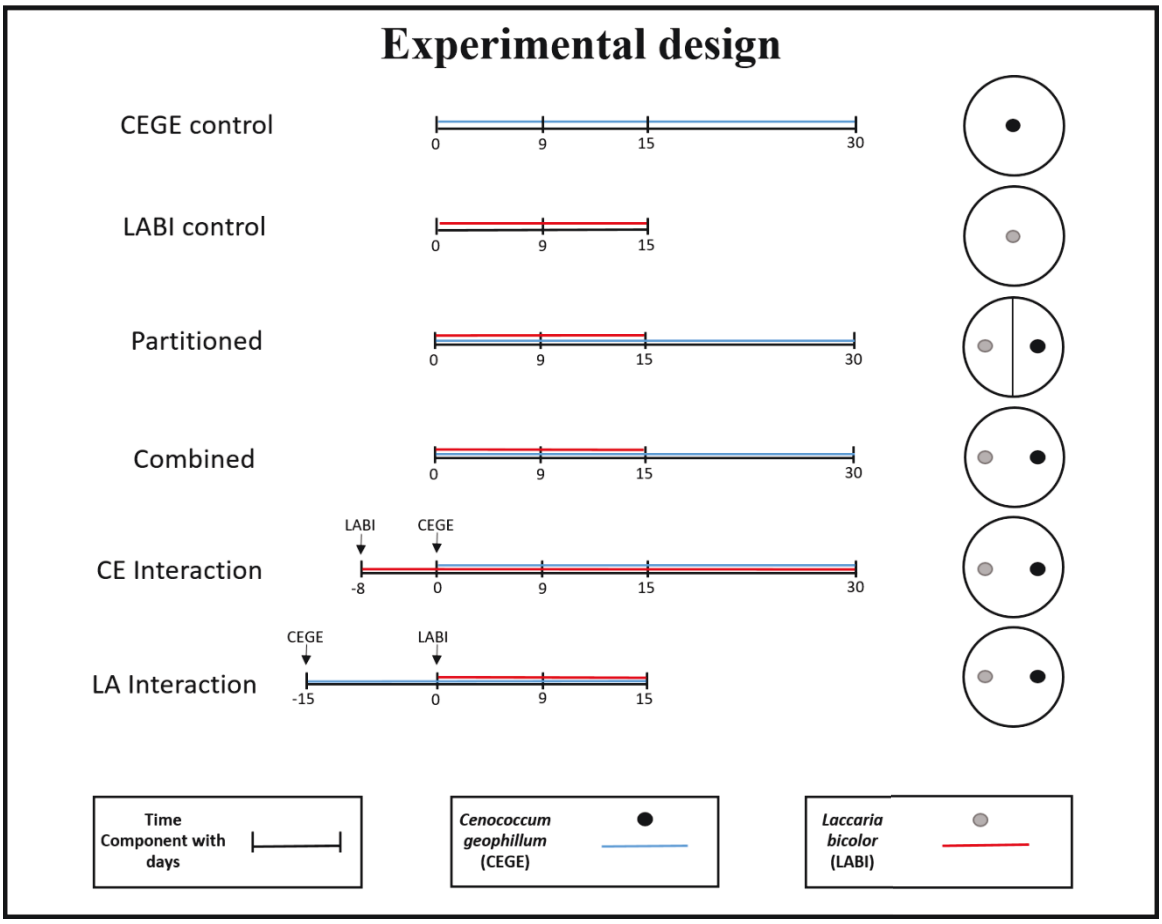


Figure 2.4. Mean (± s.e.) differences in percent root colonization of (a) *Cenococcum. geophillum* and (b) *Laccaria bicolor* and within treatments in lodgepole pine (*Pinus contorta*) seedlings. Bars with different letters are statistically different as indicated by Tukey Honest Significant difference tests.

