Efficacy of various *Hypholoma* spp. and *Phlebiopsis gigantea* as biocontrol agents against root rotting fungi *Armillaria ostoyae*

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Abstract

Conifer plantations are thinned to maximise production of high-quality timber for lumber, fire wood, and utility poles. However, thinning increases susceptibility to pathogens such as *Armillaria ostoyae*, a native basidiomycete species that causes root rot. *Armillaria* root rot is detrimental to the forestry industry as it is responsible for substantial revenue loss due to tree mortality in both establishing and mature stands. Thus, finding an effective means to inhibit or reduce its impacts and spread is critical for the industry. Past studies have suggested that biocontrol treatments based on competing basidiomycete species (i.e., *Hypholoma* spp. and *Phlebiopsis gigantea*) show promise. The objectives of my study were to: (1) determine the ability of several species and strains of *Hypholoma* spp. and *Phlebiopsis gigantea* to function as biocontrol agents against two *Armillaria ostoyae* strains by examining their *in-vitro* interactions during competition trials, and (2) examine the ability of *H. fasciculare* to survive and grow during the winter months at different soil depths in a number of Simcoe County, Ontario forests to determine its potential as a biocontrol treatment for *Armillaria* root rot in northeastern temperate forest regions.

Several strains of *Hypholoma fasciculare, H. sublateritium, H. capnoides,* and one strain of *P. gigantea* were grown in both solo (absence of competition) and paired (competing with one of two strains of *A. ostoyae* - high virulence and low virulence) conditions to examine impacts of competition on radial growth. The solo and pairings were grown and monitored on 2 % malt agar and pine wood infusion medium at 15^{o}C. Radial growth was measured and compared between solo and paired conditions, and macroscopic and microscopic interactions were qualitatively assessed. *Hypholoma fasciculare* strain Pinnel B was identified as a potential candidate to develop as a biocontrol agent against *Armillaria* root rot. Radial growth was the highest from the *H. fasciculare* strain Pinnel B when paired with either of the *A. ostoyae* strains. Characterisation of the interactions at macroscopic and microscopic levels indicated that it was most effective at inhibiting *A. ostoyae* growth.

Soil temperatures and snow depth were monitored from November 4, 2017 to May 13, 2018 at thinned and non-thinned red pine (*Pinus resinosa*) plantations located in Simcoe County, Ontario, Canada. Pine blocks inoculated with *H. fasciculare* were buried in pine plantations from February 1, 2018 to May 13, 2018 at 30 and 100 cm depths to examine how winter soil temperatures, soil depth, and snow presence impacted growth. Soil temperatures at the 30 cm depth were consistently colder than at 100 cm ($F_{1,6} = 63.46$, $p < 0.001$). *Hypholoma fasciculare* continued to grow over the winter months ($F_{1,36} = 50.41$, $p < 0.001$). Soil depth did not impact growth rate ($F_{1,18} = 1.87$, $p = 0.188$). Mean growth rates were 0.25 ± 0.11 and 0.31 ± 0.10 mm per day at 30 and 100 cm depths, respectively. While snow depths were significantly lower in non-thinned plantations, this had little impact on soil temperatures. This study will aid in the development of a *H. fasciculare* biocontrol treatment against *Armillaria* root rot given *H. fasciculare's* ability to continue to grow underground throughout the winter months, a period during which *Armillaria ostoyae* has a reduced growing rate.

Key words: Armillaria root disease; *Annosus root rot*; *Phlebiopsis gigantea* (VRA 1992); *Hypholoma* spp.

Lay Summary

The mission statement of Lakehead University's Department of Biology is "Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms". My study focuses on developing a biocontrol treatment against *Armillaria* root rot. My study contributes to one of the central research themes outlined in the mission statement, the relationships between life forms and their environmental functions. It advances our knowledge on *Armillaria ostoyae* and provides a promising direction towards developing an effective biocontrol measure against this root decaying fungus. *Armillaria* root rot is responsible for significant losses in our managed mixedwood and conifer-dominated forests as it can cause widespread *Pinus* spp. mortality (McLaughlin *et al*. 2010). Therefore, the search for a viable treatment option for *Armillaria* root rot is of paramount importance.

Two major research questions were investigated to search for a viable biocontrol treatment option for *Armillaria ostoyae* through *in-vitro* (laboratory) and field testing: (1) are there particular non-pathogenic species or strains of *Hypholoma* spp. or *Phlebiopsis gigantea* capable of preventing the spread and growth of the root rot causing basidiomycete, *Armillaria ostoyae?* (2) Are winter and early spring soil temperatures suitable for *Hypholoma fasciculare* growth? A lab study was conducted to examine how *Hypholoma* spp. and *P. gigantea* compete with *A. ostoyae* in optimal growth conditions. To compliment the lab study, a field study was conducted to examine how *Hypholoma facsciculare* grows in sub-optimal conditions (during winter) when *A. ostoyae* growth slows considerably. The *in-vitro* competition experiment indicated that *H. fasciculare* strain Pinnel B was most effective at inhibiting *A. ostoyae* growth, followed by two strains of *H. sublateritium*: FP-90085-SP and HHB-11948-SP. Wooden blocks

inoculated with *Hypholoma fasciculare* strain Pinnel B buried in various Simcoe County, Ontario red pine (*Pinus resinosa*) plantations at different soil depths were able to survive and continue to grow during winter and early spring seasons. Lab and field testing confirmed that *Hypholoma fasciculare* Pinnel B could survive and grow in the presence of *A. ostoyae* during the winter season. This study demonstrates that *H. fasciculare* could be a potential biocontrol treatment option against *A. ostoyae*.

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Chapter 1: Introduction

1.1 Overview

Pine plantations contain high densities of trees that can be managed very effectively to produce high quality stems for purposes such as lumber, fire wood, and utility poles. Forest thinning is designed to expediate forest regeneration, increase commercial wood yields, and reduce impacts of prolonged competition and the prevalence of insect damage (Kariuki, 2008). However, because of thinning and poor site conditions for pine (e.g., alkaline soils, sandy soils) (McLaughlin et al. 2011), these plantations are becoming susceptible to diseases such as *Armillaria* root rot (Lockman & Kearns, 2016). Forest thinning increases the inoculum potential for *Armillaria* spp. as the remaining stumps left after thinning can act as establishment points for *Armillaria* spp. (Chapman et al. 2004; Hood et al. 2002) while an alkaline C soil horizon can result in a shorter life cycle of red pine (*Pinus resinosa*) due to nutrient deficiency and increased vulnerability to *Armillaria* root rot (McLaughlin et al. 2011).

Root diseases can have devastating effects across the landscape as spread is hard to control, given that basidiospores are transmitted by air and mycelium can spread through underground root contact (Lockman & Kearns, 2016). *Armillaria* root rot causes reduced tree growth, crown thinning, resinosis on butt and roots, and wood decay to trees (Lockman & Kearns, 2016). Managing plantations infected with a root disease is challenging because of the difficultly in detecting the occurrence of fungal infection, predicting when tree mortality will result, and deciding upon an appropriate time to thin stands to avoid high economic loss (G. Davis, personal communication, 07/07/17). Due to the high stem density within a plantation, root diseases can spread quickly through highly connected root systems. Root rot in Ontario alone is responsible for 11 million m^3 of forest decay each year (Dumas, 2013).

1.1.1 Commercially important root diseases

1.1.1.1. *Armillaria* root rot

Armillaria root rot, which is caused by various *Armillaria* spp. (e.g. *Armillaria ostoyae*), is a basidiomycete that is found within the soil (Lockman & Kearns, 2016). *Armillaria* spp. are able to infect conifer plantations rather quickly because the disease spreads through the roots and soil and attacks the cambium of the tree. This causes root and butt decay, leading to tree mortality (Lockman & Kearns, 2016; Van Der Kamp, 1995). If preventive treatment against *Armillaria* spp. establishment does not commence soon after thinning, the stand can become susceptible to *Armillaria* root rot. Currently there is no method available to stop the spread of *Armillaria* root rot should it establish after thinning. *Armillaria* root rot is also difficult to treat as the fungus can persist on infected stumps for more than 35 years (Goheen & Otrosina, 1998).

Research is ongoing to develop a commercially viable treatment option for this disease. The long-term efficacy of stump removal of infected trees has been studied. Cleary et al. (2013), for example, showed that stump removal for the prevention of *A. ostoyae* spread is only somewhat effective. Removing all roots is challenging and leaving even a few infected roots in the ground can result in the eventual reestablishment of the disease. There are other mechanical options to prevent the spread, but they too are ineffective at controlling root rot spread.

Various non-mechanical forest management options to control and limit the impact and spread of *Armillaria* root rot have also been proposed. A long-term management option could be to plant or favour more disease-tolerant species. This may involve planting trees that are not susceptible to *Armillaria* root rot, or harvesting trees that are prone to *Armillaria* root rot and leaving only the species that are tolerant to the disease (Lockman & Kearns, 2016). Another important management practice would be to limit the amount of stress trees have to deal with.

This would involve keeping the trees healthy and not wounding them during harvesting (Lockman & Kearns, 2016).

1.2.1. Biocontrols as a treatment option

Biocontrols are treatments against a disease or pathogen that use natural enemies of the infectious agent (Ministry of Forests, Lands and Natural Resource Operations, British Columbia, Canada). Biocontrols in forestry can help treat two types of pathogens: (1) insects, and (2) fungi. Biocontrols are becoming more common in disease and invasive species management due to public demand for environmentally friendly treatment alternatives. Biocontrols are considered to be less harmful to the environment than chemical treatments (Traquair, 1995). Biocontrols such as *Phlebiopsis gigantea* have been found to have little to no impact on the surrounding fungal community, thus making them a viable and environmentally friendly option to treat Annosus root rot caused by *Heterobasidion irregulare* (Terhonen et al. 2013). Biocontrols are beneficial because they have the ability to remain within an infected plant or root system for years, and therefore can continue to prevent root rot on a long term scale (Rönnberg & Cleary, 2012) . Moreover, biocontrols using fungi as the agents are some of the most cost-effective treatment strategies available for controlling root rot disease in plantations (Sivanandhan et al. 2017).

However, usage of biocontrols also has drawbacks. Introducing a biocontrol agent to the environment can be dangerous, as the biocontrol could end-up replacing an existing native species (e.g., Kang et al, 2017). Secondly, applying them can be challenging as they often require much more handling and care (e.g., need to be stored at certain temperatures before application, proper rates of coverage need to be met for effectiveness) when applying them compared to chemical treatments (Gerhardson, 2002). Other drawbacks include the shorter shelf life and the higher initial financial costs associated with biocontrol treatments relative to other

available options (Fravel, 2005). Thus, care must be taken when selecting appropriate biocontrol measures.

1.2.2.1 Effectiveness of *Hypholoma* spp. and *Phlebiopsis gigantea* as biocontrol agents

There are many different species and strains of basidiomycetes (e.g. *Hypholoma* spp.) found across North America. *Hypholoma fasciculare* and *Hypholoma capnoides* have shown promise via *in-vitro* studies (laboratory) to be possible biocontrol agents against *A. ostoyae* (Chapman & Xiao, 2000; Keča, 2009). Another *Hypholoma* species, *H. sublateritium*, has a transcontinental distribution and is commonly found in clumps on or around coniferous stumps (Parker, 1933). *Hypholoma sublateritium* has yet to be examined as a biocontrol agent against *A. ostoyae* through *in-vitro* or in field trails. The present study will focus on three different species of *Hypholoma*: *Hypholoma fasciculare, Hypholoma capnoides,* and *Hypholoma sublateritium. Hypholoma fasciculare* is found in similar environmental conditions as *H. sublateritium* and *H. capnoides*, usually on or around conifer wood stumps or logs (Parker, 1933). Thus, these *Hypholoma* species could function as ideal biocontrol candidates. The ability of these fungi to function as biocontrol agents have been tested mostly through *in-vitro* studies, with only a few examining their efficacy in field conditions. It has been demonstrated that *Hypholoma fasciculare* can survive on Douglas fir (*Pseudotsuga menziesii*) and Lodgepole pine (*Pinus contorta*) root systems. Growth of *Hypholoma fasciculare* in red pine underground root systems has not been tested in field conditions. Moreover, it is unclear if *Hypholoma fasciculare* can continue to grow and provide protection against *Armillaria* root rot during the winter months. Determining if *Hypholoma fasciculare* can survive and grow will set the groundwork for developing an effective biocontrol agent that would offer long term, year-round protection against *Armillaria* root rot.

Phlebiopsis gigantea is another species of basidiomycete that is present within coniferous forests, especially on sapwood of coniferous stumps (Hori et al. 2014). *Phlebiopsis gigantea* is currently used as a biocontrol agent (known as Rotstop C) against *Heterobasidion annosum* and *H. irregulare*, which cause Annosus root rot (Annesi et al. 2005; Berglund & Rönnberg, 2004; Dumas & Laflamme, 2013; Łakomy et al. 2014; Oliva et al. 2015). This study will examine *Phlebiopsis gigantea* (Rotstop C strain VRA 1992) as a biocontrol agent against *Armillaria ostoyae*. *Phlebiopsis gigantea* is a wood coloniser causing rapid breakdown of stumps and roots thereby capturing resources before *Armillaria* spp. Keča (2009) tested the efficacy of *Phlebiopsis gigantea in-vitro* as a biocontrol agent against *A. ostoyae* and found that it reduced the growth of *A. ostoyae* by 71 %. This suggests that there is potential for *P. gigantea* to be effective against *A. ostoyae.* However, further testing is required as current literature is limited.

1.2.3 Testing the efficacy of *Hypholoma* spp. as biocontrol agents in Simcoe County plantations

Simcoe County is where the field research was conducted as it is home to many forest plantations that consist of red pine (*Pinus resinosa*) and white pine (*P. strobus*). These plantations vary in age, from a couple years to more than 80 years old. There are over 13,400 hectares owned by the County of Simcoe that are designated as public land under Simcoe County Forests. The ongoing harvesting allows for a continuous flow of revenue that goes back into maintaining the forests and purchasing new land. The conifer plantations within the forests are impacted by root rot diseases. There is no data available to indicate since when *Armillaria* root rot first became a concern within this County. *Armillaria* root rot leads to a loss in revenue and tough management decisions. Mortality and tree decline due to *Armillaria* root rot forces the early removal of red pine trees from plantations to salvage the timber (G. Davis, personal

communication, 07/07/17). The plantations are thinned on a regular basis (approximately once in every 9 years) to increase the productivity and species diversity (Kariuki, 2008; Thomas et al. 1999; G. Davis, personal communication, 07/07/17). One of the main management objectives for these plantations is to transition them (ideally after 80-90+ years) into a mixedwood stand consisting of red and/or white pine with some deciduous species, or a deciduous stand consisting of various tree species. Simcoe County is a prime location for *Armillaria* root rot research because the disease exists throughout the county-owned and private land conifer plantations.

1.2.4 Objectives

This research addresses several knowledge gaps associated with *Armillaria* root rot. While *Hypholoma* spp. have been examined against *Armillaria* root rot in some areas of North America, there is uncertainty as to how *H. fasciculare* grows in Simcoe County climates, particularly if it remains dormant or can continue to grow during the winter months. This study will test different native fungi species as candidates to develop as biocontrol treatment options against *Armillaria* root rot through *in-vitro* and field examination. Thus, the objectives of my study are to:

1) *Determine if Hypholoma fasciculare, Hypholoma capnoides, Hypholoma sublateritium, and Phlebiopsis gigantea are capable of inhibiting and/or preventing the growth of Armillaria ostoyae – an in-vitro study*
2) *Determine whether winter soil temperatures in Simcoe County are suitable for the survival and growth of Hypholoma fasciculare – a field study*

Chapter 2: Efficacy of *Hypholoma fasciculare*, *Hypholoma sublateritium*, *Hypholoma capnoides*, and *Phlebiopsis gigantea* as biocontrol agents against *Armillaria ostoyae*.

2.1 Abstract

Armillaria root rot is caused by *Armillaria* spp. It impacts various tree species, especially red pine (*Pinus resinosa*), within plantations. Basidiomycetes (e.g., *Hypholoma* spp.) have shown promise as biocontrol agents that replace and thus reduce the growth of *Armillaria ostoyae*. Several strains of *Hypholoma fasciculare, H. sublateritium, H. capnoides,* and one strain of *Phlebiopsis gigantea* were grown in both solo (absence of competition) and paired conditions (competing with one of two strains of *A. ostoyae* -high virulence and low virulence) to examine impacts of competition on radial growth. All species and strains were grown on both 2% malt agar and pine wood infusion media at 15ºC. Radial growth was measured and compared between solo and paired conditions, and macroscopic and microscopic interactions were qualitatively assessed. *Hypholoma fasciculare* strain Pinnel B, *H. sublateritium* strain FP-90085-SP, and *H. sublateritium* strain HHB-11948-SP were identified as potential candidates to develop as biocontrol agents against *Armillaria* root rot, of which, *H. fasciculare* Pinnel B was the most promising. Radial growth was the highest from Pinnel B when paired with both *A. ostoyae* strains. Characterisation of the interactions at macroscopic and microscopic levels indicated that *H. fasciculare* strain Pinnel B was most effective at inhibiting *A. ostoyae* growth.

2.2 Introduction

Red pine (*Pinus resinosa*) and white pine (*Pinus strobus*) plantations are grown to naturally transform into mixedwood forests and provide a substantial economic return during this transition period (Bradford & Palik, 2009; Kariuki, 2008; McLaughlin, 2001). Root diseases caused by *Armillaria* spp. (*Armillaria* root rot) are impacting these red and white pine

plantations and are leading to tough management decisions for foresters from an ecological and economic standpoint (G. Davis, R.P.F., personal communication, 07/07/17). *Armillaria* root rot causes substantial tree mortality within plantations across North America. Currently there is no effective means to manage it, biocontrol or otherwise. Mechanical treatments such as ring-barking (Chapman & Schellenberg, 2015) and stump removal (Cleary et al. 2013), for example, have had limited success.

Treatment with biocontrol agents have shown more promise, with some trials suggesting that they may be the most effective treatment option for preventing *Armillaria* spp. establishment and spread within root systems (Chapman & Xiao, 2000; Hood et al. 2015; Keča, 2009). Biocontrol fungi such as *Hypholoma* spp., for example, have shown some success as a treatment method against *Armillaria* spp. (Chapman & Xiao, 2000; Chapman et al. 2004; Hood et al. 2015; Keča, 2009; Pearce, 1990). *Hypholoma* spp. are wood decaying cord forming basidiomycetes that are naturally present within forests on decaying coniferous stumps or logs (Baroni, 2017; Parker, 1933). *Hypholoma* spp. such as *Hypholoma fasciculare* and *Hypholoma capnoides* can reduce or eliminate the production of *Armillaria* spp. rhizomorphs, reduce *Armillaria* spp. growth, and can overgrow and replace *Armillaria* spp. colonies with hyphal tissue (Chapman & Xiao, 2000; Keča, 2009). Past studies have examined *in-vitro* competitive interactions between *Hypholoma* spp. and important root decay fungi such as *Armillaria* spp. and *Heterobasidion* spp. (Chapman & Xiao, 2000; Hood et al. 2015; Nicolotti et al. 1999). For example, Keča (2009) paired *Hypholoma fasciculare* and *H. capnoides* with five different species of *Armillaria*: *A. mellea, A. gallica, A. ostoyae, A. cepistipes,* and *A. tabescens*. It was found that *H. fasciculare* and *H. capnoides* were effective at suppressing *Armillaria* spp. growth. *Hypholoma sublateritium* is another species of *Hypholoma* that is common across North America and is

present on decaying logs or stumps (Parker, 1933). However, competitive interaction trials between *Hypholoma sublateritium* and *Armillaria ostoyae* have yet to be attempted. Thus, testing is needed to determine its effectiveness as a biocontrol agent against *Armillaria* spp. Moreover, while different *Hypholoma* species have been tested as biocontrol agents against *Armillaria* root rot (e.g., Chapman & Xiao, 2000; Cox & Scherm, 2006; Keča, 2009; Pearce, 1990), there have been no studies comparing different strains of one particular species of *Hypholoma* against *Armillaria* root rot. This can be important as Baldrian & Gabriel (2002) observed variation in growth rates among strains of the same fungus species while Bruce & Highley (1991) observed differences in responses among strains in competitive interaction trials. Thus, limiting examination of the competitive ability to only a single strain of a particular *Hypholoma* species may not accurately represent the competitive ability of that species as a whole. Therefore, multiple strains should be tested under identical conditions to understand whether there is variation in growth among *Hypholoma* spp. strains.