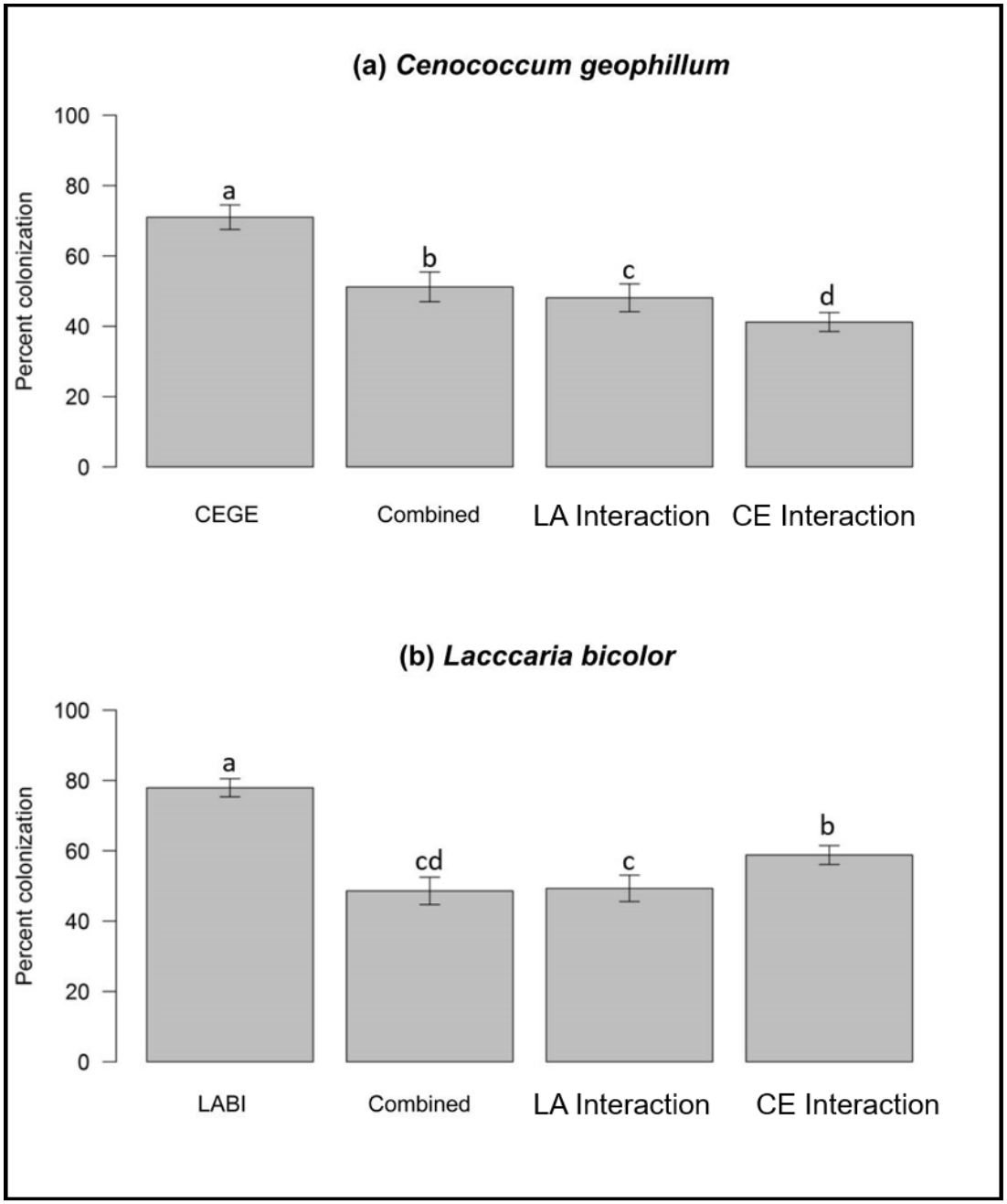


Figure 2.5. Mean (± s.e.) differences in total biomass (g) within treatments in lodgepole pine (*Pinus contorta*) seedlings. Where in, CEGE: *Cenococcum geophillum* was inoculated alone; LABI: *Laccaria bicolor* was inoculated alone; Comb.: Both the fungi were inoculated together; LA Interaction.: *C. geophillum* was inoculated first and then *L. bicolor;* CE Interaction.: *L. bicolor* was inoculated first and then *C. geophillum;* Control: No fungi were inoculated and were served as control.

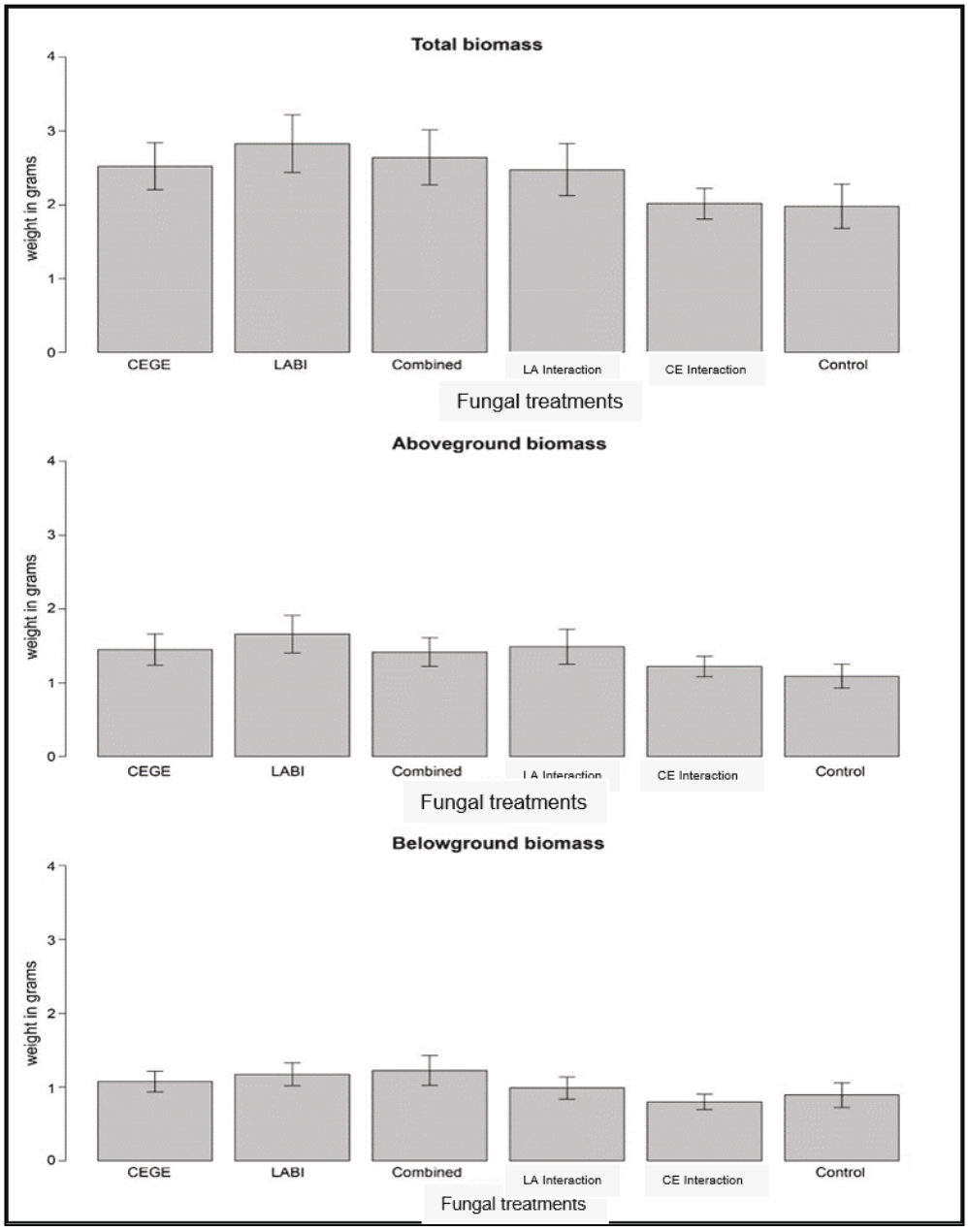


Figure 2.6. Mean (± s.e.) differences in monoterpene compounds between fungal treatments and seedling class in methyl jasmonate treated lodgepole pine (*Pinus contorta*) seedlings. Where in, CEGE: *Cenococcum geophillum* was inoculated alone; LABI: *Laccaria bicolor* was inoculated alone; Combined: Both the fungi were inoculated together; LA Interaction: *C. geophillum* was inoculated first and then *L. bicolor;* CE Interaction: *L. bicolor* was inoculated first and then *C. geophillum;* Control: No fungi were inoculated and were served as control. Bars with different letters are statistically different as indicated by Tukey Honest Significant difference tests.

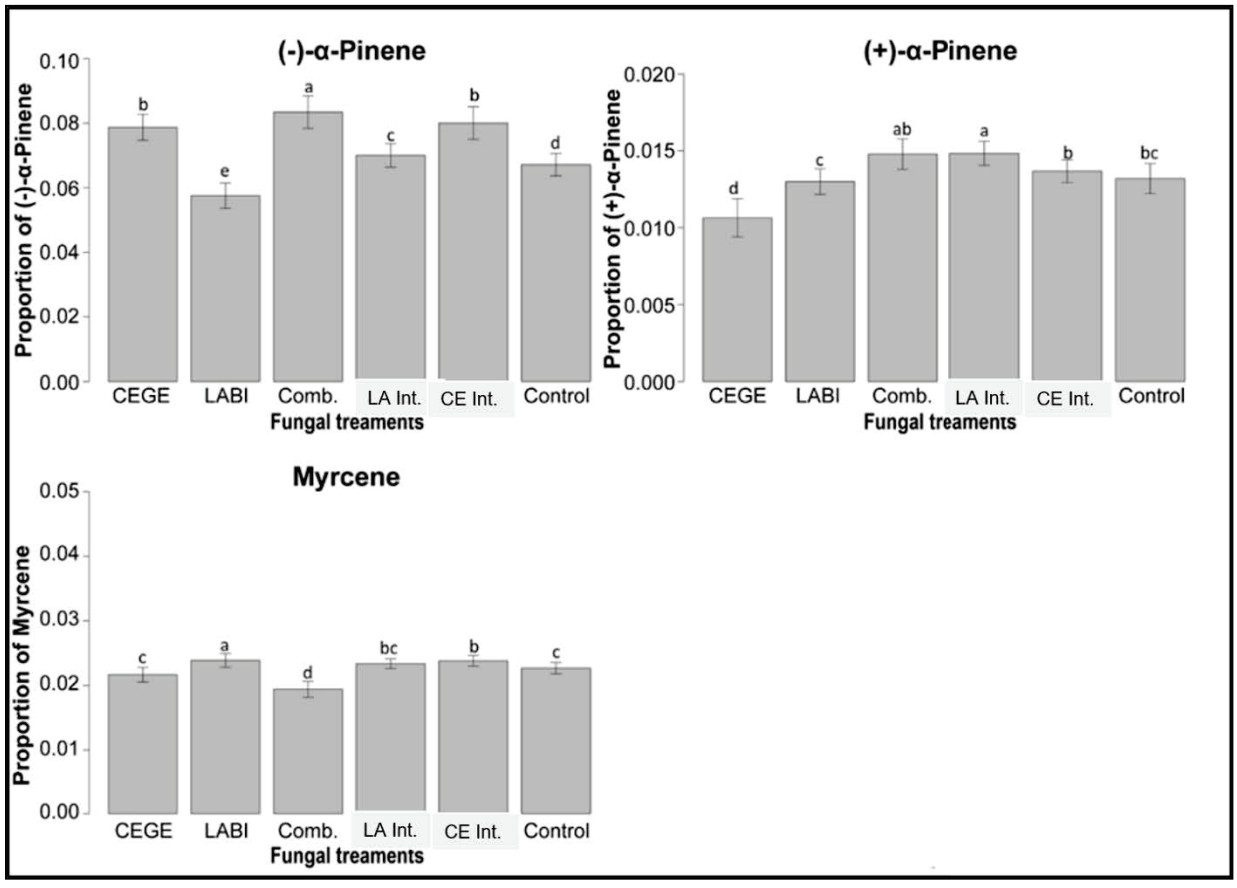


Figure 2.7. Mean (± s.e.) differences in monoterpene compounds between fungal treatments in Methyl salicylate treated lodgepole pine (*Pinus contorta*) seedlings. Where in, CEGE: *Cenococcum geophillum* was inoculated alone; LABI: *Laccaria bicolor* was inoculated alone; Combined: Both the fungi were inoculated together; LA Interaction: *C. geophillum* was inoculated first and then *L. bicolor;* CE Interaction: *L. bicolor* was inoculated first and then *C. geophillum;* Control: No fungi were inoculated and were served as control. Bars with different letters are statistically different as indicated by Tukey Honest Significant difference tests.