Phlebiopsis gigantea is currently an effective biocontrol species for the basidiomycete *Heterobasidion* spp. and is able to prevent the spread and establishment of this root and butt rot disease-causing fungi (Annesi et al. 2005; Dumas & Laflamme, 2013; Łakomy et al. 2014; Rönnberg & Cleary, 2012). Various strains of *Phlebiopsis gigantea* have been tested against *Heterobasidion irregulare* with positive results. Dumas and Laflamme (2013) noted that there was little variation between *P. gigantea* strains in terms of preventing *H. irregulare* establishment. Recently, *P. gigantea* strain VRA 1992 was isolated from Quebec, Canada, and is registered as a biocontrol against *H. irregulare* (Government of Canada, 2002). Previous literature has suggested that *Phlebiopsis gigantea* is a fungi that should be further tested as a biocontrol against *Armillaria* root rot (Hood et al. 2015; Keča, 2009). One reason for testing

Phlebiopsis gigantea against *Armillaria* spp. is that *P. gigantea* is already being used commercially to protect pine plantation trees against *Heterobasidion irregulare* and *H. annosum* and therefore it would be beneficial to know whether or not *P. gigantea* is effective against *Armillaria* spp. Very little research is available on how *P. gigantea* strain VRA 1992 interacts with *Armillaria* spp. such as *Armillaria ostoyae.* Keča (2009) does suggest that *P. gigantea* is ineffective at suppressing *Armillaria ostoyae* growth compared to other biocontrol agents such as *Hypholoma spp.* However, Keča (2009) did not indicate the specific strain of *Armillaria ostoyae* used. Further, the interactions were monitored at 22ºC, which is within the optimum temperature range for *A. ostoyae* growth, which may also explain why *P. gigantea* did not compete well. Schulze and Bahnweg (1998) demonstrated that there is a high degree of genetic variation among strains of *A. ostoyae*, which suggests that variation in response to competition may exist. Examining competitive interactions at a lower temperature for various *A. ostoyae* strains may lead to different conclusions than what Keča (2009) reported.

Most lab studies are conducted at room temperature (Chapman & Xiao, 2000; Keča, 2009). While room temperature (21ºC) is typically the optimum growth temperature for fungi such as *Armillaria* spp*., Heterobasidion* spp*., P. gigantea,* and *Hypholoma* spp. (Rishbeth, 1968, 1978), this does not accurately represent field conditions as these fungi would be exposed to soil conditions that change both daily and seasonally. An exception to testing at room temperature was completed by Oliva et al. (2015) wherein the ability of *P. gigantea* to compete with *H. annosum* at various temperatures (10, 20, and 25ºC) was examined. There have been no past studies that have examined *Armillaria* spp*., P. gigantea,* and *Hypholoma* spp. growth at 15ºC. Examining competitive interactions at 15ºC is important as it is believed to be a temperature that would represent field conditions (i.e. average annual soil temperature) within southern Ontario,

Canada. Examining temperatures outside of optimal growth temperatures may result in alternate findings compared to previous studies.

Lab studies typically use 1.5, 2, and 3 % malt extract agar artificial growth media for comparing competitive interactions and radial growth between fungi (Chapman & Xiao, 2000; Keča, 2009; Łakomy et al. 2014; Pearce, 1990). While these media may be the most suitable options for monitoring basidiomycete growth, they do not accurately represent field conditions. However, an effective, standardised medium consisting of natural ingredients sourced from their native habitats is currently insufficient. There have been no past studies that have examined a growth medium consisting of nutrients from red pine where these fungi would typically colonize and decay. Thus, a medium composed of a pine wood infusion could be more representative of field conditions and may provide different insight with respect to fungi competitive interactions and radial growth rates compared to an artificial media. Therefore, in this study a pine wood infusion medium was also used in addition to the common artificial growth medium mentioned above.

Previous research undertaking competition trials with pairings of *Armillaria* spp. and *Hypholoma* spp. have described how they grow and interact visually (macroscopic level) on petri plates, but very few examine growth and interactions at a microscopic level. Microscopic examination is important as there may be novel characteristics and/or mycelium and hyphal interactions not observable by the naked eye that could aid in understanding how two fungi such as *Hypholoma* spp. and *Armillaria* spp. grow and interact. For example, it was noted that the hyphae in some *Armillaria* spp. colonies became swollen at the point of interaction with *H. fasciculare* (Chapman & Xiao, 2000). This response did not occur without competition (solo growth) and could only be observed at a microscopic level. Cox & Scherm (2006) also observed

that *Armillaria tabescens* and *A. mellea* experienced swelling of the hyphae along the points of interaction when paired with *H. fasciculare*. Microscopic interactions may also assist in identifying signs of chemical activity produced by the fungi (Boddy, 2000; Rayner & Boddy, 1988). Microscopic examination of the *Hypholoma* spp. x *Armillaria ostoyae* as well the *P. gigantea* x *Armillaria ostoyae* interactions for responses not observable at the macroscopic level is knowledge that is currently insufficient, and further research is needed in this direction to help determine if *Hypholoma* spp. and/or *P. gigantea* can act as effective biocontrol agents against *A. ostoyae*.

Using macroscopic and microscopic tools are important avenues to improve understanding of radial growth and competitive interactions between fungi. There currently is no standardised ranking system in place that classifies the relative strength of a biocontrol. Keča (2009) does describe the ability of *Hypholoma* spp. to reduce the growth of *Armillaria* as the percentage of growth in the presence of competition relative to growth in its absence. However, there were no other *Hypholoma* spp. strains used to provide a direct comparison in his study, which would provide some measure of relative effectiveness against *Armillaria* root rot. Developing a simple ranking system (e.g., strong, mid-grade, or poor) that could be universally applied would expediate the process of finding suitable biocontrols against *Armillaria* root rot.

Armillaria root rot is highly detrimental to the forestry industry across North America (Lachance, 1996; Williams et al. 1986). There have been multiple attempts to find effective treatment options for *Armillaria* root rot, with biocontrols appearing to be the most promising to date (Chapman & Xiao, 2000; Chapman et al. 2004; Hood et al. 2015; Keča, 2009; Pearce, 1990). Previous studies identified *Hypholoma fasciculare* and *H. capnoides* as potential biocontrol candidates against *A. ostoyae* (Chapman & Xiao, 2000; Chapman et al. 2004; Keča,

2009). However, further testing is required to understand how different strains of *H. fasciculare* and *H. capnoides* act against *Armillaria*. Baldrian & Gabriel (2002) and Bruce & Highley (1991) described how different strains of fungi vary in terms of growth rates and competitive interactions. Further examination is needed to understand how various strains within a *Hypholoma* spp. react to various *Armillaria ostoyae* strains at both macroscopic and microscopic levels. Currently there is no information available on how various strains of *Hypholoma sublateritium* compete with *A. ostoyae.* Moreover, there is little understanding as to how *A. ostoyae, Hypholoma* spp., and *P. gigantea* interact and grow at sub-optimal temperatures that are naturally observed in forest plantation soils of southern Ontario.

The objective of this chapter is to examine a suite of strains from *H. fasciculare, H. capnoides, H. sublateritium*, and *P. gigantea* on artificial and natural growth media to determine if variability exists among strains of the same species with respect to their ability to reduce or prevent spreading of *Armillaria ostoyae.* It is hypothesized that strains of *H. fasciculare* will be more effective biocontrol agents against *A. ostoyae* than those of *P. gigantea* and other *Hypholoma* species because *H. fasciculare* have been shown to more effectively (1) reduce the growth of *Armillaria* spp., (2) reduce or eliminate rhizomorph production from *Armillaria* spp., and (3) overgrow *Armillaria* colonies on petri plates (Chapman & Xiao, 2000; Hood et al., 2015; Keča, 2009). It was also hypothesized that the pine wood infusion medium would result in higher radial growth due to the medium containing natural ingredients that increase foraging ability (Dowson et al. 1989).

2.3 Methods

This study involved isolating and maintaining cultures of *H. fasciculare* (8 strains), *H. sublateritium* (5 strains), *H. capnoides* (4 strains), *P. gigantea* (VRA 1992), and *A. ostoyae* (2

strains) (Table 1.). Lab experiments involved monitoring and comparing growth rates of each fungi and examining species interactions on standard petri plates (100 x 15 mm).

Table 1. Fungal species, strains, region, and country of original collection all under examination for *in-vitro* studies. This collection was obtained from BioForest Technologies Inc. and the United States Forest Service.

Species	Strain	Region	Country
Hypholoma fasciculare	OKM-2932-T	Idaho	USA
Hypholoma fasciculare	OKM-7107-Sp	Maryland	USA
Hypholoma fasciculare	RLG-11562-Sp	Arizona	USA
Hypholoma fasciculare	RLG-12668-Sp	Arizona	USA
Hypholoma fasciculare	HHB-14801-Sp	Washington	USA
Hypholoma fasciculare	JPL-62-Sp	Arizona	USA
Hypholoma fasciculare	Pinnel B	British Columbia	Canada
Hypholoma fasciculare	FP-133566-Sp	Oregon	USA
Hypholoma sublateritium	OKM-6192-Sp	Virginia	USA
Hypholoma sublateritium	HHB-11948-Sp	Michigan	USA
Hypholoma sublateritium	49-1107	New Hampshire	USA
Hypholoma sublateritium	FP-90085-Sp	New York	USA
Hypholoma sublateritium	OKM-6947-Sp	Maryland	USA
Hypholoma capnoides	OKM-1523-T	Idaho	USA
Hypholoma capnoides	TAK2	Ontario	Canada
Hypholoma capnoides	TAK5	Ontario	Canada
Hypholoma capnoides	TAK6	Ontario	Canada
Hypholoma capnoides	SSM	Ontario	Canada
Armillaria ostoyae	B249-28	Ontario	Canada
Armillaria ostoyae	P162-7	Ontario	Canada
Phlebiopsis gigantea	VRA 1992	Quebec	Canada

2.3.1 Solo growth (no competition)

Solo growth consisted of examining the radial growth (growth protruding from the inoculum plug) of the various strains of *H. fasciculare*, *H. sublateritium*, and *H. capnoides*, along with *P. gigantea*, and *A. ostoyae.* The growth was performed on two types of media: 2% malt agar (2 % MA) which was an artificial media and pine-wood infusion (PWI) medium which was made from red pine wood chips with the addition of sucrose and was determined to be more aligned with natural growth conditions. Plates were poured with approximately 20 ml of media per plate. Plates were inoculated with 7 mm plugs on the edge of the plates and incubated at 15^{o}C in the dark (mean annual soil temperature in Simcoe County). Three replicates of each species and strains were used (Oliva et al. 2015) and three measurements of radial growth of mycelium were taken per plate at a 24-hour interval starting from day 5 (to allow for an establishment period) to day 30 for the PWI medium and to day 36 for 2 % MA medium. This study ended when radial growth of mycelium of the first *Hypholoma* spp. reached the opposite end of the plate. This study was completed to compare/contrast the solo growth of these species/strains relative to growth when paired with each *A. ostoyae* strain. This would help to determine if radial growth of the biocontrol agents (growth before interacting with *A. ostoyae* in the same petri plates) differed when competing with *A. ostoyae* for resources. Comparing radial growth between the biocontrol species/strains will also help to determine which biocontrol agent(s) can quickly interact with *A. ostoyae*.

2.3.2 *Hypholoma* spp. and *P. gigantea* paired with *A. ostoyae* (competitive interactions)

Various strains of *H. fasciculare, H. sublateritium, H. capnoides*, and *P. gigantea* were paired with *A. ostoyae* strains to assess competitive interactions (macroscopic and microscopic) and radial growth. Pairings were performed on 2 % MA medium + ETOH and PWI medium +

ETOH on plates (Chapman & Xiao, 2000) with approximately 20 ml of media per plate. Ethanol was added to the growth medium to stimulate *A. ostoyae* rhizomorph production (Chapman & Xiao, 2000). The ethanol concentration in both media was set at 3 ml/L, as initial testing concluded that 3 ml/L is a concentration that allows *A. ostoyae* rhizomorph production with little to no impact on *Hypholoma* spp. radial growth of mycelium. Two strains of *A. ostoyae* were used for this pairing study, a high virulence/high rhizomorph production (B249-28) and a low virulence/low rhizomorph production (P162-7) strain, both of which were isolated from Simcoe County Forests, Ontario, Canada. Similar to Chapman and Xiao (2000), the *A. ostoyae* strains were plated weeks before the *Hypholoma* spp. and *P. gigantea*. Chapman and Xiao (2000) and Keča (2009) plated the *Hypholoma* spp. after two weeks of *A. ostoyae* growth. This was to account for the slow growth rate and establishment of *A. ostoyae* compared to *Hypholoma* spp. and *P. gigantea*. This delay allowed for measuring of radial growth from both the biocontrol agents and the *Armillaria* strains at the same time. Due to the lower incubation temperature of 15ºC used in this study, *A. ostoyae* needed more time to establish on the plates. Therefore, the *Hypholoma* spp. were plated on the same plates after three weeks of *A. ostoyae* growth. Three replicates were used for each species pair, along with ten *A. ostoyae* control plates (solo growth), five plates per *A. ostoyae* strain. Six measurements each were taken per plate (three total measurements per species) on a 24-hour interval. Similar to Keča (2009), the *A. ostoyae* strains were plated 3 cm from the edge of the plate, and the *Hypholoma* spp. and *P. gigantea* were plated 2 cm from the opposing side of the same plate. This allowed sufficient space for both species to establish before interacting in the middle of the plate. The radial growth of the *A. ostoyae* and the biocontrol species was monitored and measured for 45 days. Radial growth of the species pairs (under competition) was compared to the solo growth (no competition) on day

13 to examine how the presence of *A. ostoyae* impacted the growth of the *Hypholoma* spp. and *P. gigantea*. Day 13 was chosen to compare/contrast solo and paired growth conditions (prior to physical interaction) because this is one of the days when all paired species were growing well; nb., a later date would have resulted in the *A. ostoyae* and biocontrol agents physically interacting, leading to inaccurate radial growth measurements.

Along with radial growth, the physical interactions between *A. ostoyae* and the biocontrol agents were described and recorded at the macroscopic level. This was completed on day 65 after the final radial growth measurements were taken. This also provided sufficient time for both fungus species to interact. Physical interactions between *A. ostoyae* and the biocontrol species were described as per Porter (1924). Fungal interactions were first described by Porter (1924) and have become the standard for evaluating and explaining *in-vitro* interactions between fungi. Porter (1924) describes five different types of growth inhibition (Figure 1). Each biocontrol strain within each species was classified into three competitor types (Appendix A): (1) strong competitor - achieves the highest radial growth, with the biocontrol overgrowing the *A. ostoyae* colony, (2) midgrade competitor - moderate radial growth, with the biocontrol only surrounding the *A. ostoyae* colony, and (3) poor competitor - low radial growth, with the biocontrol exhibiting little to no macroscopic interaction with the *A. ostoyae* colony.

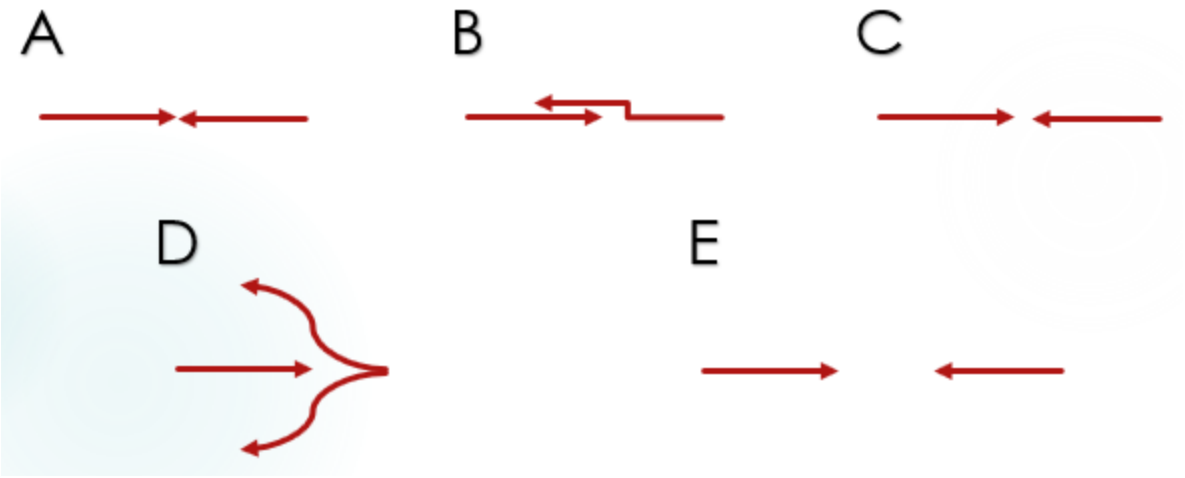

Figure 1. The five different macroscopic interactions similar to Porter (1923). (A) Mutually intermingling of both species; (B) Growth superficial over the contending organism; (C) Slight inhibition between the two species (1-4mm); (D) Growth around the contending organism, one fungi surround's the other; and (E) Mutual inhibition at a considerable distance (<5mm).

Microscopic interactions between the hyphae of *A. ostoyae* and the biocontrol agents were examined to further understand how they interact. Due to insufficient timing, *H. capnoides* SSM and *H. fasciculare* RLG-11562-SP were not included in the microscopic assessments. All other pairings were examined for their interactions. Boddy (2000) noted that microscopic examination helps to identify if there are volatile and diffusible chemicals being produced by fungi due to interactions with other species. Microscopic examination allowed viewing of any apparent swelling or bursting of the hyphae tips for the *A. ostoyae* and biocontrol agent. Cox and Scherm (2006) noted the occurrence of hyphal bursting, tip bursting, granulation, and swelling between *A. ostoyae* and other biocontrol candidates at a microscopic level. These characteristics were noted for the solo and paired interactions between the biocontrol agents and *A. ostoyae*.

Small samples of mycelium were taken from each of the solo growth cultures and from the interaction zone of the paired cultures and placed onto a glass slide along with a small drop of cotton blue dye, which allowed for easier viewing of the hyphae (John McLaughlin,

BioForest; Sylvia Greifenhagen, MNRF). The slides were viewed under an inverted microscope at 200x and 400x. Descriptions and images were taken of the hyphae appearance in both solo and paired conditions (Cox and Scherm, 2006). Hyphae characteristics/morphological responses at the growing apical tips when grown with and without competition with *A. ostoyae* were compared and contrasted.

2.3.3 Statistical Analysis

The influence of media type and the impact of presence/absence of competition with *A. ostoyae* on the radial growth of different species and strains of *Hypholoma* and *P. gigantea* was analyzed using factorial ANOVA. A post hoc test (Tukey's) was carried out to compare hyphal growth with media type, strain, and ± competition. Levene's test was run to check for homogeneity within all residuals while an Anderson Darling test was carried out to check for normality within the residuals. Macroscopic (visual) and microscopic interactions were not statistically analyzed. All statistical tests were run using R studio version 1.1.383.