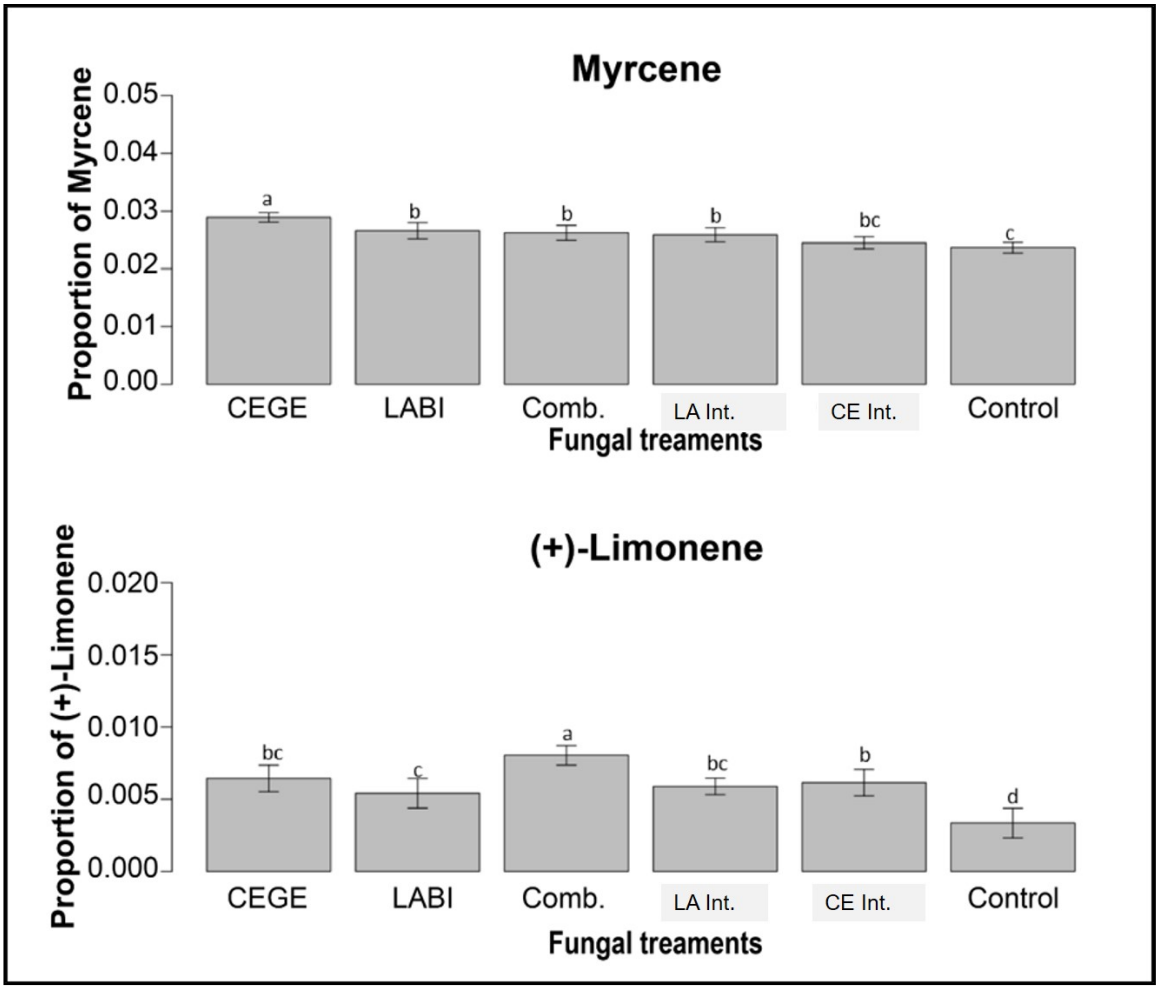


Figure 2.8. Heat map comparing percentage of secondary compounds to total monoterpenes in all fungal colonised lodgepole pine (*Pinus contorta*) seedlings with untreated control lodgepole pine seedlings treated with methyl jasmonate. Where in black color is amount less, dark gray is amount equal and light gray is amount more than in untreated control lodgepole pine seedlings.

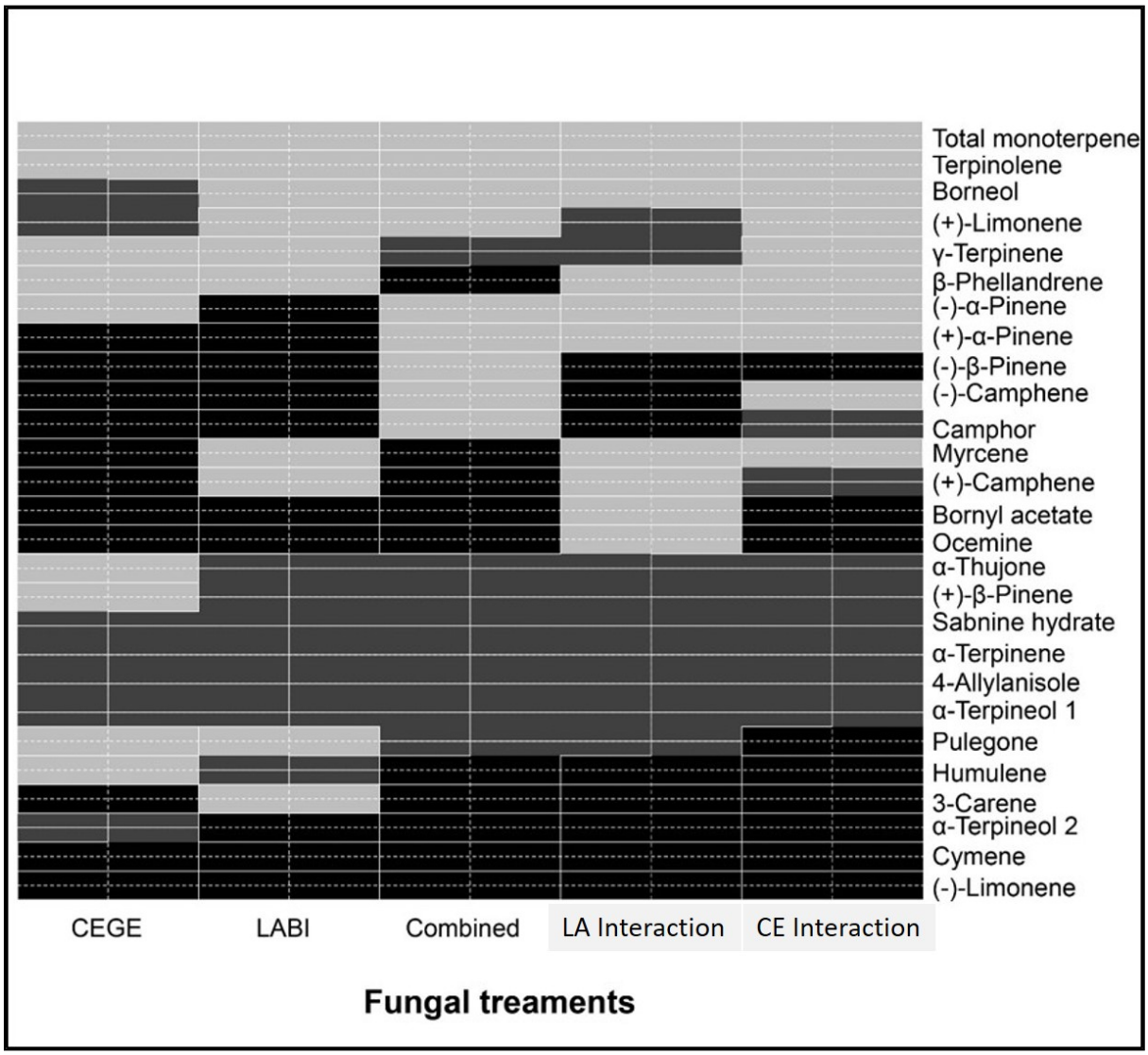


Figure 2.9. Heat map comparing percentage of secondary compounds to total monoterpenes in all fungal colonised lodgepole pine (*Pinus contorta*) seedlings with untreated control lodgepole pine seedlings treated with methyl salicylate. Where in black color is amount less, dark gray is amount equal and light gray is amount more than in untreated control lodgepole pine seedlings.