2.4 Results

2.4.1 *Hypholoma* spp. and *P. gigantea* - solo growth and paired growth with *A. ostoyae*

2.4.1.1 *Hypholoma fasciculare* paired with *A. ostoyae*

Growth rates were significantly higher on the PWI medium compared to the 2 % MA ($F_{1,96} = 161.80$, $p < 0.001$). Depending on strain, there was a significant increase or decrease in *H. fasciculare* growth in the presence of competition with *A. ostoyae* strains ($F_{14,96} = 14.38$, $p < 0.001$). Growth between each *H. fasciculare* strain was significantly different ($F_{7,96} = 590.64$, $p < 0.001$). Growth of each *H. fasciculare* strain was significantly impacted by the growth medium ($F_{7,96} = 20.79$, $p < 0.001$) (Figures 2 & 3). A post hoc test (Tukey's) showed there were

significant differences in the radial growth of *H. fasciculare* strains when under solo versus paired growth conditions. There was a significant decrease in *H. fasciculare* growth when competing with *A. ostoyae* on 2 % MA (Figure 2) for the following pairs: Pinnel B solo & B249-28 paired ($p = 0.01$), Pinnel B solo & P162-7 paired ($p = 0.001$), HHB-14801-SP solo & B249-28 paired ($p = 0.01$), JPL-62-SP solo & B249-28 paired ($p < 0.001$), JPL-62-SP solo & P162-7 paired ($p < 0.001$), OKM-7107-SP solo & B249-28 paired ($p = 0.004$), OKM-7107-SP solo & P162-7 paired ($p = 0.01$), and a significant increase between RLG-12668-SP solo & P162-7 paired ($p = 0.04$) (Figure 2). There was a significant decrease in *H. fasciculare* growth when competing with *A. ostoyae* on PWI medium for the following pairs: FP-133566-SP solo & B249-28 paired ($p < 0.001$), FP-133566-SP solo & P162-7 paired ($p < 0.001$), HHB-14801-SP solo & B249-28 paired ($p < 0.001$), HHB-14801-SP solo & P162-7 paired ($p < 0.001$), JPL-62-SP solo & B249-28 paired ($p < 0.001$), JPL-62-SP solo & P162-7 paired ($p < 0.001$), OKM-2932-T solo & B249-28 paired ($p < 0.001$), OKM-2932-T solo & P162-7 paired ($p < 0.001$), OKM-7107-SP solo & B249-28 paired ($p < 0.001$), OKM-7107-SP solo & P162-7 paired ($p < 0.001$), Pinnel B solo & B249-28 paired ($p < 0.001$), Pinnel B solo & P162-7 paired ($p < 0.001$), and a significant increase between RLG-11562-SP solo & B249-28 paired ($p < 0.001$) and RLG-11562-SP solo & P162-7 paired ($p < 0.001$) (Figure 3).

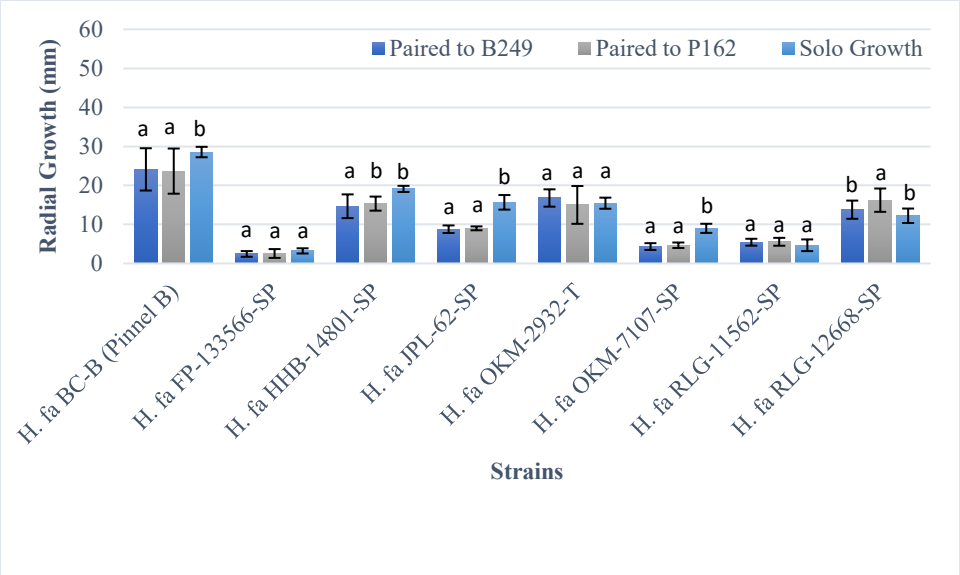

Figure 2. Mean radial growth of different *Hypholoma fasciculare* strains (2 % MA medium) on day 13 with and without competition with *A. ostoyae* strain B249-28 or P162-7. Black bars indicate standard error of the mean. For each strain, letters above bars that differ indicate significant differences, matching letters indicate no significant difference in radial growth.

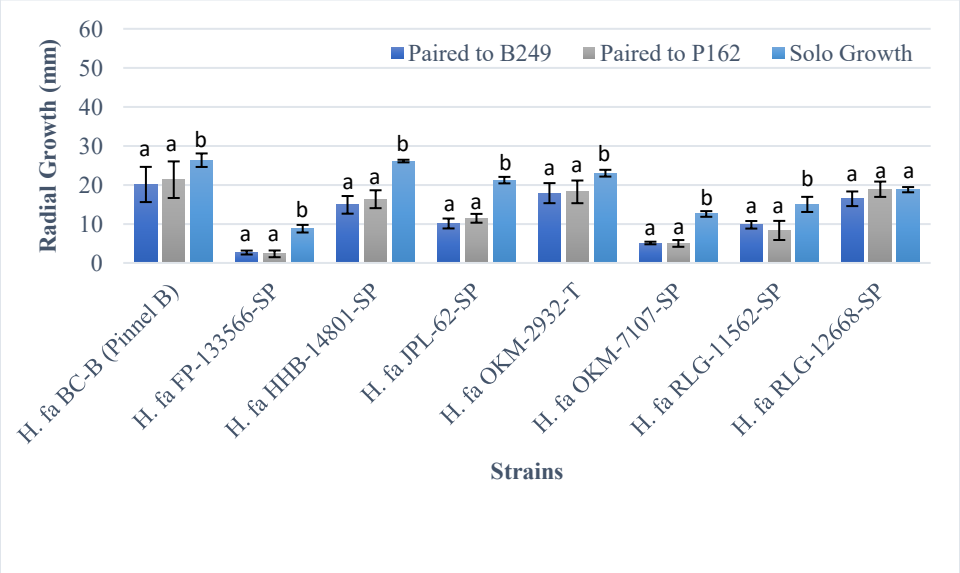

Figure 3. Mean radial growth of different *Hypholoma fasciculare* strains (PWI medium) on day 13 with and without competition with *A. ostoyae* strain B249-28 or P162-7. Black bars indicate standard error of the mean. For each strain, letters above bars that differ indicate significant differences, matching letters indicate no significant difference in radial growth.

2.4.1.2 *Hypholoma sublateritium* paired with *A. ostoyae*

Hypholoma sublateritium strains growing on PWI medium showed significantly higher radial growths compared to growing on 2 % MA ($F_{1,60} = 255.30$, $p < 0.001$). There was a significant difference in growth among the different *H. sublateritium* strains ($F_{4,60} = 88.10$, $p < 0.001$). Depending on strain, there was a significant increase or decrease in *H. sublateritium* growth in the presence of competition with *A. ostoyae* strains B249-28 & P162-7 ($F_{8,60} = 16.58$, $p < 0.001$). A post hoc test (Tukey's) showed there was significant differences in growth between the *H. sublateritium* strains solo growth and paired growth. There was a significant increase in *H. sublateritium* growth when competing with *A. ostoyae* on 2 % MA for: 49-1107 solo & P162-7 ($p = 0.007$), HHB-11948-SP solo & B249-28 ($p < 0.001$), HHB-11948-SP solo & P162-7 ($p < 0.001$) (Figure 4). There was a significant decrease in *H. sublateritium* growth when competing with *A. ostoyae* on PWI medium for: FP-90085-SP solo & B249-28 paired ($p < 0.001$), FP-90085-SP solo & P162-7 paired ($p < 0.001$), OKM-6192-SP solo & B249-28 paired ($p < 0.001$), OKM-6192-SP solo & P162-7 paired ($p < 0.001$) (Figure 5). *Hypholoma sublateritium* strain 49-1107 was the only strain that had a significant increase in radial growth when paired with B249-28 *A. ostoyae* ($p < 0.001$).

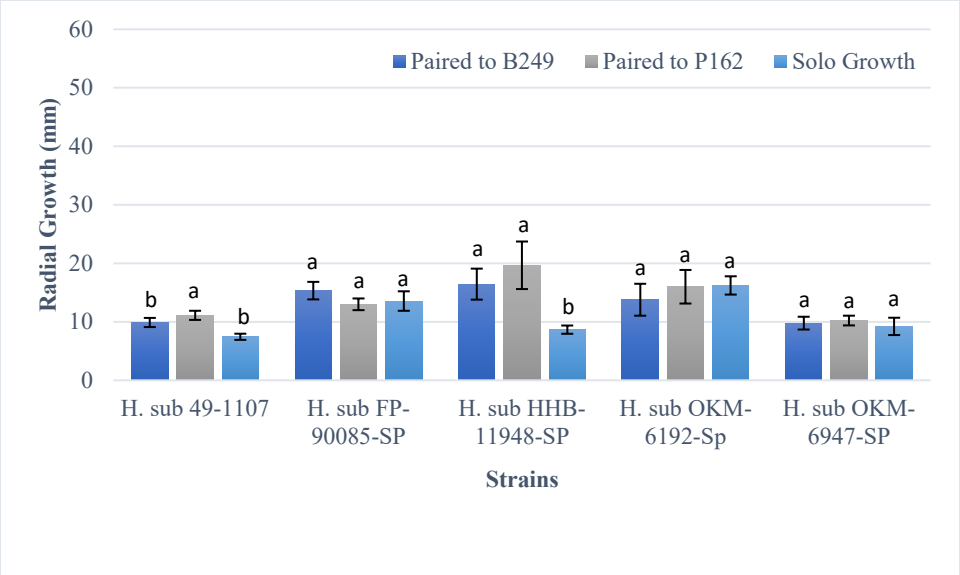

Figure 4. Mean radial growth of different *Hypholoma sublateritium* strains (2 % MA medium) on day 13 with and without competition with *A. ostoyae* strain B249-28 or P162-7. Black bars indicate standard error of the mean. For each strain, letters above bars that differ indicate significant differences, matching letters indicate no significant difference in radial growth.

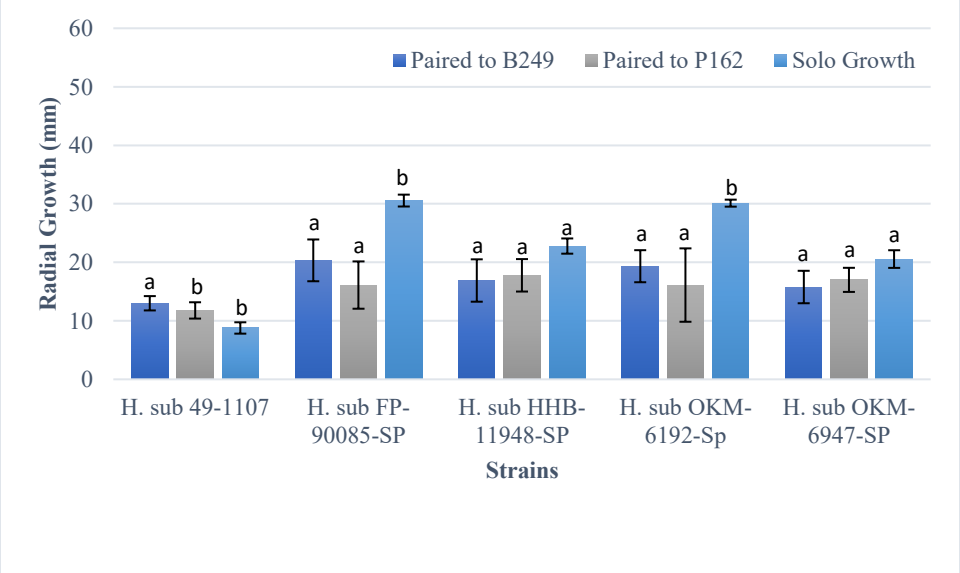

Figure 5. Mean radial growth of different *Hypholoma sublateritium* strains (PWI medium) on day 13 with and without competition with *A. ostoyae* strain B249-28 or P162-7. Black bars indicate standard error of the mean. For each strain, letters above bars that differ indicate significant differences, matching letters indicate no significant difference in radial growth.

2.4.1.3 *Hypholoma capnoides* paired with *A. ostoyae*

Growth with *H. capnoides* strains when paired against *A. ostoyae* strains was significantly higher on the PWI medium than on 2 % MA medium ($F_{1,60} = 127.23$, $p < 0.001$). There was a significant decrease in *Hypholoma capnoides* growth when competing with both *A. ostoyae* strains ($F_{8,60} = 15.01$, $p < 0.001$). There were significant differences in growth between the *H. capnoides* strains ($F_{4,60} = 27.15$, $p < 0.001$). A post hoc test (Tukey's) found there was no significant difference in radial growth with or without competition with *A. ostoyae* on 2 % MA (Figure 6). There was a significant decrease in *Hypholoma capnoides* growth when competing with *A. ostoyae* on the PWI medium for: OKM-1523-T solo & P162-7 paired ($p = 0.014$), TAK 2 solo & B249-28 paired ($p < 0.001$), TAK 2 solo & P162-7 paired ($p < 0.001$), TAK 5 solo & B249-28 paired ($p = 0.017$), and TAK 5 solo & P162-7 paired ($p < 0.001$) (Figure 7).

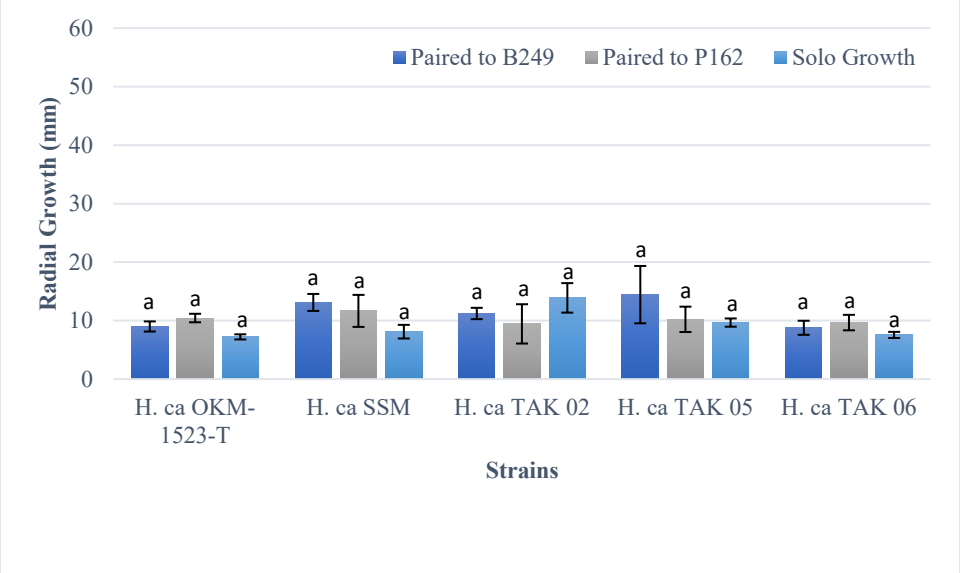

Figure 6. Mean radial growth of different *Hypholoma capnoides* strains (2 % MA medium) on day 13 with and without competition with *A. ostoyae* strain B249-28 or P162-7. Black bars indicate standard error of the mean. For each strain, letters above bars that differ indicate significant differences, matching letters indicate no significant difference in radial growth.

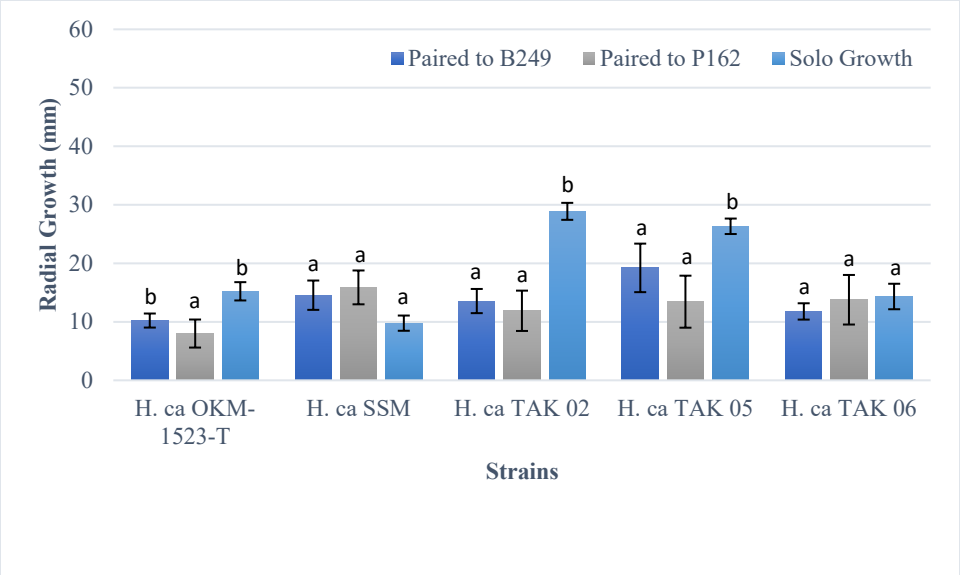

Figure 7. Mean radial growth of different *Hypholoma capnoides* strains (PWI medium) on day 13 with and without competition with *A. ostoyae* strain B249-28 or P162-7. Black bars indicate standard error of the mean. For each strain, letters above bars that differ indicate significant differences, matching letters indicate no significant difference in radial growth.

2.4.1.4 *Phlebiopsis gigantea* paired with *A. ostoyae*

There was a significant decrease in *Phlebiopsis gigantea* growth when competing with *A. ostoyae* ($F_{2,12} = 57.23$, $p < 0.001$). There were no differences in *P. gigantea* radial growth rates between 2 % MA and PWI growth media (Figure 8) ($F_{1,12} = 0.20$, $p = 0.65$). The 2 % MA and PWI growth media did not influence the solo and paired growth of *P. gigantea* with the *A. ostoyae* strains ($F_{2,12} = 0.74$, $p = 0.49$).

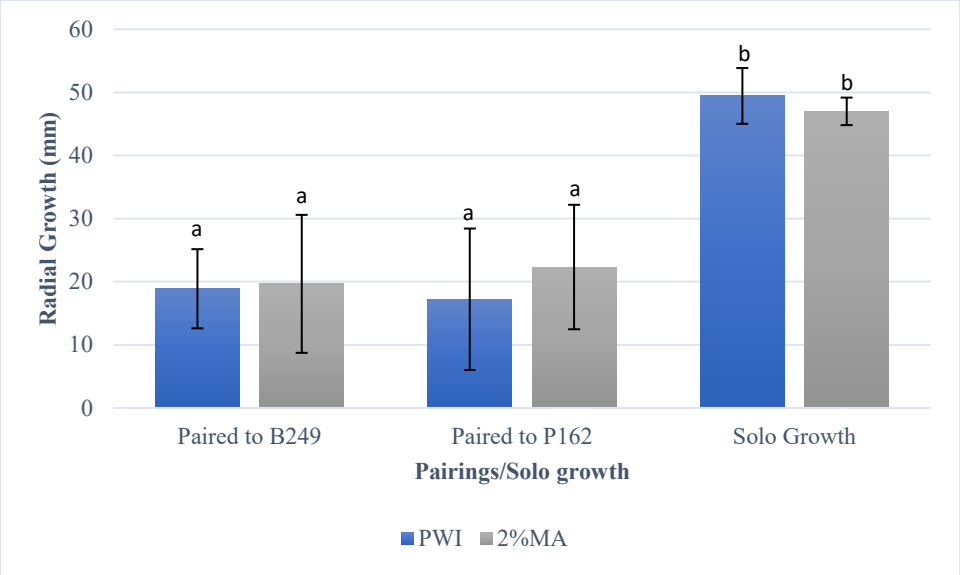

Figure 8. Mean radial growth of *Phlebiopsis gigantea* (PWI and 2 % MA medium) on day 13 with and without competition with *A. ostoyae* strain B249-28 or P162-7. Black bars indicate standard error of the mean. For each strain, letters above bars that differ indicate significant differences, matching letters indicate no significant difference in radial growth.

2.4.2 Macroscopic interactions between *Hypholoma* spp. and *A. ostoyae*

2.4.2.1 *Hypholoma fasciculare* paired with *A. ostoyae*

Each of the *H. fasciculare* strains had different types of interactions with the two strains of *A. ostoyae* (Figure 9). Four of eight strains of *H. fasciculare* were identified as strong competitors, one as midgrade, and three as poor competitors against *A. ostoyae* (Table 2). *Hypholoma fasciculare* strains HHB-14801-SP, Pinnel B, OKM-2932-T, and RLG-12668-SP were able to overgrow *A. ostoyae* strains B249-28 and P162-7 on both growth media. These *H. fasciculare* strains were all determined to be strong competitors towards *A. ostoyae*. *Hypholoma fasciculare* strains JPL-62-SP and RLG-11562-SP were able to grow around and surround both *A. ostoyae* strains. *Hypholoma fasiculare* strain JPL-62-SP was determined to be a midgrade competitor while RLG-11562-SP was considered to be a poor competitor. *Hypholoma fasciculare* strain OKM-7107-SP was identified as a poor competitor with *A. ostoyae* despite it

having multiple macroscopic interactions with *A. ostoyae* strains B249-28 and P162-7. There was a slight inhibition between the two fungi on PWI medium whereas on 2 % MA there was mutual intermingling with P162-7 and growth surrounding the B249-28 strain of *A. ostoyae*. *Hypholoma fasciculare* strain FP-133566-SP was determined to be a poor competitor towards *A. ostoyae* as it was intermingling with the *A. ostoyae* for most interactions.

Table 2. *Hypholoma fasciculare* growth interactions with *A. ostoyae* strains B249-28 and P162-7 on both media (2 % MA and PWI). (A) Mutual intermingling of both species (1-4 mm); (B) Growth superficial over the contending organism; (C) Slight inhibition; (D) Growth around the contending organism; (E) Mutual inhibition at a considerable distance (>5 mm).

***Hypholoma fasciculare* strains**	**P162-7 (2 % MA)**	**B249-28 (2 % MA)**	**P162-7 (PWI)**	**B249-28 (PWI)**
Pinnel B	B	B	B	B
FP-133566-SP	A	A	D	A
HHB-14801-SP	B	B	B	B
JPL-62-SP	D	D	D	D
OKM-2932-T	B	B	B	B
OKM-7107-SP	A	D	C	C
RLG-11562-SP	D	D	D	D
RLG-12668-SP	B	B	B	B

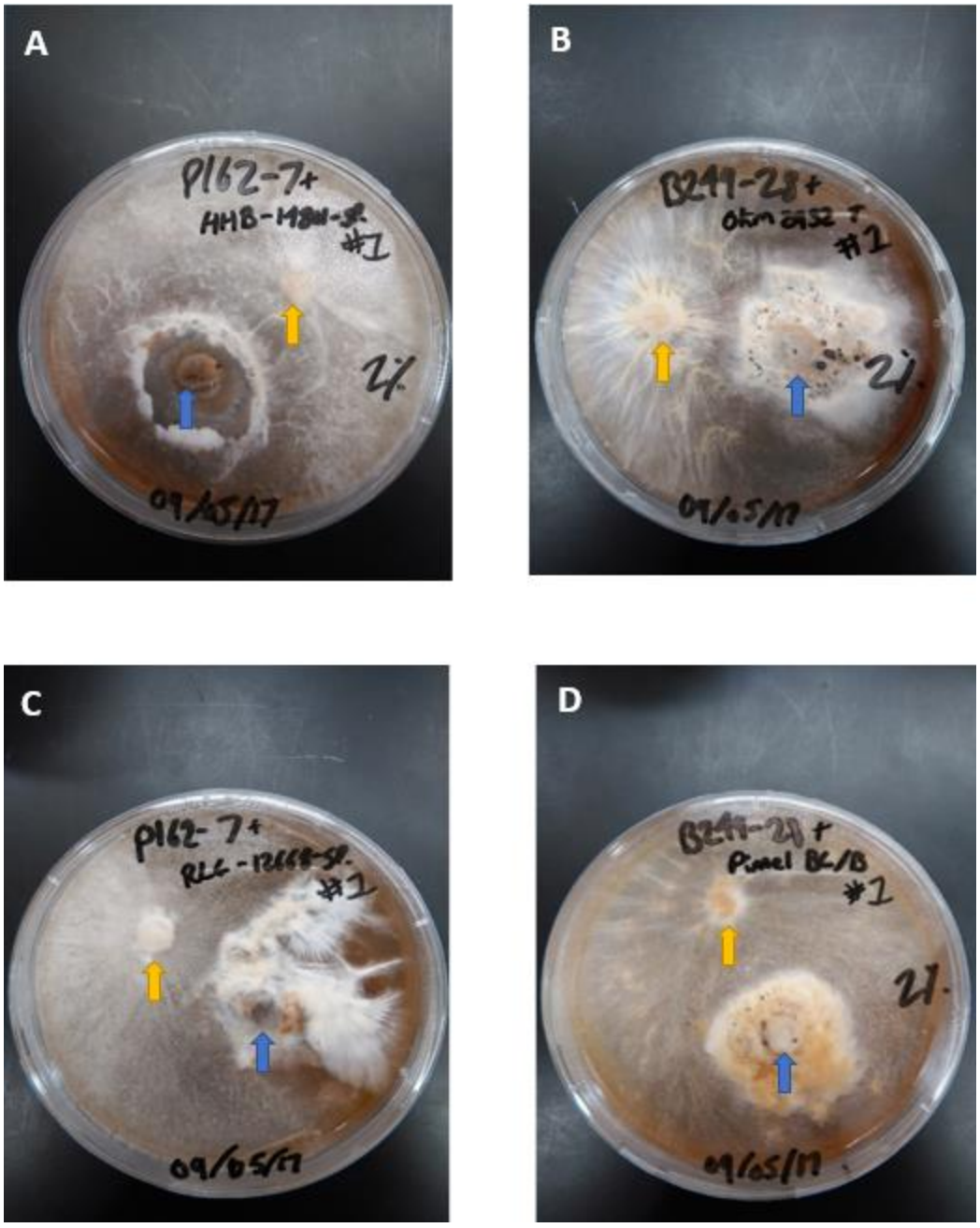

Figure 9. Photos taken from day 65 of *Hypholoma fasciculare* strains overgrowing *Armillaria ostoyae* colonies. (A) *H. fasciculare* strain HHB-14801-SP & *A. ostoyae* strain P162-7 (B) *H. fasciculare* strain OKM-2932-T & *A. ostoyae* strain B249-28 (C) *H. fasciculare* strain RLG-12668-SP & *A. ostoyae* strain P162-7, and (D) *H. fasciculare* strain Pinnel B & *A. ostoyae* strain B249-28. Yellow arrows indicate *H. fasciculare* strains and blue arrows indicate *A. ostoyae*.

2.4.2.2 *Hypholoma sublateritium* paired with *A. ostoyae*

Hypholoma sublateritium was a strong competitor species against *A. ostoyae* as three of five strains were strong competitors and two of five were midgrade competitors. There were no poor competitor strains of *H. sublateritium* (Table 3 & Figure 10). *Hypholoma sublateritium* strain HHB-11948-SP was a strong competitor against both *A. ostoyae* strains B249-28 and P162-7 as it overgrew the colonies of *A. ostoyae*. *Hypholoma sublateritium* strains FP-90085-SP, OKM-6192-SP, and OKM-6947-SP were all strong competitors and could overgrow the colonies of both *A. ostoyae* strains B249-28 and P162-7, but only on the 2 % MA medium. On the PWI medium, the *H. sublateritium* strains were growing around the *A. ostoyae* colonies. *Hypholoma sublateritium* strain 49-1107 was a midgrade competitor toward the *A. ostoyae* as it grew around and contained the *A. ostoyae* strains on both media.

Table 3. *Hypholoma sublateritium* growth interactions against *A. ostoyae* strains B249-28 and P162-7 on both media (2 % MA and PWI). (A) Mutual intermingling of both species (1-4 mm); (B) Growth superficial over the contending organism; (C) Slight inhibition; (D) Growth around the contending organism; (E) Mutual inhibition at a considerable distance (>5 mm).

***Hypholoma sublateritium* strains**	**P162-7 (2 % MA)**	**B249-28 (2 % MA)**	**P162-7 (PWI)**	**B249-28 (PWI)**
49-1107	D	D	D	D
FP-90085-SP	B	B	D	D
HHB-11948-SP	B	B	B	B
OKM-6192-Sp	B	B	D	D
OKM-6947-SP	B	B	D	D

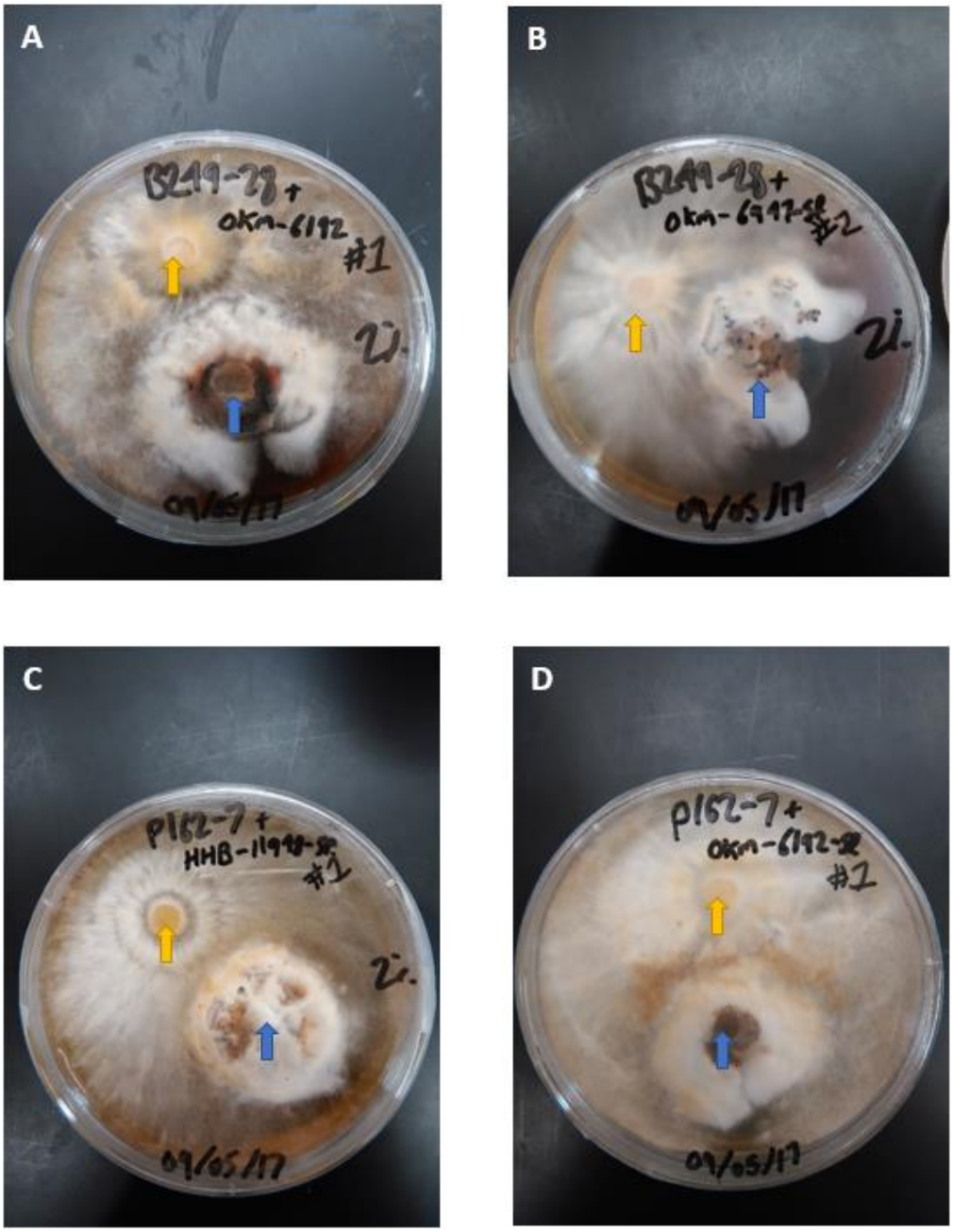

Figure 10. Photos taken from day 65 of *Hypholoma sublateritium* strains overgrowing *Armillaria ostoyae* colonies. (A) *H. sublateritium* strain OKM-6192-SP & *A. ostoyae* strain B249-28, (B) *H. sublateritium* strain OKM-6947-SP & *A. ostoyae* strain B249-28, (C) *H. sublateritium* strain HHB-11948-SP & *A. ostoyae* strain P162-7, (D) *H. sublateritium* strain OKM-61920SP & *A. ostoyae* strain P162-7. Yellow arrows indicate *H. sublateritium* strains and blue arrows indicate *A. ostoyae*.

2.4.2.3 *Hypholoma capnoides* paired with *A. ostoyae*

Hypholoma capnoides was identified as a poor to midgrade competitor with *A. ostoyae* (Table 4 & Figure 11). *Hypholoma capnoides* strain TAK 5 was a midgrade competitor as it could overgrow *A. ostoyae* strain P162-7 on the PWI medium. However, against *A. ostoyae* strain B249-28 on PWI and *A. ostoyae* strains B249-28 & P162-7 on the 2 % MA, *H. capnoides* only grew around the *A. ostoyae* colonies. *Hypholoma capnoides* strains TAK 2 and TAK 6 were poor competitors as they could grow around the B249-28 and P162-7 *A. ostoyae* colonies only partially, and not fully surround the *A. ostoyae* colony. *Hypholoma capnoides* strain SSM was a midgrade competitor as it could surround the *A. ostoyae* colony. The most variable strain was *H. capnoides* strain OKM-1523-T which was a poor competitor. This strain, when competing with *A. ostoyae* strain B249-28, the species were intermingling on both the PWI and 2 % MA media whereas with *A. ostoyae* strain P162-7 showed growth around the *A. ostoyae* colony on the PWI medium and was experiencing a slight inhibition on 2 % MA.

Table 4. *Hypholoma capnoides* growth interactions against *A. ostoyae* strains B249-28 and P162-7 on both media (2 % MA and PWI). (A) Mutual intermingling of both species (1-4 mm); (B) Growth superficial over the contending organism; (C) Slight inhibition; (D) Growth around the contending organism; (E) Mutual inhibition at a considerable distance (>5 mm).

***Hypholoma capnoides* strains**	**P162-7 (2 % MA)**	**B249-28 (2 % MA)**	**P162-7 (PWI)**	**B249-28 (PWI)**
OKM-1523-T	C	A	D	A
SSM	D	D	D	D
TAK 2	D	D	D	D
TAK 5	D	D	B	D
TAK 6	D	D	D	D

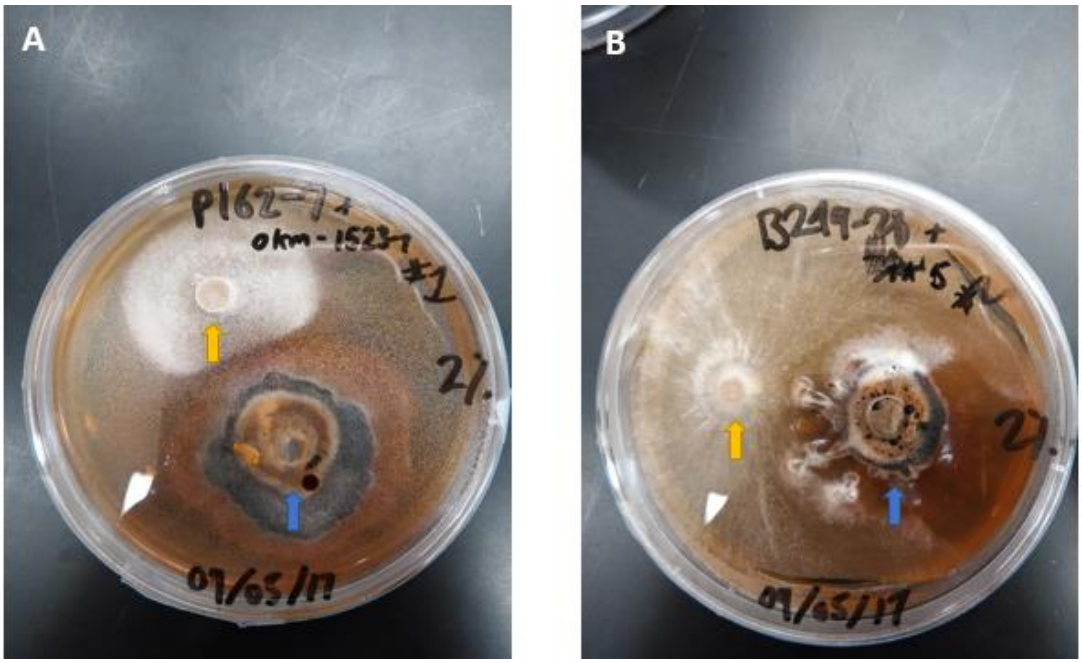

Figure 11. Photos taken from day 65 of *Hypholoma capnoides* strains intermingling and surrounding *A. ostoyae* colonies. (A) *H. capnoides* strain OKM-1523-T & *A. ostoyae* strain P162-7, (B) *H. capnoides* strain TAK 5 & *A. ostoyae* strain B249-28. Yellow arrows indicate *H. capnoides* strains and blue arrows indicate *A. ostoyae*.

2.4.2.4 *Phlebiopsis gigantea* paired with *A. ostoyae*

Phlebiopsis gigantea was a midgrade competitor as it was as dominant as some of the *Hypholoma* species against *A. ostoyae* (Table 5). When paired against both *A. ostoyae* strains B249-28 and P162-7, *P. gigantea* was able to grow around and contain the *A. ostoyae* colonies on both types of media (Figure 12). The *P. gigantea* did not allow any further growth of *A. ostoyae.*

Table 5. *Phlebiopsis gigantea* growth interactions against *A. ostoyae* strains B249-28 and P162-7 on both media (2 % MA and PWI). (A) Mutual intermingling of both species (1-4 mm); (B) Growth superficial over the contending organism; (C) Slight inhibition; (D) Growth around the contending organism; (E) Mutual inhibition at a considerable distance (>5mm).

***Phlebiopsis gigantea* strain**	**P162-7 (2 % MA)**	**B249-28 (2 % MA)**	**P162-7 (PWI)**	**B249-28 (PWI)**
VRA 1992	D	D	D	D

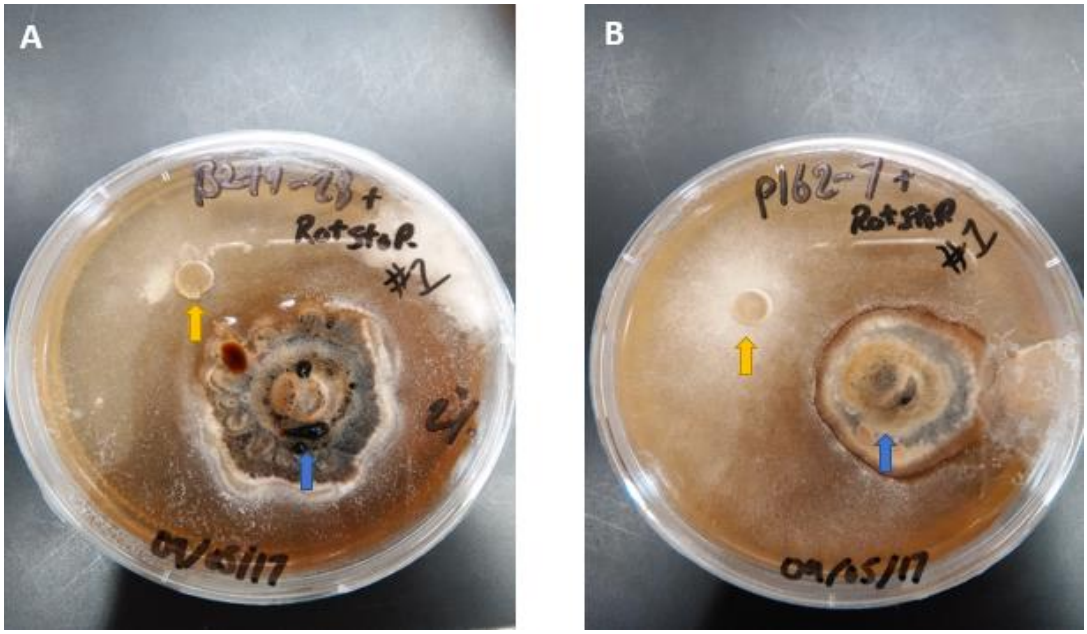

Figure 12. Photos taken from day 65 of *Phlebiopsis gigantea* surrounding *A. ostoyae* colonies. (A) *P. gigantea* & *A. ostoyae* strain B249-28, (B) *P. gigantea* & *A. ostoyae* strain P162-7. Yellow arrows indicate *P. gigantea* and blue arrows indicate *A. ostoyae*.