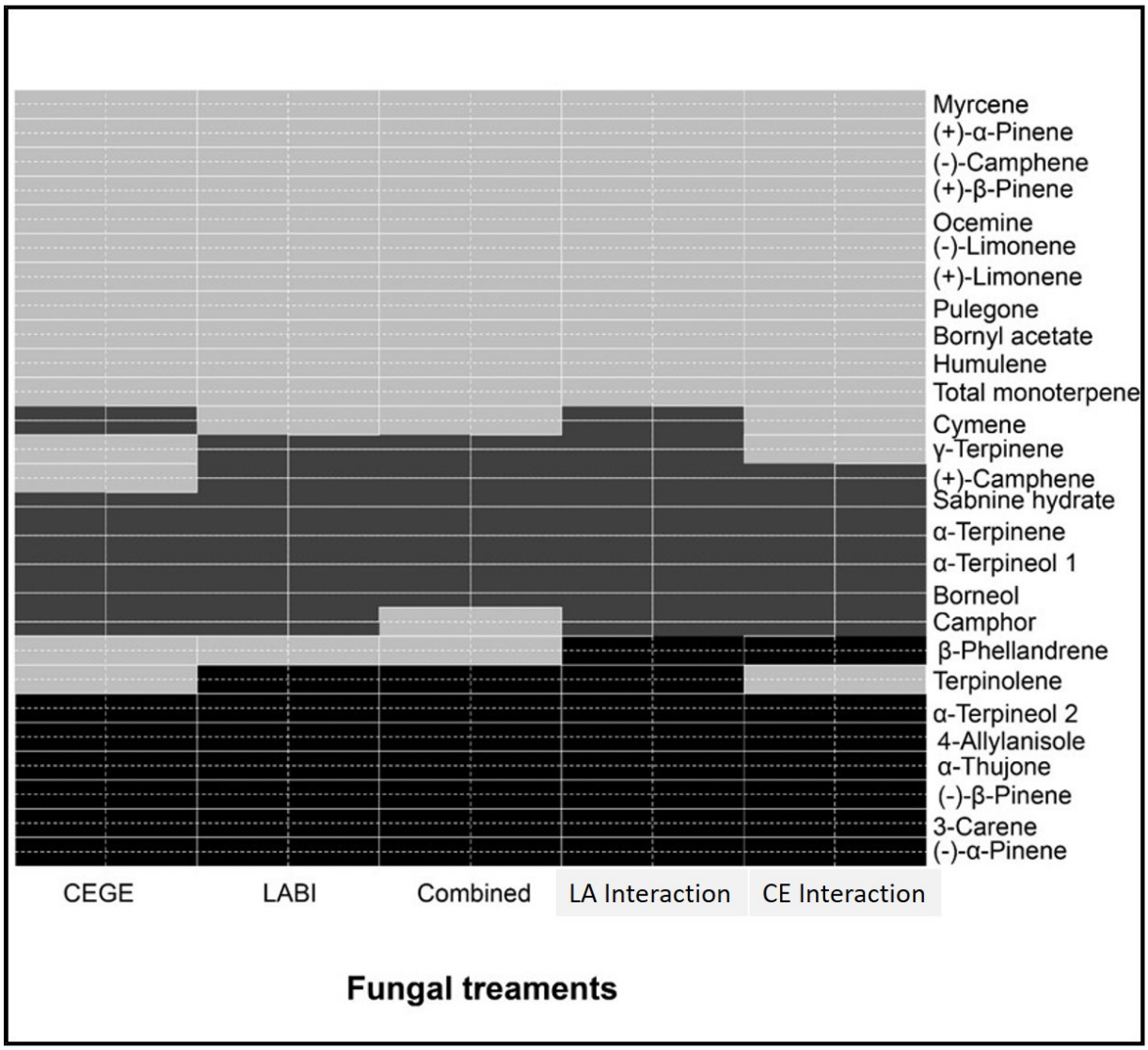


Figure 2.10. Mean (± s.e.) differences in growth of *Cenococcum geophillum* (mm^2) within treatments. Where in, CEGE control: *Cenococcum geophillum* was grown alone; LABI control: *Laccaria bicolor* was grown alone; Partitioned: Both the fungi were grown together in partitioned Petri dish; Combined: Both the fungi were grown together in non- partitioned (regular) Petri dish; CE Interaction: *C. geophillum* was grown on established *L. bicolor*. Bars with different letters are statistically different as indicated by Tukey Honest Significant difference tests.