2.4.3 Microscopic examination of *Hypholoma* spp., and *P. gigantea* paired with *A. ostoyae*

2.4.3.1 *Hypholoma fasciculare* paired with *A. ostoyae*

When *H. fasciculare* strains were paired with *A. ostoyae* there were strong interactions from both the species (Table 6, Figure 13). The hyphae at the physical contact point between these two species had swelling or tip bursting observed. However, between *H. fasciculare* strain FP-133566-SP & *A. ostoyae* strain B249-28 the swollen hyphae tips were observed only in *A. ostoyae*. On the contrary, the swelling tips or tip bursting was seen only in *H. fasciculare* hyphae (RLG-12668-SP & B249-28, OKM-2932-T & B249-28, HHB-14801-SP & P162-7, and OKM-2932-T & P162-7) when paired with *A. ostoyae*. There were also *H. fasciculare* pairings where neither the *H. fasciculare* nor *A. ostoyae* showed any visual symptoms of interaction and both fungi appeared as if they were growing solo without competition (*H. fasciculare* strain JPL-62-

SP & *A. ostoyae* strains B249-28/P162-7, *H. fasciculare* strain OKM-7107-SP & *A. ostoyae* strains B249-28/P162-7, and *H. fasciculare* strain FP-133566-SP & *A. ostoyae* strain P162-7).

Table 6. Microscopic interaction outcome of *Hypholoma fasciculare* when interacting with *A. ostoyae* strains B249-28 and P162-7. (0) No swelling from either species, (1) *Hypholoma* hyphae swelling or tip bursting, (2) *Armillaria* hyphae swelling or tip bursting, (3) Both *Hypholoma* and *Armillaria* hyphae swelling or tip bursting.

***Hypholoma fasciculare* strains**	**Microscopic interaction (B249-28 *A. ostoyae*)**	**Microscopic interaction (P162-7 *A. ostoyae*)**
RLG-12668-SP	1	3
HHB-14801-SP	3	1
JPL-62-SP	0	0
OKM-2932-T	1	1
OKM-7107-SP	0	0
Pinnel B	3	3
FP-133566-SP	2	0

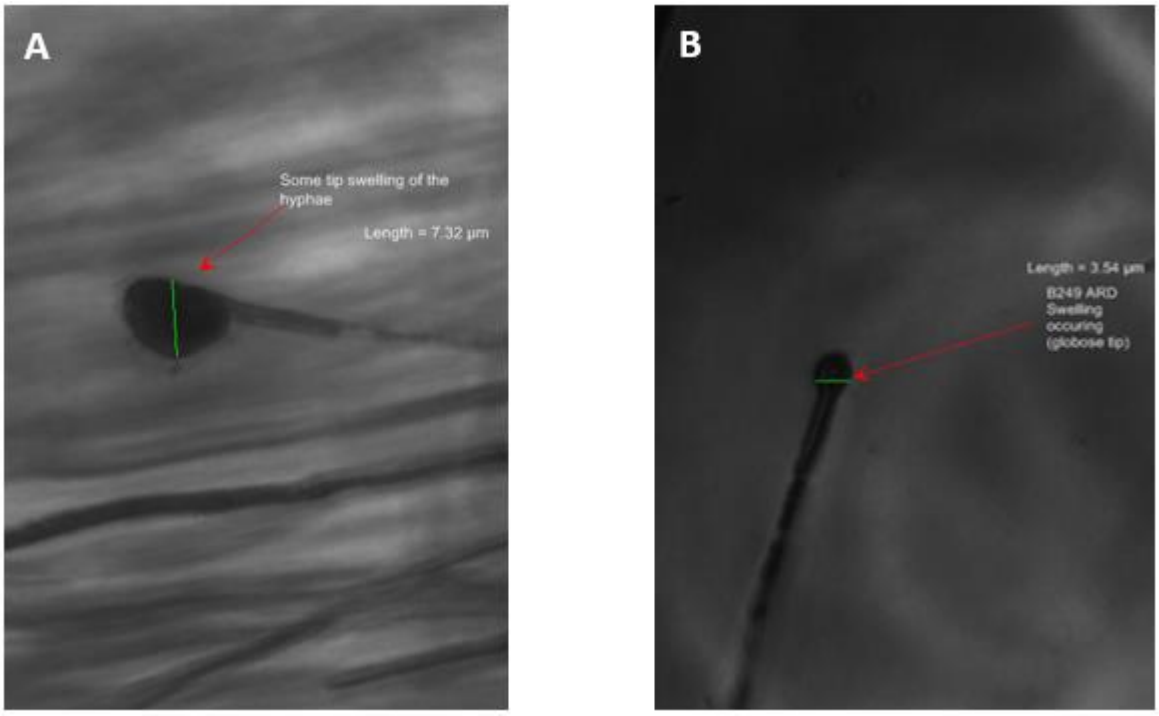

Figure 13. (A) *Hypholoma fasciculare* strain Pinnel B hyphae tip swelling when paired with *A. ostoyae* strain B249-28, (400x). (B) *Armillaria ostoyae* strain B249-28 hyphae tip swelling when paired with *H. fasciculare* strain Pinnel B, (400x)

2.4.3.2 *Hypholoma sublateritium* paired with *A. ostoyae*

Hypholoma sublateritium had hyphal interactions with both *A. ostoyae* strains B249-28 and P162-7 (Table 7). Interactions where both the *H. sublateritium* and *A. ostoyae* hyphae tips were swelling or bursting were noted for pairings *H. sublateritium* strain 49-1107 & *A. ostoyae* strain B249-28, *H. sublateritium* strain FP-90085-SP & *A. ostoyae* strain B249-28, and *H. sublateritium* strain OKM-6947-SP & *A. ostoyae* strain B249-28 (Figure 14). Among the pairings, *H. sublateritium* strain HHB-11948-SP & *A. ostoyae* strains B249-28/P162-7, *H. sublateritium* strain OKM-6192-SP & *A. ostoyae* strains B249-28/P162-7, *H. sublateritium* strain 49-1107 & *A. ostoyae* strain P162-7, and *H. sublateritium* strain FP-90085-SP & *A. ostoyae* strain P162-7, only *H. sublateritium* hyphae produced swelling or some rare hyphal tip bursting. Between *H. sublateritium* strain OKM-6947 & *A. ostoyae* strain P162-7 there was no unique interaction response noted for *H. sublateritium* or for *A. ostoyae*, as hyphae apical tips were similar to that observed under solo growth conditions (no swelling or tip bursting of the hyphae).

Table 7. Microscopic interaction of hyphal tips of *Hypholoma sublateritium* interacting with *A. ostoyae* strains B249-28 and P162-7. (0) No swelling from either species, (1) *Hypholoma* hyphae swelling or tip bursting, (2) *Armillaria* hyphae swelling or tip bursting, (3) Both *Hypholoma* and *Armillaria* hyphae swelling or tip bursting.

***Hypholoma sublateritium* Strain**	**Microscopic interaction (B249-28 *A. ostoyae*)**	**Microscopic interaction (P162-7 *A. ostoyae*)**
49-1107	3	1
FP-90085-SP	3	1
HHB-11948-SP	1	1
OKM-6192-Sp	1	1
OKM-6947-SP	3	0

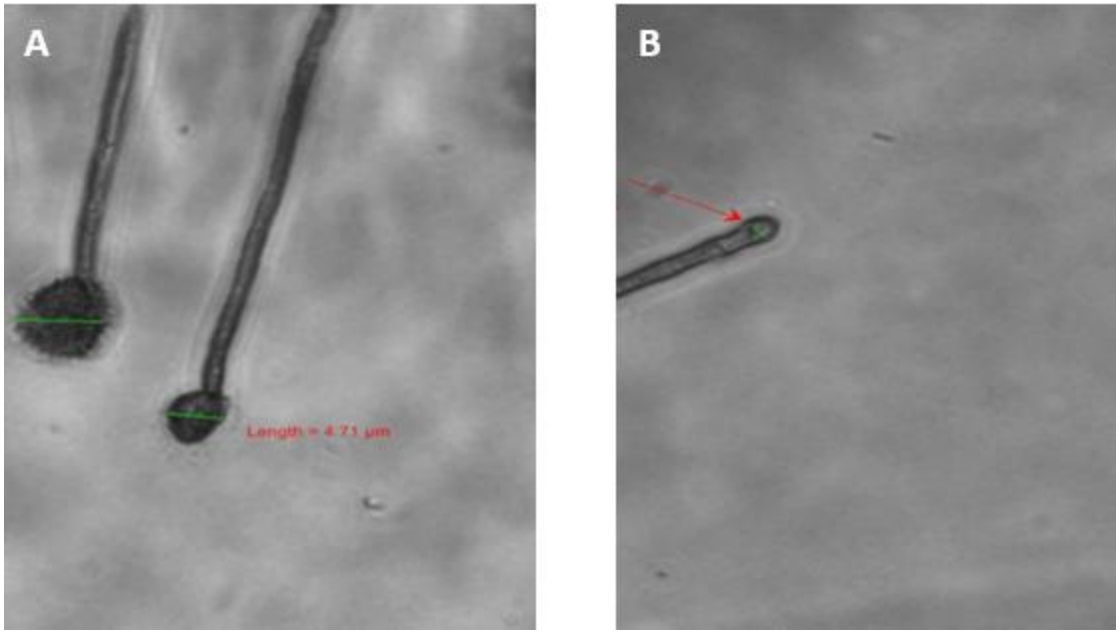

Figure 14. (A) *Hypholoma sublateritium* strain OKM-6947-SP hyphae swelling and bursting when paired with *A. ostoyae* strain B249-28, (400x). (B) *Armillaria ostoyae* strain B249-28 hyphae slight swelling when paired with *H. sublateritium* strain OKM-6947-SP, (400x)

2.4.3.3 *Hypholoma capnoides* paired with *A. ostoyae*

Hypholoma capnoides experienced the fewest interactions with *A. ostoyae* out of all *Hypholoma* species examined (Table 8). There were no *H. capnoides* and *A. ostoyae* pairings where both *H. capnoides* and *A. ostoyae* hyphae showed swelling or expressed a novel response when interacting. There were pairings that had only *A. ostoyae* hyphae with swelling which included: *H. capnoides* strain TAK 2 & *A. ostoyae* strain B249-28, *H. capnoides* strain TAK 5 & *A. ostoyae* strain P162-7 (Figure 15), and *H. capnoides* strain TAK 6 & *A. ostoyae* strain P162-7. With *H. capnoides* strain OKM-1523-T & *A. ostoyae* strain B249-28, observations of *H. capnoides* hyphae swelling were rare. Beyond this, no unique hyphae growth responses to competition were observed. Hyphae appearance was similar with and without competition (no swelling). The remaining pairings showed no unique hyphae growth responses, as the characteristics exhibited by both fungus species when competing were similar to that observed

when growing on its own. These pairings included: *H. capnoides* strain TAK 5 & *A. ostoyae* strain B249-28, *H. capnoides* strain TAK 6 & *A. ostoyae* strain B249-28, *H. capnoides* strain OKM-1523-T & *A. ostoyae* strain P162-7, and *H. capnoides* strain TAK 2 & *A. ostoyae* strain P162-7.

Table 8. Microscopic interaction of *Hypholoma capnoides* hyphal tips when interacting with *A. ostoyae* strains B249-28 and P162-7. (0) No swelling from either species, (1) *Hypholoma* hyphae swelling or tip bursting, (2) *Armillaria* hyphae swelling or tip bursting, (3) Both *Hypholoma* and *Armillaria* hyphae swelling or tip bursting.

***Hypholoma capnoides* Strains**	**Microscopic interaction (B249-28 *A. ostoyae*)**	**Microscopic interaction (P162-7 *A. ostoyae*)**
OKM-1523-t	1	0
TAK 02	2	0
TAK 05	0	2
TAK 06	0	2

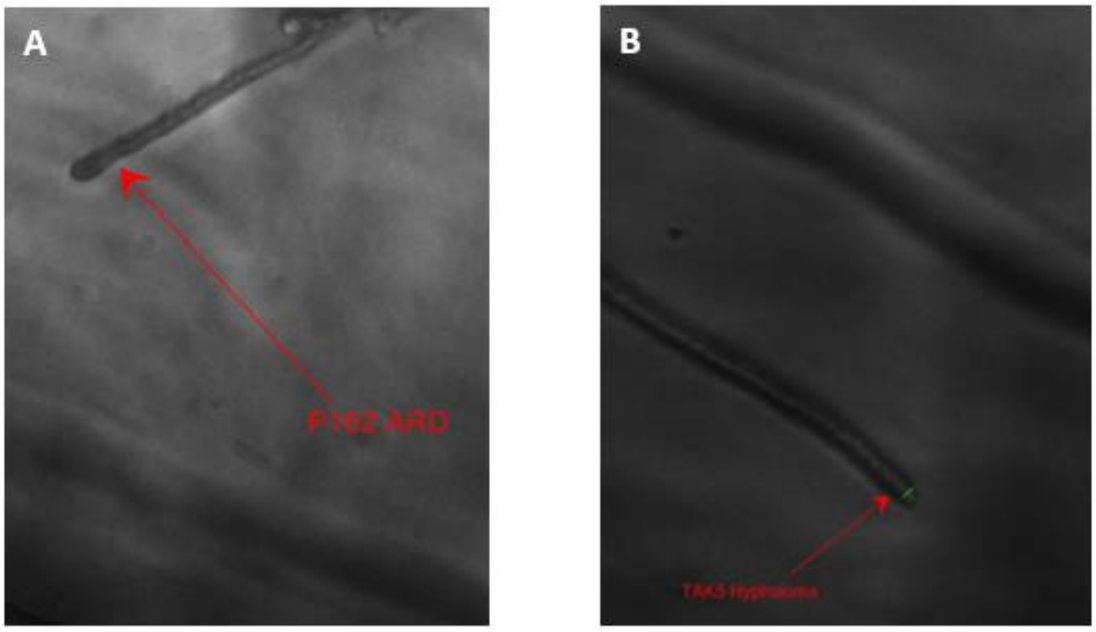

Figure 15. (A) *Armillaria ostoyae* strain P162-7 hyphae swelling when paired with *H. capnoides* strain TAK 5, (400x). (B) *Hypholoma capnoides* strain TAK 5 hyphae appears normal and no swelling when paired with *A. ostoyae* strain P162-7, (400x).

2.4.3.4 *Phlebiopsis gigantea* paired with *A. ostoyae*

Phlebiopsis gigantea was found to have frequent hyphal tip swelling when paired with *A. ostoyae* strain B249-28 but rare tip swelling was noted with *A. ostoyae* strain P162-7 (Table 9, Figure 16). The *A. ostoyae* had no reaction towards the *P. gigantea*.

Table 9. Microscopic interaction of *Phlebiopsis gigantea* hyphal tips when interacting with *A. ostoyae* strains B249-28 & P162-7. (0) No swelling from either species, (1) *P. gigantea* hyphae swelling or tip bursting, (2) *Armillaria* hyphae swelling or tip bursting, (3) Both *P. gigantea* and *Armillaria* hyphae swelling or tip bursting.

***Phlebiopsis gigantea* Strain**	**Microscopic interaction (B249-28 *A. ostoyae*)**	**Microscopic interaction (P162-7 *A. ostoyae*)**
P.G VRA 1992	1	1

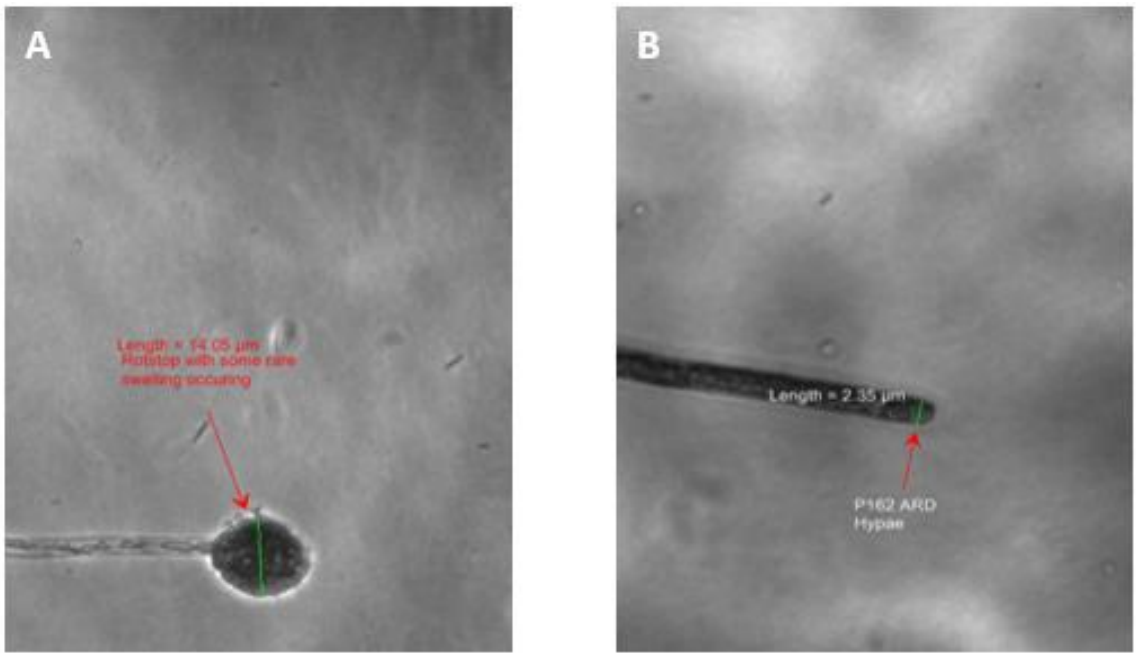

Figure 16. (A) *Phebiopsis gigantea* (VRA 1992) hyphae tip swelling when paired with *A. ostoyae* strain P162-7, (400x). (B) *Armillaria ostoyae* strain P162-7 hyphae appearing normal with no swelling occurring when paired with *P. gigantea*, (400x).

2.5 Discussion

2.5.1 *Hypholoma* spp. and *P. gigantea* paired with *A. ostoyae*

Among *H. fasciculare* strains, Pinnel B maintained the highest radial growth on both media and was a strong competitor with *A. ostoyae* strains B249-28 and P162-7. However, radial growth was significantly less than when grown in the absence of competition. *Hypholoma fasciculare* strain Pinnel B reduced the growth of *A. ostoyae* by over 70 % compared to when *A. ostoyae* was growing on its own. This implies that when the two fungi came into contact the *H. fasciculare* prevented further growth of *A. ostoyae*. Chapman and Xiao (2000) found that the Pinnel B strain could outcompete *A. ostoyae* in both *in-vitro* and field conditions. They also noted that there was a period of reduced growth by both species when first paired, but over time *H. fasciculare* would begin to replace the *A. ostoyae*. We did not experience a slow period of initial growth from either species. However, we did see the *H. fasciculare* grow over and replace the *A. ostoyae* colonies. The other *H. fasciculare* strains exhibited much slower radial growth compared to the Pinnel B strain when in competition with *A. ostoyae*. However, it is also important to note that growth medium type did impact the radial growth of the *Hypholoma* species and strains. This suggests that it is important to utilise the correct strain and growth medium when trying to develop biocontrol agents for *Armillaria* root rot through *in-vitro* testing. Selecting only a single strain of a species, or only one particular medium may result in erroneous conclusions about a species' potential efficacy.

An example of differences in morphological responses at macroscopic versus microscopic levels, and thus, the importance of investigating response at multiple scales, was present with *H. fasciculare* strain OKM-7107-SP in competition with *A. ostoyae* strains B249-28 and P162-7. *Hypholoma fasciculare* strain OKM-7107-SP was a poor competitor towards *A.*

ostoyae strains B249-28 and P162-7. This is interesting because the pairing growth test demonstrated macroscopically that the presence of *A. ostoyae* reduced the growth of *H. fasciculare* strain OKM-7107-SP and that *A. ostoyae* radial growth increased towards the *H. fasciculare* strain OKM-7107-SP compared to when *A. ostoyae* was in the absence of competition. However, examining their interaction at a microscopic level, neither *A. ostoyae* or the *H. fasciculare* strain OKM-7107-SP had swelling of the hyphae or any other evidence of physical interaction. At the macroscopic level, mutual intermingling and slight inhibition between the two fungi were apparent. This implies that there may be a chemical reaction occurring or other microclimate explanations that is slowing *H. fasciculare* growth (Boddy, 2000; Cox & Scherm, 2006; Schoeman et al. 1996). Boddy (2000) noted that interactions are affected by non-enzymatic metabolites (antibiotics), whereas Cox & Scherm (2006) noted that when swelling of *A. ostoyae* hyphae occurred, overgrowth from *H. fasciculare* was always present. For our study, this was not the case.

Another example of a strong competitor was with *H. fasciculare* strain RLG-12668-SP in competition with *A. ostoyae* strains B249-28 and P162-7 on the PWI medium. But rather than reducing radial growth, radial growth was stimulated by the presence of *A. ostoyae.* This is a defensive response to the presence of the *A. ostoyae* competitor. *Hypholoma fasciculare* strain RLG-12668-SP had no significant change in growth when competing with *A. ostoyae* strains B249-28 and P162-7. *Armillaria ostoyae* strains B249-28 and P162-7 had little change in growth when paired with *H. fasciculare* strain RLG-12668-SP compared to when competition was absent. *Hypholoma fasciculare* strain RLG-12668-SP at the macroscopic level can overgrow both strains of *A. ostoyae* whereas the microscopic interaction showed different results between the *A. ostoyae* strains B249-28 and P162-7. With *A. ostoyae* strains B249-28, *H. fasciculare*

strain RLG-12668-SP is the only strain that showed swelling and bursting of the hyphal tips compared to the *A. ostoyae* strain P162-7 pairing where both the *H. fasciculare* and *A. ostoyae* showed hyphal tip swelling. These results agree with the findings from Cox and Scherm (2006) as they noted that when *A. ostoyae* hyphae had swelling or granulation, the *A. ostoyae* was overgrown by the antagonist. This suggests that microscopic examination of hyphae may not be accurate indicators of the macroscopic interaction as the macroscopic interactions were similar despite observed differences at the microscopic level.

Hypholoma sublateritium strain HHB-11948-SP is a strong competitor that showed promise as a potential biocontrol agent towards *A. ostoyae*. *Hypholoma sublateritium* strain HHB-11948-SP had the highest radial growth against *A. ostoyae* strains B249-28 and P162-7 on the 2 % MA. The paired radial growth of *H. sublateritium* strain HHB-11948-SP is nearly double the radial growth in absence of competition. This suggests that the presence of *A. ostoyae* is a stimulant and caused the rate of *H. sublateritium* strain HHB-11948-SP growth to increase. The macroscopic interaction between *H. sublateritium* strain HHB-11948-SP with both *A. ostoyae* strains B249-28 and P162-7 showed that *H. sublateritium* could overgrow both strains of *A. ostoyae.* The microscopic interaction between *H. sublateritium* strain HHB-11948-SP and both *A. ostoyae* strains showed *H. sublateritium* hyphae tip bursting whereas no similar response was observed from the *A. ostoyae* strains. It seems that *H. sublateritium* increased their growth rate to combat the *A. ostoyae* and that *A. ostoyae* showed no signs of defense. The *A. ostoyae* had very little change in radial growth when paired with *H. sublateritium* strain HHB-11948-SP. This is interesting as Chapman and Xiao (2000) noted that when only the *A. ostoyae* hyphae were swelling it caused the *H. fasciculare* growth to be reduced until the swelling of *A. ostoyae* began to lyse after a few days. This suggests that the *H. sublateritium* was trying to attack the *A.*

ostoyae colony and there is possible chemical reactions occurring as the *H. sublateritium* could increase the rate of growth to reach the *A. ostoyae* colony more rapidly. These findings suggest that *H. sublateritium* strain HHB-11948-SP should be considered as a potential biocontrol candidate as it was able to reach the *A. ostoyae* colony quickly and was able to overgrow the colonies.

The PWI medium was designed to be a more natural medium as it contained nutrients found within the roots of *Pinus resinosa*, which is important as these biocontrols live within the decaying stumps and roots of coniferous tree species (Parker, 1933). Growth media such as 2 % MA and 2 % malt extract agar are artificial media and are used in mycology studies due to their high nutrient availability (Harold et al. 2005; Keča, 2009). The PWI medium lead to higher radial growth rates for the *Hypholoma* strains and different macroscopic interactions. Radial growth was significantly higher for *Hypholoma* spp. on the PWI compared to the 2 % MA medium. This agrees with our initial hypothesis that the PWI medium was anticipated to achieve higher radial growth compared to 2 % MA at the same time frame due to the strong foraging ability of the biocontrol agents. *Armillaria ostoyae* had very little differences in radial growth on either medium. This justifies the importance of using multiple media to test for biocontrol candidates. In terms of macroscopic interactions, the 2 % MA medium resulted in the most visible interactions whereas the PWI medium was unclear and difficult to examine and had some slight variations in macroscopic interactions compared to the 2 % MA for some strains of *Hypholoma* spp. The two media demonstrated how each species and strain can vary in radial growth with different nutrient levels.

Phlebiopsis gigantea was a midgrade competitor whose growth was significantly lower in the presence of *A. ostoyae* strains B249-28 and P162-7. This response was similar to those of

the *Hypholoma* spp. While examining the macroscopic interactions between *P. gigantea* and the *A. ostoyae* strains it showed that *P. gigantea* could grow around the *A. ostoyae* colony but could not overgrow the *A. ostoyae.* Our findings agree with Keča (2009) who found that there was a thick barrage of mycelium surrounding the *A. ostoyae* colony. At the microscopic level, the *P. gigantea* hyphae had swelling near the tips when in contact with *A. ostoyae* hyphae. This interaction may have caused reduction in growth and may also suggest the possibility of chemical interactions resulting in the growth of *P. gigantea* around the *A. ostoyae* colonies. This suggests that *P. gigantea* is not as effective a biocontrol agent as some of the *Hypholoma* species/strains. Further field testing would need to be completed to come to an accurate conclusion on the efficacy of *P. gigantea* as a biocontrol agent against *A. ostoyae*. It may be possible for *P. gigantea* to colonize resources such as root systems before *A. ostoyae* due to its high non-competitive growth, meaning that *P. gigantea* may decay a root system quickly enough to prevent *A. ostoyae* from establishing or at least reduce resource availability and limit its growth.

2.5.2 Macroscopic Interactions

Boddy (2000) has noted that there are essentially two types of combative interactions, one is replacement where one fungus takes over another, or deadlock where both fungi come into contact and nothing further ensues. Hynes et al. (2007) suggested that fungi produce volatile organic compounds that may affect the ability to defend territory when invaded by other fungi. Cox and Scherm (2006) found that *H. fasciculare* could suppress the growth of *A. ostoyae* colonies and prevent rhizomorph expansion on wood blocks. Chapman and Xiao (2000) concluded that when *H. fasciculare* and *A. ostoyae* are paired on root segments, *Hypholoma* were able to overtake the *Armillaria* and fully colonize the root with no further evidence of *A. ostoyae*.

Our study found that there was variability with how each *Hypholoma* species and *P. gigantea* responded to the presence of *A. ostoyae,* which was in some cases also dependent on the growth medium used. With *Hypholoma fasciculare* strains, there were four out of eight strains that were strong competitors and could overgrow the *A. ostoyae* mycelium when the two came into contact. This finding agrees with Keča (2009) as this study also found that *H. fasciculare* could overgrow the *A. ostoyae* mycelium/colony. Our findings also agree with Chapman and Xiao (2000) as they noted that after a period of time the *H. fasciculare* could grow over the *A. ostoyae* colony with a dense mat of *Hypholoma* mycelium. *Hypholoma fasciculare* strain Pinnel B was one of the strongest competitors towards *A. ostoyae* showing overgrowth of both *A. ostoyae* strains tested. This finding agrees with the initial hypothesis that *H. fasciculare* would be a strong competitor towards *A. ostoyae* as the Pinnel B strain was able to overtake both strains of *A. ostoyae* colonies on both growth media. This may be due to the presence of volatile organic compounds decreasing the ability of *A. ostoyae* to defend its territory, leading to replacement by Pinnel B (Hynes et al. 2007). Our study found that not all strains of *H. fasciculare* could overgrow the *A. ostoyae* mycelium as *H. fasciculare* strains RLG-11562-SP (poor competitor) and JPL-62-SP (midgrade competitor) could only surround the *A. ostoyae* colony; i.e., there was containment but not over growth. *Hypholoma fasciculare* strains FP-133566-SP and OKM-7107-SP were both poor competitors and had very mixed results as the interaction depended on the strain of *A. ostoyae* and the growth medium.

Our study found *H. sublateritium* to be a very effective competitor against *A. ostoyae*. There were two visual interactions that were expressed by *H. sublateritium* when paired to *A. ostoyae*. There was overgrowth and growth surrounding the *A. ostoyae* colonies. Different interactions were noted for each growth medium as with the 2 % MA, *H. sublateritium* strains

FP-90085-SP, HHB-11948-SP, OKM-6192-SP were all strong competitors and were all able to overgrow the *A. ostoyae* mycelium with thick dense *Hypholoma* mycelium. *Hypholoma sublateritium s*train 49-1107 was a midgrade competitor and was able to surround the *A. ostoyae* colony on both the 2 % MA and the PWI growth medium. On the PWI growth medium all strains except for *H. sublateritium* strain HHB-11948-SP were able to grow around *A. ostoyae* colony. With *H. sublateritium* strain HHB-11948-SP the *Hypholoma* was able to overgrow the *A. ostoyae* on the PWI medium. The difference in *Hypholoma* behaviour between growth media could mean that behaviour is related to nutrient levels present, or could be due to the production of volatile organic compounds leading to less overgrowth from the *H. sublateritium* strains on the PWI medium (Hynes et al. 2007). Our study indicates that *H. sublateritium* should be considered in future field competition trials with *A. ostoyae* given the positive outcomes in the *in-vitro* pairing studies here.

Hypholoma capnoides has been paired *in-vitro* with *A. ostoyae* by Keča (2009). The study found that from a visual interaction perspective *H. capnoides* was just as effective as *H. fasciculare*, as it could overgrow the *A. ostoyae* colony. Our study did not fully support the findings of Keča (2009) as there was only one instance where an *H. capnoides* strain could overgrow *A. ostoyae.* This was with *H. capnoides* strain TAK 5 which was a midgrade competitor when paired with *A. ostoyae* strain P162-7 on PWI growth medium. All other strains except *H. capnoides* strain OKM-1523-T had interactions where they would grow or partially surround the *A. ostoyae* colony. *Hypholoma capnoides* strain OKM-1523-T was a poor competitor and had different interactions depending on growth medium and *A. ostoyae* strain. When paired with *A. ostoyae* strain B249-28 on the 2 % MA and PWI medium, *H. capnoides* strain OKM-1523-T was intermingling with the *A. ostoyae* and no further growth was seen from

either species after contact. When *H. capnoides* strain OKM-1523-T was paired with *A. ostoyae* strain P162-7 on the 2 % MA, the two fungi had an area of slight inhibition where the two species would not come near one another and the growth stopped. These findings imply that *A. ostoyae* strain and nutrient levels within the growth media impact the effectiveness of *H. capnoides strain* OKM-1523-T and that this strain would not be a viable biocontrol agent due to its high variability in growth and interactions with *A. ostoyae*.

2.5.3 Microscopic Examination of Fungal Pairings

Cox and Scherm (2006) examined the microscopic interactions between *Armillaria mellea* and *A. tabescens* when paired to *H. fasciculare*. *Armillaria mellea* and *A. tabescens* were paired to *H. fasciculare* strain OKM-7107-SP which was used in our study. They found that the *A. mellea* and *A. tabescens* had hyphal swelling a majority of the time with some tip bursting and granulation. Our study disagrees with their findings as we found that with *H. fasciculare* strain OKM-7107-SP, when paired to *A. ostoyae* strains B249-28 and P162-7, there was no unique interaction present between either species, as the hyphae appeared morphologically similar to fungi growing in the absence of competition. Chapman and Xiao (2000) found that *A. ostoyae* hyphae were swelling to a globose shape at the tips when the *H. fasciculare* Pinnel B strain was approaching, but no interaction response was noted from the *H. fasciculare.* Our study disagrees with the findings of Chapman and Xiao (2000) as we found that when *H. fasciculare* strain Pinnel B and the *A. ostoyae* were nearing each other that many of the hyphae tips of *A. ostoyae* were swelling to a globose shape as were some of the Pinnel B hyphae. Within the *H. fasciculare* species, a majority of the strains had different microscopic responses. Cox and Scherm (2006) reported that when *A. ostoyae* hyphae were swollen and had granulation of the hyphae tips, they were always overgrown by another fungi, indicating that the *A. ostoyae* was under some stress,

which we also observed but only in scenarios where the *Hypholoma* strains had hyphal tip swelling or tip bursting.

Similar to *H. fasciculare* strains, *H. sublateritium,* and *H. capnoides* strains had a variety of microscopic responses when paired with both strains of *A. ostoyae*. However, many of the visual interactions were similar among species indicating that microscopic examination does not provide any additional information already provided at the macroscopic level. These results would indicate that the *H. sublateritium* strains, a majority of the time, are aware they are in a competition for resources. The swelling and tip bursting of the hyphae is a defence mechanism towards the *A. ostoyae* (Cox and Scherm, 2006). Note that there may be a chemical response present from the *H. sublateritium* strains as well.

Our study found that the microscopic interaction results tended to differ between the *A. ostoyae* strains B249-28 and P162-7. Conversely, the macroscopic interaction results were similar for most of the strains. Chapman and Xiao (2000) state that microscopic examination may be important when first pairing species together and for explaining why sometimes a decrease in growth or a dormancy may be present when two fungi approach. Further, there are other factors besides microscopic interactions that should be tested when developing biocontrols, such as examining chemical competition between interacting fungi (Hynes et al. 2007).

2.6 Conclusions

This study found that the radial growth for each *Hypholoma* species in response to competition with *A. ostoyae* is influenced by the strain of the species and the growth medium used. There was a high degree of variability in radial growth among the *Hypholoma* species and strains. This study demonstrates the importance of testing various strains of a species when

choosing a biocontrol candidate for a root disease such as *Armillaria* root rot. Multiple strains should be tested *in-vitro* on 2 % MA to monitor the macroscopic interaction and growth rates. For example, when the biocontrol agents were grown solo the fastest and highest radial growth was seen in *P. gigantea*. With the *Hypholoma* spp., *H. fasciculare* strain Pinnel B achieved the fastest and highest radial growth among the *Hypholoma* species/strains tested. When pairing the biocontrol agents with *A. ostoyae*, it was found that the *H. fasciculare* strain Pinnel B had the greatest radial growth towards the *A. ostoyae* colony, as it was able to reach the *A. ostoyae* colony first. However, it is important to note that *A. ostoyae* has been found to have a high amount of strain variability (Schulze and Bahnweg, 1998), which implies that further testing with other strains of *A. ostoyae* should be completed to confirm its effectiveness.

Hypholoma fasciculare was the species most capable of overgrowing and replacing the *A. ostoyae* colony, irrespective of the media tested. *Hypholoma fasciculare* also had more novel interaction responses towards *A. ostoyae* at the microscopic level. There were multiple strains of *H. fasciculare* that exhibited hyphal tip swelling and/or bursting when paired with both strains of *A. ostoyae*, while the *A. ostoyae* also exhibited similar responses.

Chapter 3: Impact of Simcoe County winter soil temperatures at various depths on the growth of *Hypholoma fasciculare* in pine plantations

3.1 Abstract

Hypholoma fasciculare has been examined as a potential biocontrol agent against *Armillaria* root rot. However, these trials examined the efficacy of *H. fasciculare* either under lab conditions or in field conditions during summer months only. Our study examined the ability of *H. fasciculare* to survive and grow during the winter months buried in six red pine (*Pinus resinosa*) plantations located in Simcoe County, Ontario, Canada. Soil temperatures and snow depth were monitored from November 4, 2017 to May 13, 2018 at each site. Pine blocks inoculated with *H. fasciculare* were buried in the three thinned pine plantations at 30 and 100 cm depths from February 1, 2018 to May 13, 2018 to examine how winter soil temperatures and depth impacted growth. Soil temperatures at the 30 cm depth were consistently colder than at 100 cm ($F_{1,6} = 63.46$, $p < 0.001$). *Hypholoma fasciculare* continued to grow over the winter months ($F_{1,36} = 50.41$, $p < 0.001$). Soil depth did not impact growth rate ($F_{1,18} = 1.87$, $p = 0.188$). Mean growth rates were 0.25 ± 0.11 and 0.31 ± 0.10 mm per day at 30 and 100 cm depths, respectively. While snow depths were significantly lower in non-thinned plantations ($t_4 = 9.95$, $p = 0.001$), this had little impact on soil temperatures. This study will aid in the development of using *H. fasciculare* as a biocontrol treatment against *Armillaria* root rot given *H. fasciculare*'s ability to grow underground throughout the winter months, a period during which *Armillaria* has a reduced growing capacity.

3.2 Introduction

Soil temperature is a vital factor when it comes to fungi establishment, growth, and survival (Pietikäinen et al. 2005; Rishbeth, 1978; Voříšková et al. 2014; Wells & Boddy, 1995). Growth rates of fungi slow down as soil temperatures decrease, thus impacting their ability to decay wood (Rishbeth, 1978; Pearce & Malajczuk, 1990; Wells & Boddy, 1995; Pietikäinen et al. 2005). *Hypholoma fasciculare*, for example, is able to decay wood at 5ºC. However, the rate is approximately half of that observed when temperatures approach 15 to 20ºC (Wells & Boddy, 1995). Coutts & Nicoll (1990) discovered that fungi such as *Thelephora terrestris* can grow 3 mm per day when the soil temperature is 15ºC compared to winter growth of 0.4 mm per day at 2ºC.

The ability to continue to grow in colder soils is species specific. Some fungi such as *Heterobasidion parviporum*, a species that causes tree root rot, is able to grow in ambient temperatures as low as -4ºC (Müller et al. 2014) while species such as *Armillaria luteobubalina*, another tree root rot fungus, remains dormant in the soil at 4ºC (Pearce & Malajczuk, 1990). Growth, as evidenced by the production of rhizomorphs, seems to stall at soil temperatures approaching 10ºC for this species; nb., optimal growing conditions are between 16 and 20ºC (Pearce & Malajczuk, 1990; Rishbeth, 1978).