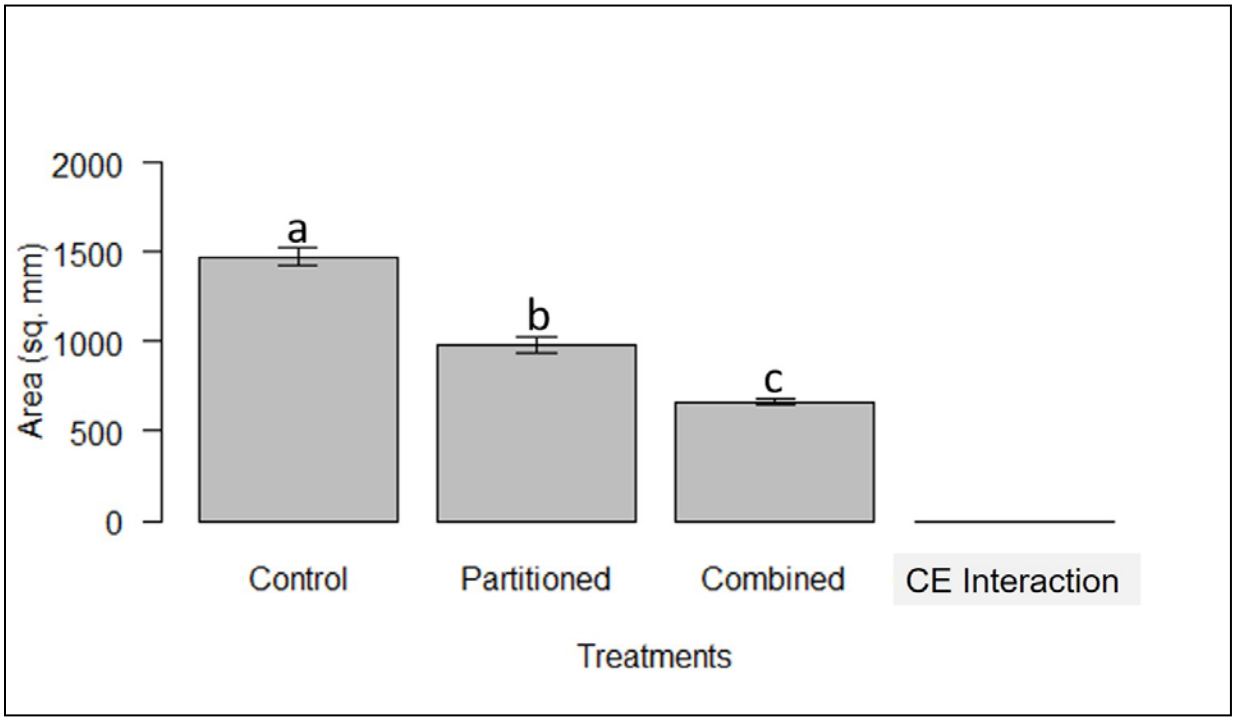


Figure 2.11. Mean (± s.e.) differences in growth of *Laccaria bicolor* (mm^2) within treatments. Where in, CEGE control: *Cenococcum geophillum* was grown alone; LABI control: *Laccaria bicolor* was grown alone; Partitioned: Both the fungi were grown together in partitioned Petri dish; Combined: Both the fungi were grown together in non- partitioned (regular) Petri dish; LA Interaction: *L. bicolor* was grown on established *C. geophillum.* Bars with different letters are statistically different as indicated by Tukey Honest Significant difference tests.

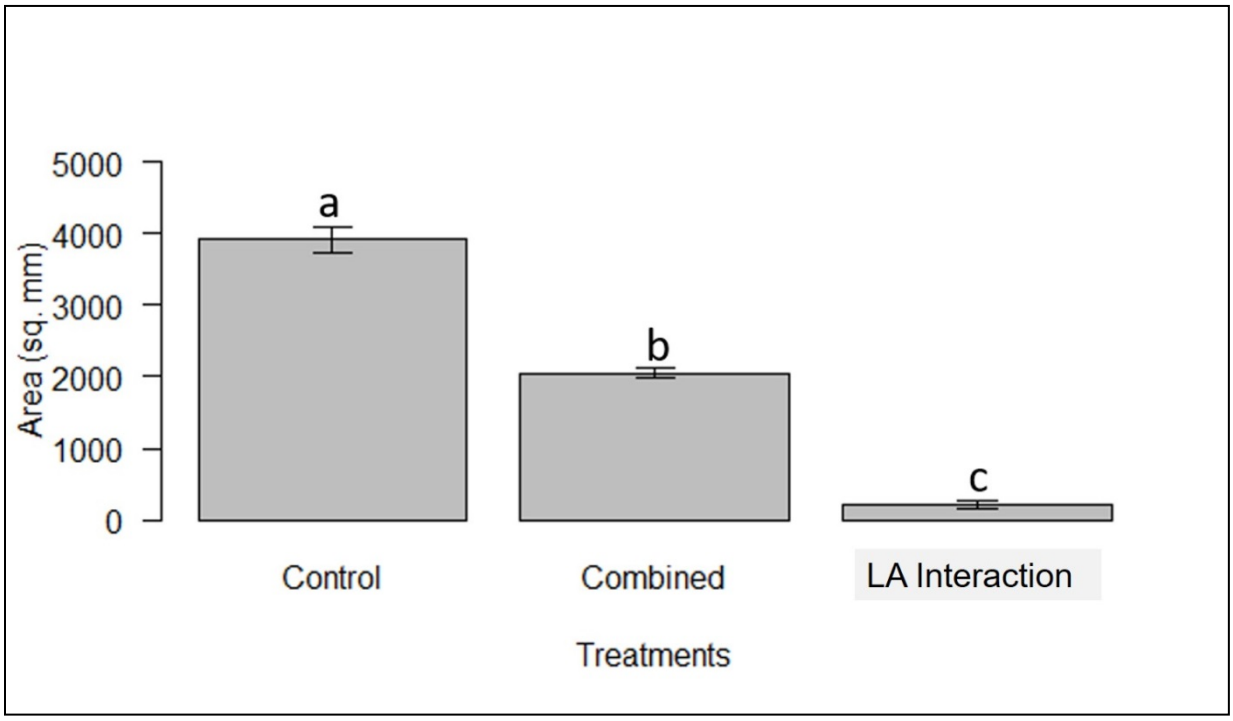


Figure 2.12. Mean (± s.e.) differences in growth rate of *Cenococcum geophillum* (mm^2 per day) within treatments. Where in, CEGE control: *Cenococcum geophillum* was grown alone; LABI control: *Laccaria bicolor* was grown alone; Partitioned: Both the fungi were grown together in partitioned Petri dish; Combined: Both the fungi were grown together in non- partitioned (regular) Petri dish; CE Interaction: *C. geophillum* was grown on established *L. bicolor*. Bars with different letters are statistically different as indicated by Tukey Honest Significant difference tests.

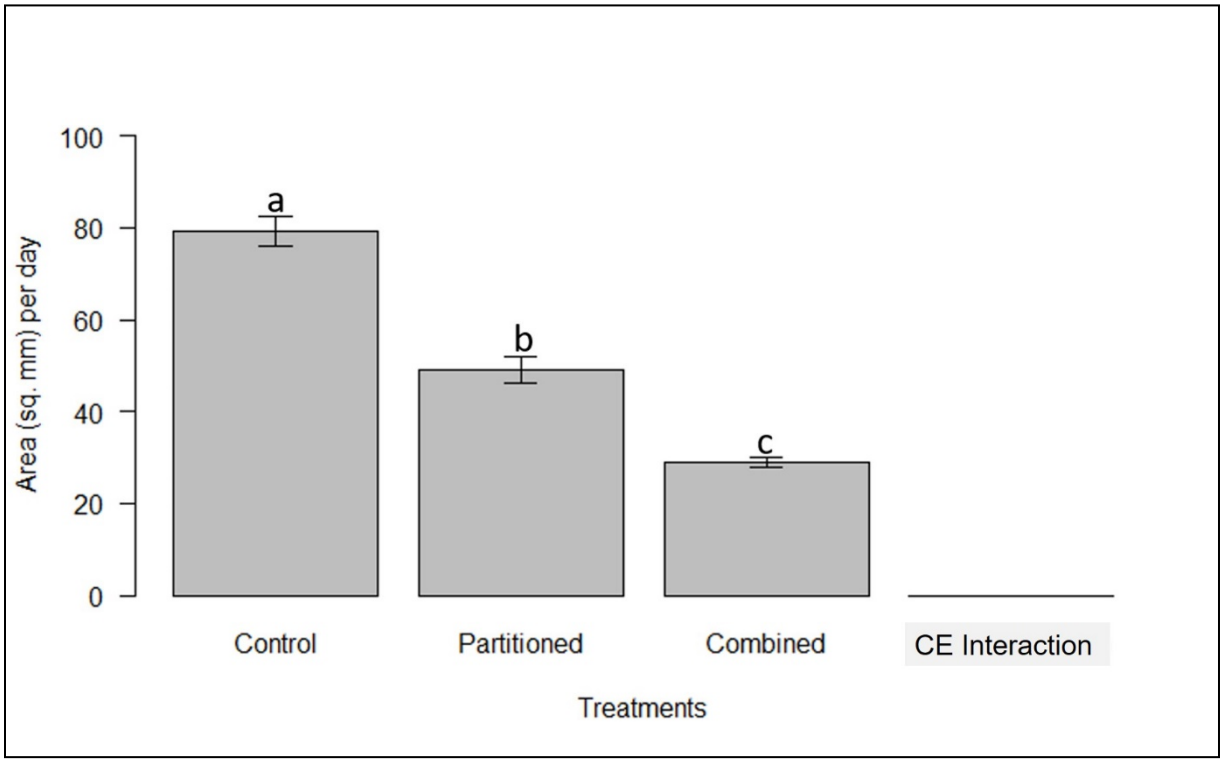
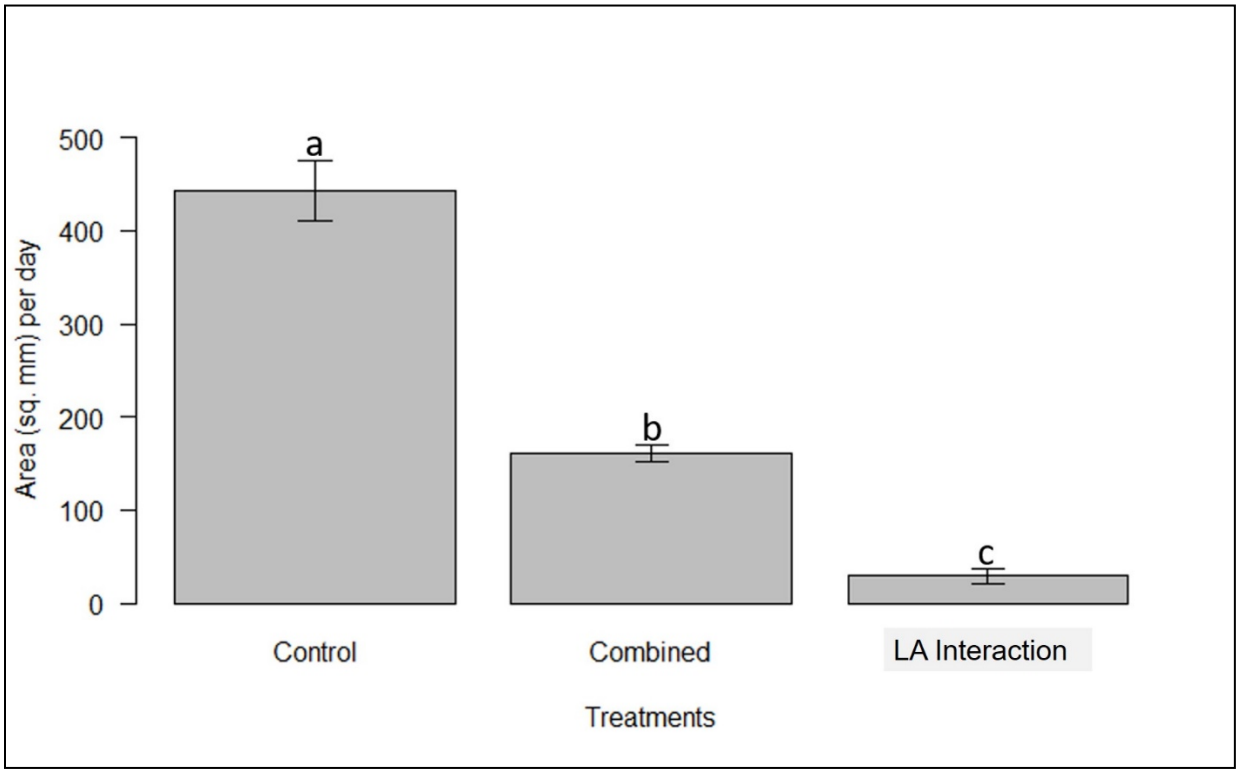


Figure 2.13. Mean (± s.e.) differences in growth rate of *Laccaria bicolor* (mm^2 per day) within treatments. Where in, CEGE control: *Cenococcum geophillum* was grown alone; LABI control: *Laccaria bicolor* was grown alone; Partitioned: Both the fungi were grown together in partitioned Petri dish; Combined: Both the fungi were grown together in non- partitioned (regular) Petri dish; LA Interaction: *L. bicolor* was grown on established *C. geophillum.* Bars with different letters are statistically different as indicated by Tukey Honest Significant difference tests.