Growth may also be impacted by soil depth given the differences in seasonal and diurnal fluctuations in temperature at different depths within the soil solum (Baker, 1971; Carreiro & Koske, 1992). *Armillaria luteobubalina*, for example, produces significantly more rhizomorphs at 12 cm depth compared to 28 cm, particularly during the summer months (Pearce & Malajczuk, 1990). Moreover, aboveground conditions such as snow cover can influence the surface and underground soil temperatures. Decker et al. (2003) observed that sites with snow cover

moderated soil temperatures relative to sites without it, but that this influence was only evident at soil depths greater than 15 cm. Greater snow cover provides better soil insulation, leading to warmer soil temperatures, a shallower frost layer depth (Baker, 1971; Buckeridge & Grogan, 2008; Zhang et al. 2008), and thus, a larger refuge during the winter months for many organisms. Kristinsson (1976), for example, observed that fungi can survive and continue to decay organic matter when under snow cover during winter; however, the species present were dependent on the length of time there was snow cover and whether the ground was frozen. Mundra et al. (2016) discovered that as snow cover depth increased the total fungal richness increased.

Only a handful of studies have examined soil fungus growth rates during the winter months in north temperate forests (e.g., Coutts & Nicoll, 1990; Kuhnert et al. 2012). This minimal understanding of fungi growth during winter, especially those belonging to the basidiomycetes division, is an obstacle for the development of biocontrol agents against root rot diseases, as knowledge of winter growth and survival is crucial for effective root rot disease management. Thus, studies on fungi growth during winter will help us determine if a biocontrol agent can persist year-round in soils and be able to actively prevent establishment of root diseases including the winter season. Therefore, understanding *Hypholoma* species' ability to colonize wood or root segments at various soil temperatures is an important precursor to testing their efficacy as biocontrol agents for *Armillaria* and *Heterobasidion* spp.

Several *Hypholoma* species have been demonstrated to be viable candidates for *Armillaria* and *Heterobasidion* control in both lab and field conditions. *Hypholoma fasciculare*, in particular, has shown promise towards controlling the establishment and spread of *Armillaria* spp. For example, Keča (2009) found that *H. fasciculare* growth could be stimulated by the presence of *Armillaria* spp. and ultimately decrease the rhizomorph production in *Armillaria* spp.

In other lab trials, *H. fasciculare* successfully outcompeted *Armillaria ostoyae* on root segments placed in sand at 20ºC (Chapman & Xiao, 2000). In their subsequent field trials in areas with heavy *A. ostoyae* infection, *H. fasciculare* was able to colonize Douglas fir (*Pseudotsuga menziesii*) root systems and stumps and prevented further growth of *A. ostoyae* (Chapman & Xiao, 2000). Field trials using *Hypholoma* spp. against the establishment and spread of *Heterobasidion* spp. have also been performed. For example, *H. fasciculare* was inoculated on Norway spruce (*Picea abies*) stumps to examine its efficacy against *Heterobasidion annosum* (Nicolotti et al. 1999). In this instance, *H. fasciculare* was of low efficacy towards reducing the establishment of *H. annosum.* However, this was only examined during the summer months. This relationship should be tested during winter months to determine if *H. fasciculare* can survive on *Pinus* spp. during winter and whether *H. fasciculare* has a competitive advantage over *Armillaria* in colder temperatures. There is no current literature on if/how *H. fasciculare* will grow in red pine (*Pinus resinosa*) and white pine (*Pinus strobus*) plantations during the winter months and into the middle of spring.

The objective of this study was to determine if winter soil temperatures within Simcoe County are suitable for *H. fasciculare* growth. The hypotheses tested were that soil temperatures would be warmer at 100 cm relative to 30 cm depths due to the insulation gains associated with soil depth (Baker, 1971). Soil temperatures were also expected to be warmer in thinned versus non-thinned sites because of increased snow cover. Lastly, *H. fasciculare* growth at 100 cm depth was expected to exceed the growth at 30 cm depth.

3.3 Methods

3.3.1 Site conditions

Simcoe County is located in the Mixedwood Plains Ecozone of Ontario, Canada (Lee et al. 1998). Much of the natural ecosystems have been converted for agriculture and urban development. However, deciduous, coniferous, and mixedwood stands still persist in the County. Approximately 50% of the County is forested, of which approximately 25% are managed as plantations consisting of mostly red (*Pinus resinosa*) and white pine (*Pinus strobus*). Climate in the region is relatively cool as mean annual temperature for 2018 was 6.6 ± 10.4ºC. Mean January and July temperatures are -8.8 ± 8.0ºC and 20.3 ± 2.9ºC, respectively (Environment and Climate Change Canada, 2011). Annual precipitation is 78.3 ± 14.6 mm, with 25 % falling as snow (Environment and Climate Change Canada, 2011). Major disturbances impacting plantations include root rot diseases, insect outbreaks, and infrequent windstorms (G. Davis, personal communication, 07/07/17; McLaughlin et al. 2010).

Soil conditions were similar among the plantations, consisting mainly of two types of sandy loams; Vasl-s and Tisl. Vasl-s is a steep phase soil consisting of calcareous and non-calcareous sandy loam till. These soils have good drainage, are moderate to very stony, and have a slight to medium acidic surface. Tisl is a calcareous outwash sandy loam having good drainage, is stone free to moderately stony, and has a medium acidic surface (Canada Department of Agriculture, 1959).

3.3.2 Soil temperature and depth

Six mature red pine plantations were selected within Simcoe County, Ontario, Canada, of which three were owned by the County, one by the province, and two were a private holding (Figure 17). Two sample plots were established in each plantation. Three of the plantations have

never been thinned while the other three were thinned in 2009, 2013, and 2015, respectively. Soil temperatures in thinned vs non-thinned plantations were compared to determine if thinned canopies, which allow for more sunlight and potentially more precipitation to reach the ground, would result in higher soil temperatures than non-thinned sites.

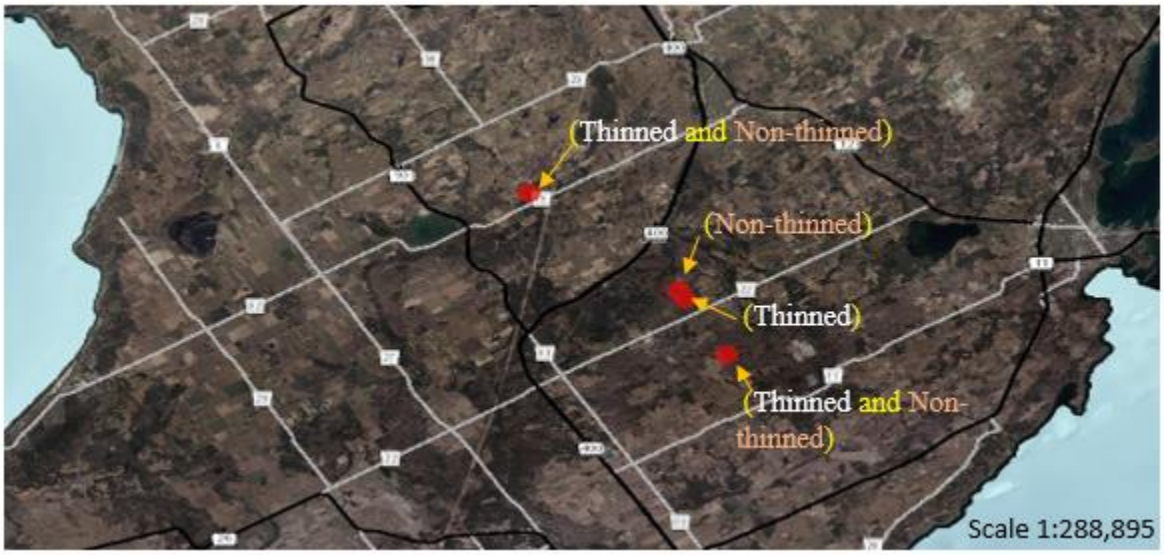

Figure 17. Location of soil temperature data logger sample plots (indicated in red) within Simcoe County, ON.

Within each stand, two temperature data loggers (OMEGA® OM-SP-MICROTEMP) were randomly placed in the soil, one at a depth of 100 cm, the other at a depth of 30 cm and approximately 2 m away. These two depths were used because red pine have many shallow horizontal roots (10-30 cm deep) and deep vertical anchor roots (100 ± 50 cm) to allow for tree anchoring and nutrient uptake (Fayle, 1975). The data loggers were set to collect daily temperatures at 02:00 and 14:00 (12-hour rotation) from November 4, 2017 to May 13, 2018. Thus, 380 measurements were obtained from each soil data logger over the study period. Snow depth was measured at all sites on January 9, 2018 to determine if any differences in depth between thinned and non-thinned stands were evident.

3.3.3 *Hypholoma fasciculare* growth

Our lab test results indicated that *Hypholoma fasciculare* strain Pinnel B had the greatest potential to reduce and/or replace *Armillaria ostoyae* growth. Therefore, it was selected for field trials. Forty wooden blocks (2.5 x 2.5 x 20 cm) were inoculated with *H. fasciculare* strain Pinnel B in lab conditions. An inoculum plug of *H. fasciculare* (10 mm) was obtained from a fresh culture and placed on one end of each wooden block (1 plug per block). The blocks were then placed in a container and incubated in the dark at 24ºC. The blocks were incubated for 9.5 weeks (67 days) to ensure sufficient mycelium establishment for the field experiment. Only 24 of 40 blocks showed sufficient growth of *H. fasciculare*. Therefore, only these 24 blocks were used for the field study. The low numbers did not allow us to compare growth between thinned and non-thinned sites. Thus, we decided to install the blocks only within the thinned sites to examine potential differences in growth at 100 vs 30 cm depths.

Blocks were placed in the ground on February 1, 2018 (mid-winter) and were collected on May 13, 2018 (mid-spring). Mycelium growth of *H. fasciculare* was measured prior to burial. *Hypholoma fasciculare* growth was measured on each side of the block (4 sides and marked) before being placed horizontally in the ground and labelled. Growth on blocks placed at 100 cm depth ranged from 63.3 to 126.3 mm while blocks placed at 30 cm depth ranged from 70.0 to 152.5 mm. Eight blocks were installed at each thinned site. Four were buried at a depth of 100 cm and four at 30 cm soil depth. At the time of the block placements, each site had less than 30 cm of snow on the forest floor, and there was only one site (Amos) where the first 10 cm of the soil was frozen, the rest were still thawed. When the blocks were extracted on May 13 there was no snow left on the forest floor.

3.3.4 Statistical analyses

Soil temperatures were averaged for each day as an initial analysis concluded that there were no significant differences in temperature readings between 2:00 and 14:00 time periods at both the thinned (t = -0.225, df = 2278, p = 0.822) and non-thinned sites (t = -0.090, df = 2278, p = 0.929). We compared mean monthly and bi-weekly soil temperatures between thinned and non-thinned sites at each soil depth using factorial ANOVA and repeated measure ANOVA (α = 0.95). A Levene's Test for Homogeneity of Variance indicated that both the weekly and bi-weekly data were not homogeneic. However, ANOVA has been shown to be robust enough to use with non-homogeneic data (Zuur et al. 2010). Moreover, Anderson Darling normality tests indicated that the data were normally distributed. Impacts of soil depth (100 and 30 cm) on *H. fasciculare* total growth were examined using factorial ANOVA (α = 0.95). Snow depths were also compared between the thinned and non-thinned sites using an independent t-test. All statistical tests were run using R studio version 1.1.383.

3.4 Results

3.4.1 Soil temperature and depth

Soil temperature decreased from November until late to mid-April and then increased rapidly into May. On November 4, 2017 when the probes were first placed in the ground the temperatures at 100 cm depth were 10.4 ± 0.02 SE (standard error) and 10 ± 0.2 SE ºC in the thinned and non-thinned sites, respectively. At the 30 cm depth, temperatures were 8.1 ± 0.68 SE and 8 ± 0.35 SE ºC in the thinned and non-thinned sites, respectively (Figure 2). On May 13, 2018 when the soil probes were removed the temperatures at 100 cm were 4.9 ± 2.19 SE and 4.8 ± 0.79 SE ºC in the thinned and non-thinned sites, respectively. At 30 cm depth on May 13, 2018, temperatures were 7.5 ± 1.56 SE and 6.4 ± 0.76 SE ºC at the thinned and non-thinned sites,

respectively (Figure 18 & 19). There was a significant difference in temperature between the 100 and 30 cm depths throughout the winter and into the middle of the spring between each month ($F_{1,56} = 63.468$, $p < 0.001$). A significant interactive effect of soil depth and month on soil temperature ($F_{5,56} = 14.481$, $p < 0.001$) was observed. Similar patterns of a gradual temperature decline over the winter season were observed at both soil depths (Figure 18). During this time temperatures at the 100 cm depth were consistently warmer than at 30 cm ($F_{1,6} = 63.46$, $p < 0.001$). However, as the season transitioned into spring, temperatures at 30 cm depths rose more rapidly than at 100 cm depths and were ultimately warmer. Further, there were no differences in soil temperatures between the first two weeks and the last two weeks for each month examined ($F_{1,56} = 2.08$, $p = 0.155$). Thus, we did not run a second analysis examining temperature variation in function of bi-monthly readings x soil depth.

3.4.2 Impacts of stand thinning on soil temperature over time

No significant difference in monthly soil temperatures were observed between thinned and non-thinned sites from November to May ($F_{1,56} = 0.083$, $p = 0.774$). Mean soil temperatures in thinned and non-thinned sites were 3.8 ± 2.1ºC at 100 cm and 2.6 ± 2.2ºC at 30 cm, and 3.9 ± 1.9ºC at 100 cm and 2.6 ± 1.8ºC at 30 cm, respectively.

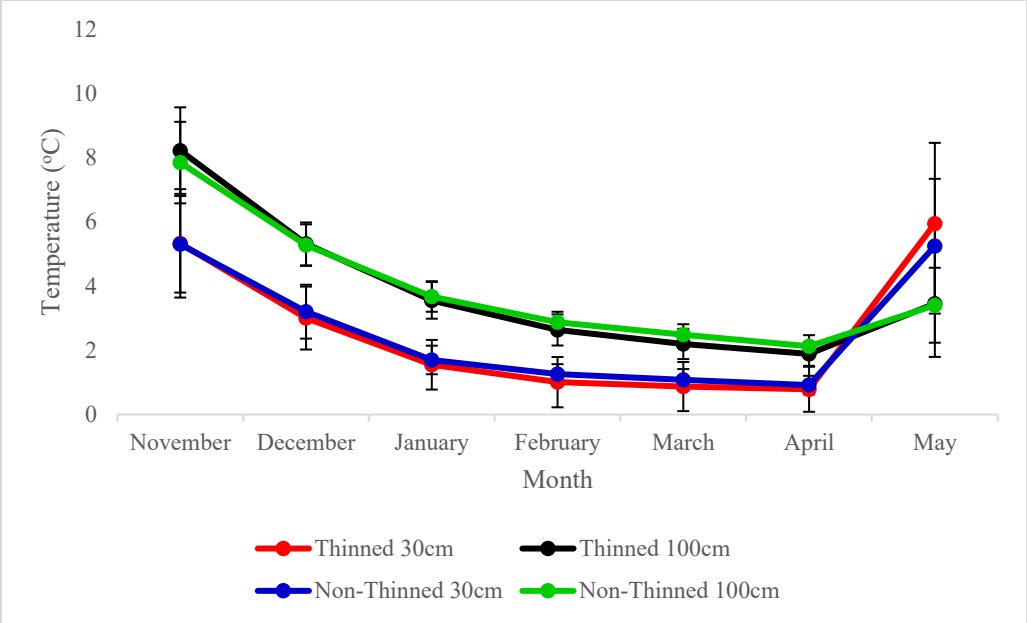

Figure 18. Soil temperatures from November 4, 2017 to May 13, 2018 at 30 cm and 100 cm depths under thinned and non-thinned tree canopies. Black bars indicate standard error of the mean.

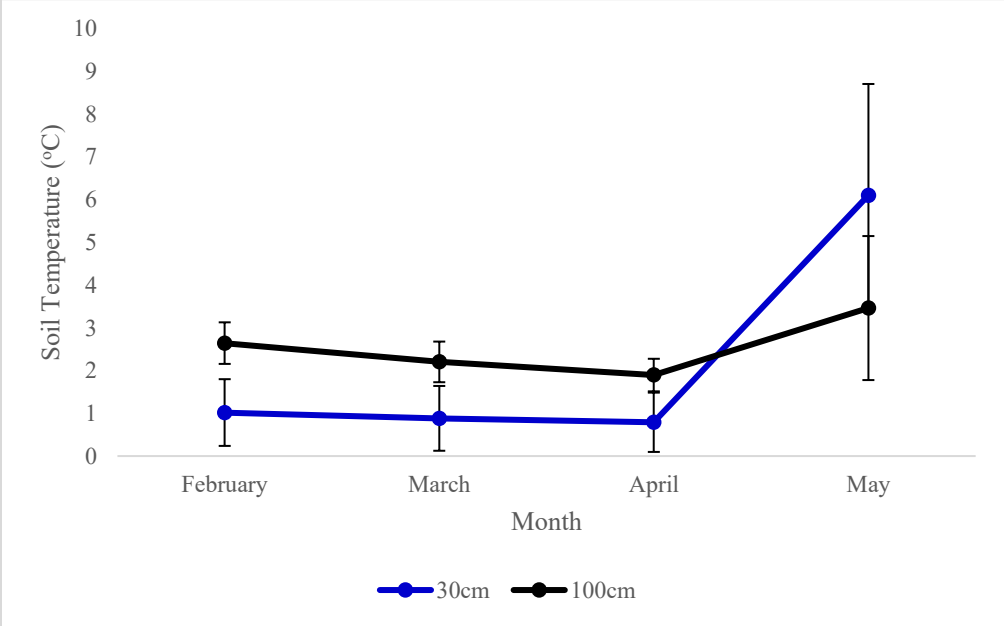

Figure 19. Soil temperatures at 30 cm and 100 cm from February 1, 2018 to May 13, 2018 where wooden blocks with *H. fasciculare* were buried. Black bars indicate standard error of the mean.

3.4.3 Snow depth

The thinned sites had significantly more snow compared to the non-thinned sites (t_4 = 9.95, p = 0.001). Thinned sites had 55.3 ± 1.15 SE cm of snow compared to 44.3 ± 1.52 SE cm for non-thinned sites. Despite these differences in snow cover, it had little impact on soil temperatures given there was no significant difference in temperatures between thinned and non-thinned sites.

3.4.4 *Hypholoma fasciculare* growth in response to burial depth

Hypholoma fasciculare continued to be active from mid-winter to mid-spring as a significant increase in growth was observed ($F_{1, 36}$ = 50.41, p < 0.001). No significant differences in *H. fasciculare* growth were observed at the different burial depths ($F_{1,18}$ = 1.87, p = 0.188) (Figure 20). Some blocks at both depths were very moist indicating that the soil was also very moist. The hyphae were very visible on the woodblocks that were damp (Figure 21).

Examining *H. fasciculare* growth as a daily rate from February 1, 2018 to May 13, 2018, at 100 cm depth the mycelium growth was similar to 30 cm depth (t_{22} = 1.25, p = 0.223). The *H. fasciculare* at 100 cm grew on average 0.31 ± 0.10 SE mm/day whereas the *H. fasciculare* at 30 cm grew an average of 0.25 ± 0.11 SE mm/day.