Chapter 3: Thesis discussion

To improve the health of seedlings in nursery and reforestation settings, pine seedlings are often inoculated with ectomycorrhizal fungi. Species of root-colonizing EM fungi can affect the defense-related induced chemistry of conifer trees. Monoterpenes are a major class of defensive induced chemicals. These symbiotic fungi affect their host plant's nutrition (Goodsman et al., 2013; Karst et al., 2015; Pec et al., 2017), and monoterpene production is dependent on plant nutritional status (Bennett et al., 2006, 2009; Bennett & Bever, 2007). It is essential to understand how EM fungi influence monoterpene composition, as this change directly affects the host defense. In the seedlings treated with individual fungi, the changes in monoterpene concentrations increased beneficial compounds that are involved in plant resistance. Further, fungal treatments increased seedling biomass

In nature, colonization by EM fungi is dynamic and often colonizing fungi are out-competed and displaced by other EM fungi species (Benecke & Gobl, 1974; Lamb, 1979; Wu et al., 1999). Which EM fungi successfully colonize roots when many EM fungi are present depends on the dominance of one fungus over others. Competition between two EM fungal species that share a host substrate can affect the growth of the interacting fungi. For example, competing fungi may experience the effects of negative or asymmetric competition whereby the growth rate is reduced for either both fungi or the least competitive fungi, respectively (Kennedy, 2010; Kennedy et al., 2011). In case of *C. geophillium* and *L. bicolor*, *L. bicolor* is the dominant species and can have inhibitory effects on *C. geophillium*, whose ability to colonize seedling roots is inhibited. The superior competitive ability of *L. bicolor* may be due to inherently high growth rate compared to that of *C. geophillium*, as indicated in the single-fungus treatments.

However, the growth rate of both fungi was reduced when the other fungus was present. Such negative competition may be a fairly common interaction between co-occurring EM fungi in nature (Kennedy & Bruns, 2005; Kennedy et al., 2009; Kennedy, 2010). The negative competition effects are not just on the EM fungi species themselves, but can extend to the host plant as well, where co-colonization may not be beneficial. Colonization by a single species can provide greater seedling growth and nitrogen uptake. In multi-species treatment, fungi might utilise nitrogen for producing nitrogen based defensive compounds which are used for direct antagonistic interactions, leading to a lower provision of nitrogen to the host plant (Kennedy et al., 2007). Thus, the benefits that the plant receives from the EM fungi may be determined or limited by the dominant EM fungal species and the presence of competing fungi. Overall, these factors and my investigation indicate that different EM fungi can also differentially affect seedling induced monoterpenes. Whether these fungi individually or in combination colonize host plants can differentially benefit host plant defenses. Colonization by individual EM fungi can promote defense, whereas colonization by a combination of EM fungi leads to competition which does not benefit the plant defense. Although the effect of EM fungi on induced chemicals is mixed, their effect on plant biomass is completely beneficial.

The differential effect of EM fungi on induced compounds of lodgepole pine seedlings differs with phytohormone. This indicates that EM fungi might affect differently defenses involved in during pest and pathogen attack as these phytohormones elicit similar responses. In MJ treated seedlings, I found that the concentration of 3-carene was higher in some of the seedlings, the same trend was also seen in the case of terpinolene. The increase in 3-carene is justified as it acts as an anti-feedant to reduce the activities of attacking beetles. The trend observed for terpinolene does not directly enhance the defense mechanism, in-fact it acts

synergistically with beetle aggregation pheromones. Myrcene, α-pinene and limonene were found to be higher in some seedlings. Myrcene and α-pinene promote beetle aggregation, female beetles utilise α-pinene which acts as a precursor to produce the aggregation pheromone trans-verbenol. Limonene has an antifeedant action, thus helping in the defense of the seedlings. Similarly, in MS treated seedlings, in some seedlings, concentrations of (+)-α-pinene, myrcene and limonene were increased and (−)-α-pinene, 3-carene and terpinolene were found in increased concentration in some seedlings.

The findings of this study provide evidence that EM fungal symbionts can affect the induced defense chemistry of conifers depending on a variety of factors. The factors which mainly affect induced chemistry include the fungal species, their competitive interactions, the order in which they colonize host roots, and the type of biotic stressor inducing defensive monoterpenes.

3.1 Management implications

Ectomycorrhizal associations can affect the composition of plant secondary compounds (Gershenzon, 1994; Smith & Read, 2008; Karst et al., 2015). Yet, the role EM fungi play in the synthesis and production of secondary compounds has not been investigated (Gershenzon, 1994; Smith & Read, 2008; Karst et al., 2015). Studies have been conducted to determine how EM fungal species can alter constitutive defense related compounds (Karst et al., 2015). There was still a critical need to determine how EM fungal species will affect the induced defense chemicals (Bennett et al., 2006; Gehring & Bennett, 2009; Koricheva & Jones, 2009). My study provides the first evidence that induced monoterpenes in lodgepole pine are sensitive to variation

in ectomycorrhizal fungi. My research focused on two EM fungal species which increased the proportion of certain monoterpenes, such as the anti-feedants 3-carene and (+)-limonene. More research is required to look for EM fungi that increase the proportion of compounds which adversely affect pest insects and pathogens. This can be done by studying pine and its EM fungi occurring in natural settings. Once such symbionts are known and their effects on pine chemistry has been studied, one EM fungus or combination of EM fungi may be chosen and utilised to develop seedling stock resistant to MPB attack.

This strategy needs to be employed individually on plants to identify and develop resistant seedlings which are found in different ecosystems. The optimal choice of EM fungi to promote resistant trees in one ecosystem may be different for a different ecosystem; hence it is essential to choose EM fungal species that would act the best for a particular ecosystem. The same method of studying induced defensive chemicals can also be used to develop EM fungal systems against pathogens or insect pests other than MPB. Also, on comparing the relative contribution of individual symbiotic fungus to host plant defenses, I recommend that single-EM fungus inoculation be done to obtain maximal benefits. If seedlings are grown in greenhouse conditions, the number of inoculations should also be increased; this will increase the percent colonization of inoculated seedlings. When these seedlings are later used in a reforestation program, other EM fungi may not easily colonize or displace the original inoculated EM fungi. However, based on time and need, combination of EM fungi can also be utilised to target increase in specific secondary compounds. Research can also be done using non-competitive species and their effects on plant secondary compounds can be measured and compared.

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