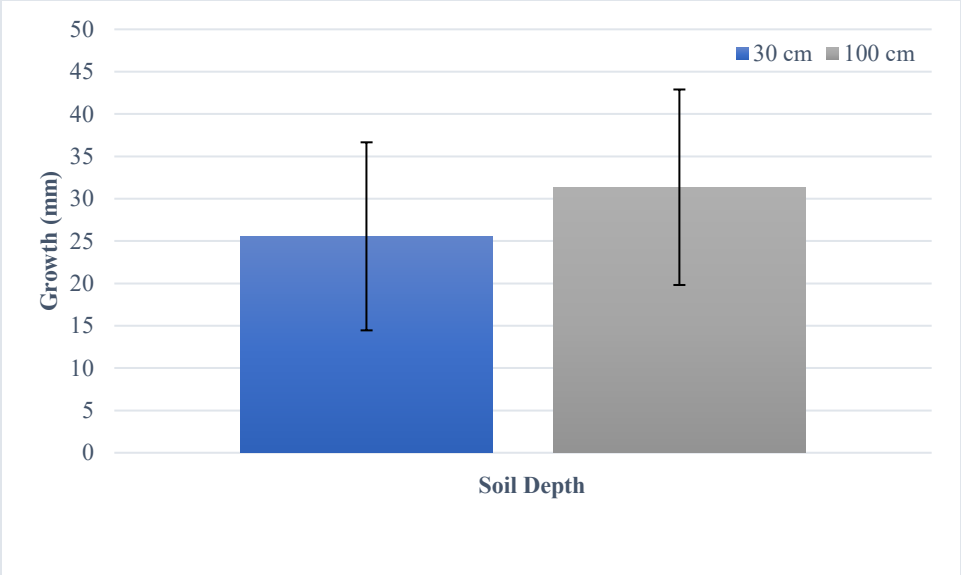

Figure 20. The growth of *H. fasciculare* at 30 cm and 100 cm soil depth from February 1, 2018 to May 13, 2018. Black bars indicate standard error of the mean.

Figure 21. Picture of woodblock from DRI on May 13, 2018, white hyphae from *H. fasciculare* visible.

3.5 Discussion

3.5.1 Soil temperature and depth

Soil temperatures are influenced by various factors including climate, soil type, depth, moisture, vegetation, litter and snow cover, and time of the year (MacKinney1929). We examined a number of these factors that could potentially impact fungi growth during winter and into early spring. There was a significant difference in temperature at deeper (100 cm) *versus* shallower (30 cm) soil depths. Soil temperatures were warmer at deeper depths throughout the winter season; thus, agreeing with the initial hypothesis. We anticipated that shallower soils would fluctuate more with respect to daily and seasonal ambient temperatures, which is possibly why the temperatures were still decreasing at 100 cm in April while they started to increase at 30 cm. At the shallower depth in May, soil temperatures increased to higher levels than what was observed during winter. This is interesting as it appears that shallower soils are very susceptible to changes in air temperature and can change quite rapidly in these plantations. Soil temperatures were higher at deeper depths during winter until the middle of spring when shallower soils warmed more quickly. This suggests that deeper soils tend to have a higher insulation value leading to warmer temperatures over winter, and a slower increase in temperatures once spring starts. Fungi growing at deeper soil depths would tend to grow better due to the higher temperatures over winter but as spring starts the growth would take much longer due to the slower increase in soil temperature. Minimum soil temperatures were reached in April at both shallow and deep soil depths, with soils at 100 cm depth recording 2^{o}C compared to 1^{o}C at 30 cm depth. The slow decline in temperature during winter months and slow increase during spring at deeper depths suggests that fungi at these depths will not experience as rapid an increase or decrease in temperature compared to those in shallower soils, possibly leading to differences in

survivability. These results were anticipated as the air temperatures were still fluctuating considerably during this time period and there was no snow cover, so more impacts on shallower soils would be expected.

Soil temperatures found within the pine plantations during the winter months were warm enough to maintain fungi growth. Soil temperatures in November and May were as high as 10ºC, which is more than adequate for growth of fungi such as *Armillaria* spp., *Hypholoma* spp., and *Heterobasidion* spp. For example, *Armillaria luteobubalina* is still able to produce some rhizomorphs at 10ºC (Pearce & Malajczuk, 1990; Rishbeth, 1978) while *Heterobasidion parviporum* can grow at temperatures as low as -4ºC (Müller et al., 2014). The soil temperature readings observed within our sites suggest that many root rot disease-causing basidiomycetes such as *Armillaria* spp. and *Heterobasidion* spp. may still be able to grow in Simcoe County, Ontario during the winter months, especially at deeper soil depths. This could create challenges for the usage of certain fungi biocontrol agents as they may not be as effective during winter months. *Phlebiopsis gigantea*, for example, is not as effective at controlling *Heterobasidion* spp. at temperatures below 10ºC (Oliva et al., 2015).

3.5.2 Impacts of stand thinning on soil temperature over time

The difference in soil temperatures were not statistically different between thinned and non-thinned canopy sites. This suggests that canopy cover from late fall to midspring within red pine plantations has little influence on the soil temperatures and that from a biocontrol perspective, each stand type should be treated equally in terms of treatment application due to the similarities. This is interesting as the non-thinned stands had nearly 100% canopy closure compared to the thinned stands that had 50%, meaning that sunlight on the forest floor has little impact on the soil temperatures and that ambient temperatures appeared more influential from

late fall to mid-spring. This finding disagrees with Boggs and McNulty (2010) who found that greater canopy closure (100%) leads to colder forest floor temperatures during winter compared to 45% or lower canopy closure in the absence of snow cover. The coldest soil temperatures reached at deep and shallow soil depths were 1.3°C and 0.1°C, respectively. The 100 cm depth was able to stay over a degree warmer than the 30 cm depth. Examining air temperatures during April when soil temperatures were the coldest, the minimum daily ambient temperatures ranged from -3.9 ± 3.7 SE °C and the maximum daily temperatures ranged from 5.2 ± 5.81 SE °C (Environment Canada, 2018). This suggests that the soil temperatures have a lag in response relative to the ambient temperatures as air temperatures were warmer than they were during the winter months.

3.5.3 Snow depth

Snow depths measured in January confirmed that there was significantly less snow accumulating in the non-thinned sites compared to the thinned sites. The non-thinned sites had approximately 10 cm less snow compared to the thinned sites. This finding agrees with the initial hypothesis. However, this difference did not impact soil temperatures in our sites. This finding was unexpected as Buckeridge & Grogan (2008, 2010) observed that deeper snow cover generally resulted in warmer soil temperatures. Perhaps the overstory of the non-thinned sites compensated for the differences in snow cover and provided some measure of thermal cover to compensate for differences in snow depth. Zhang (2005) did note that snow depth beyond 40 cm has little effect on further insulating the soil, and that this depth is essentially the maximum insulating threshold. This agrees more with our findings as the lowest snow depth recorded was 43 cm in January which would have been enough to insulate the soil from other factors such as

ambient temperature. It remains unclear what differences in snow cover depth are required to impact soil heat exchange.

3.5.4 *Hypholoma fasciculare* growth in response to burial depth

Hypholoma fasciculare was able to grow in the soil from February to May (midwinter to midspring). Thompson and Body (1983) suggest that basidiomycetes such as *Hypholoma fasciculare* may not persist in deeper root systems whereas *Armillaria* spp. can. However, our study discovered that *Hypholoma fasciculare* can grow on red pine wood at deep and shallow soil depths. No significant differences in *H. fasciculare* growth among blocks placed at the different soil depths were observed. This disagrees with the initial hypothesis that *H. fasciculare* growth at 100 cm depth would exceed the growth at 30 cm because of the potentially higher temperatures at the deeper soil depth. Thus, differences in soil temperatures between 100 cm and 30 cm were not great enough to impact *H. fasciculare* growth. There may have been differences in growth if the *H. fasciculare* blocks were placed at 5 cm depth rather than 30 cm, due to a higher possibility of the ground being frozen and even colder temperatures closer to the surface. Further study is required to determine this.

At 5ºC *H. fasciculare* has been shown to be able to grow at 0.2 mm/day when grown *in-vitro* on 2 % malt agar (Dowson et al. 1989). We observed slightly different results under field conditions using pine blocks. Our study found that the *H. fasciculare* could grow slightly faster than what Dowson et al. (1989) reported, and our temperatures were slightly colder than 5ºC during our testing period. Our increased growth rate could be related to the strain of *H. fasciculare* chosen for our study as Dowson et al. (1989) did not mention the *H. fasciculare* strain tested in their study. The *H. fasciculare* (Pinnel B strain) grew on average 0.31 ± 0.10 SE mm/day at 100 cm whereas at 30 cm, it grew an average of 0.25 ± 0.11 SE mm/day. Since the *H.*

fasciculare was able to grow on the red/white pine woodblocks at these temperatures it suggests that *H. fasciculare* (Pinnel B strain) has the potential to grow on red pine roots during the winter months.

Visual inspection of wood blocks upon retrieval during spring suggest that decay over the course of winter was minimal. Wood decay of stumps is the goal for *H. fasciculare* to be an effective biocontrol against *Armillaria* root rot as resource capture would mean that *Armillaria* spp. would have no means of growth, and if *H. fasciculare* can replace *Armillaria* spp. within a root system then that would lead to mortality of *Armillaria*. While the pine blocks were not measured for decay, future research should examine the ability of *H. fasciculare* to decay wood during the winter months, as growth and decay rates are two important aspects in finding an effective biocontrol (Chapman & Xiao, 2000; Wells & Boddy, 1995). The blocks excavated from one site (DRI) at both depths were very dark and moist and hyphae from *Hypholoma* were visible. This suggests that moist soils increase moisture within the wood which is needed for *H. fasciculare* to forage and decay wood (Dowson et al. 1989). The wood blocks also had a distinct odor where the *H. fasciculare* growth established, similar to when *H. fasciculare* is found on decaying roots, indicating that the *Hypholoma* were quite active and healthy. The blocks from all other sites were relatively dry and the hyphae were not visible. Carreiro & Koske (1992) discovered that soil moisture can impact fungal activity within the soil. This is promising as over a period of time (i.e. one year) the blocks may become further decayed, and it may be possible for the *H. fasciculare* to decay wood over the winter season and prevent *Armillaria* establishment.

3.6 Conclusion

Soil temperature is dependent on soil depth and the time of the year. Soil temperatures reach lower minimum temperatures shallower versus deeper soil depths throughout the winter months in pine plantations located in Simcoe County, Ontario, Canada. However, temperatures remained above freezing at both depths studied (0 and 2ºC at 30 and 100 cm depths, respectively). Temperatures were more variable in shallow versus deeper soils, particularly at the onset of the winter and the beginning of spring where temperatures rapidly declined and rose, respectively. *Hypholoma fasciculare* (Pinnel B strain) was able to grow equally well at temperature conditions observed at both depths examined from midwinter to midspring. This study concludes that winter and early to midspring soil temperatures are suitable for fungi growth and wood decaying fungi such as *H. fasciculare* are able to continue to grow, albeit at a reduced rate. Future research could focus on testing the growth and interactions of *H. fasciculare* and *Armillaria ostoyae* at colder temperatures to better understand the biocontrol implications of using *H. fasciculare* across North America, and whether *H. fasciculare* has a competitive advantage during winter. Future climate conditions suggest that surface temperatures will increase leading to lower snowfall amounts and higher precipitation as rain annually. It is further predicted that there will be more days with no snow cover, potentially leading to colder soil temperatures during winter (Boland et al. 2004; Templer, 2012). However, mean annual soil temperature may increase or decrease due to shorter winters with less snow cover, or due to a lower snow insulating threshold (Jungqvist et al. 2014). Summers are expected to be warmer with more precipitation and higher occurrences of drought (Boland et al. 2004). These changes in climate suggest that *H. fasciculare* may have decreased growth rates during the shorter winters and may even become dormant, and during the summer the growth rates may increase due to the

increased temperatures which will also lead to an increase in *Armillaria* growth rates. There will be a longer growing season due to the increase in temperatures and shorter winters which will lead to *Armillaria* root rot becoming more of a concern, and also the increased occurrence of drought will lead to more stress and increased mortality for pine species, which will further increase resource availability for *Armillaria ostoyae*.

Chapter 4: Conclusions

Conifer plantations in Simcoe County, Ontario, Canada consist of mostly red pine and white pine. These plantations are designed to turn old unproductive agricultural land into highly productive (ecologically and economically) plantations. The end goal for these plantations is to achieve a mixedwood or deciduous dominant forest as thinning the plantation every 9 years after the stand is 30-40 years old allows natural seed-in of deciduous and coniferous species (G. Davis, personal communication, 07/07/17). *Armillaria* root rot is present within these plantations and is causing tree decline and mortality in multiple *Pinus* species stands across North America (Dumas, 1988; Łakomy et al. 2014; McLaughlin, 2001). This disease is leading to tough management decisions for forest managers and is also impacting the long-term stand goals of achieving a mixedwood or deciduous dominant stand. Currently, there is no effective treatment for the disease. Mechanical treatment (e.g., stump removal) is not effective (Cleary et al. 2013), and ways to minimize the establishment of *Armillaria ostoyae* such as: (1) care when tree harvesting to reduce damage to the uncut trees, and (2) planting or managing species that are not susceptible to the disease (Lockman & Kearns, 2016) do not really help maintain *Pinus spp.* across the landscape. Biocontrol treatment options for *Armillaria ostoyae* have been briefly tested in British Columbia, Canada using wood decaying basidiomycetes such as *Hypholoma fasciculare* (Chapman & Xiao, 2000) and in the lab using *Phlebiopsis gigantea* (Keča, 2009). Further testing is required to find an effective biocontrol candidate that is suitable for the northeastern US and Canada. Finding a treatment for *A. ostoyae* is significant to areas such as Simcoe County, Ontario, Canada where nearly half of the County-owned forest landscape alone is red and white pine plantations that are impacted by the disease, not including privately-owned plantations (Simcoe County Forest Management Plan 2011-2030). *Armillaria* root rot leads to

substantial revenue losses for private and public land owners. Long-term management objectives (i.e. natural transition to a mixed wood forest) are in jeopardy due to the scale of red pine decline, causing forest managers to change their objectives and try to salvage remaining healthy trees (i.e. clear-cutting red pine) (G. Davis, personal communication, 07/07/17). To address the challenge of finding a viable biocontrol agent against *Armillaria ostoyae*, a laboratory (*in-vitro*) and field study were completed.

Various strains of *Hypholoma fasciculare, H. sublateritium, H. capnoides*, and *Phlebiopsis gigantea* were subjected to laboratory (*in-vitro*) competition trials with *A. ostoyae* to examine their efficacy as biocontrol agents against *A. ostoyae.* The main objective for the lab study was to examine a suite of strains from *H. fasciculare, H. capnoides, H. sublateritium*, and *P. gigantea* on artificial and natural growth media to determine if variability exists among strains of the same species with respect to their ability to reduce or prevent the spread of *Armillaria ostoyae.*

Macroscopic and microscopic examination determined that *H. fasciculare* Pinnel B strain is the most promising biocontrol agent for *A. ostoyae*. *Hypholoma fasciculare* strain Pinnel B was the fastest growing strain when paired with *A. ostoyae* on both the growth media and reduced the growth of *A. ostoyae* by over 70% compared to when *A. ostoyae* was growing on its own. *Hypholoma fasciculare* strain Pinnel B consistently overgrew the *A. ostoyae* colony and suppressed its rhizomorph production. Microscopic observation of their interaction revealed swelling of the hyphal tips at their points of contact. These findings agreed with the hypotheses that *H. fasciculare* strain Pinnel B is the most effective biocontrol agent against *A. ostoyae* to date. Future work could include the examination of Pinnel B versus other strains of *A. ostoyae* as it has been shown that there is a high amount of variability within the *A. ostoyae* species

(Schulze and Bahnweg, 1998). Field trials representing various growth seasons need to be conducted to determine *H. fasciculare* strain Pinnel B's effectiveness in pine plantations against *A. ostoyae*.

A field study was conducted with the *Hypholoma fasciculare* strain Pinnel B to further understand its growth and survival during winter season in central Ontario, Canada. The objective for this study was to determine if winter soil temperatures in Simcoe County are suitable for the growth of *H. fasciculare*. Soil temperatures were monitored as well as the growth of *H. fasciculare* strain Pinnel B was studied in the field during the winter months of 2018 at plantations in Simcoe County, ON. Our results demonstrated that temperatures at 30 cm depth were significantly cooler over the winter months compared to 100 cm depth. This agreed with our initial hypothesis that warmer temperatures exist at deeper soil depths. Thinned versus non-thinned plantations and snow thickness above the soil had no effect on the soil temperatures as the temperatures were similar between the two plantation types that had different snow thickness. However, the amount of snow present was enough to eliminate the influence of ambient temperatures on the soil temperatures. This finding disagreed with our hypothesis. *Hypholoma fasciculare* Pinnel B had a significant increase in growth from February 1, 2018 to May 13, 2018. However, soil depth did not influence fungal growth due to very low temperature variation between the two soil depths. This finding also disagreed with our hypothesis. The finding that *H. fasciculare* is able to grow in field conditions during winter months complimented the lab findings. We found that *H. fasciculare* strain Pinnel B can grow and survive during winter. Another important finding was that the *H. fasciculare* strain Pinnel B could grow on the red/white pine blocks used for the study. This means that there is potential for *H. fasciculare* Pinnel B to establish and grow on red pine stumps year around. Further testing is needed to

determine if/how well *Armillaria ostoyae* grows during the winter months and with and without the presence of a biocontrol agent. Testing should also include field trials examining the competitive interactions between *H. fasciculare* strain Pinnel B and *A. ostoyae*.

This research has potential benefits for forest managers in terms of working to achieve their long-term stand objectives and make the plantations more productive. A biocontrol product consisting of *H. fasciculare* could potentially be applied to the stump tops during harvesting as it has the capacity to establish and grow year-round on the stumps and thus to provide long-term protection against *A. ostoyae* infection. Treatment for *A. ostoyae* might also allow increased revenue for land owners as it results in sustaining larger merchantable pine trees overtime.

Challenges still exist for using *H. fasciculare* strain Pinnel B as a biocontrol treatment against *A. ostoyae*. This includes more intense study to understand its long-term survival in other parts of North America (i.e. eastern Canada) and whether it can establish and grow on red and white pine root systems. There will be an ongoing challenge as to how to apply *H. fasciculare* to a site. Applying *H. fasciculare* to a plantation site needs to be both cost effective and non-labour intensive otherwise there may be no market for such a product. This will be one of many major barriers in trying to implement *H. fasciculare* as a biocontrol agent in the future.

Climate change is another factor that will lead to challenges in developing a biocontrol for *A. ostoyae*. It is predicted that the future will experience an increase in summer temperatures with periods of more precipitation, and prolonged periods of drought (Boland et al. 2004). This increase in temperature and periods of drought will undoubtedly lead to increased stress for red and white pine leading to higher levels of tree mortality as trees become more susceptible to *Armillaria* root rot. The increased temperatures will also lead to an increased *Armillaria ostoyae*

growth throughout the summer months. Thus, steps to use *H. fasciculare* as a biocontrol for *A. ostoyae* need to be implemented sooner rather than later.

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Appendices

Appendix A

Fungal species, strains, region, and country of original collection examined for *in-vitro* studies. Species/strains highlighted in green are strong competitors, blue are midgrade competitors, and red are poor competitors when competing with *A. ostoyae*.

Species	Strain	Region	Country
Hypholoma fasciculare	OKM-2932-T	Idaho	USA
Hypholoma fasciculare	OKM-7107-Sp	Maryland	USA
Hypholoma fasciculare	RLG-11562-Sp	Arizona	USA
Hypholoma fasciculare	RLG-12668-Sp	Arizona	USA
Hypholoma fasciculare	HHB-14801-Sp	Washington	USA
Hypholoma fasciculare	JPL-62-Sp	Arizona	USA
Hypholoma fasciculare	Pinnel B	British Columbia	Canada
Hypholoma fasciculare	FP-133566-Sp	Oregon	USA
Hypholoma sublateritium	OKM-6192-Sp	Virginia	USA
Hypholoma sublateritium	HHB-11948-Sp	Michigan	USA
Hypholoma sublateritium	49-1107	New Hampshire	USA
Hypholoma sublateritium	FP-90085-Sp	New York	USA
Hypholoma sublateritium	OKM-6947-Sp	Maryland	USA
Hypholoma capnoides	OKM-1523-T	Idaho	USA
Hypholoma capnoides	TAK2	Ontario	Canada
Hypholoma capnoides	TAK5	Ontario	Canada
Hypholoma capnoides	TAK6	Ontario	Canada
Hypholoma capnoides	SSM	Ontario	Canada
Armillaria ostoyae	B249-28	Ontario	Canada
Armillaria ostoyae	P162-7	Ontario	Canada
Phlebiopsis gigantea	VRA 1992	Quebec	Canada