Effect of different temperatures and pH on the growth and interactions of *Hypholoma species* and *Phlebiopsis gigantea* with *Armillaria ostoyae* and *Heterobasidion irregulare*

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Abstract

Root and butt rot diseases caused by the fungal pathogens *Armillaria ostoyae* and *Heterobasidion irregulare,* result in substantial economic and ecological losses to pine plantations across southcentral Ontario, Canada. Biocontrol agents like *Hypholoma* species and *Phlebiopsis gigantea* offer potential solutions for managing these diseases. However, their effectiveness against these pathogens is highly influenced by key abiotic factors such as temperature and soil pH. I investigated the impact of temperature and pH on the growth of fungal pathogens *A. ostoyae* (strains: B249-28 and P162-7) and *He. irregulare*, in the presence and absence of biocontrol species and strains of *Hypholoma* spp. (*Hypholoma fasciculare, Hy. sublateritium, Hy. capnoides*) and *Phlebiopsis gigantea.*

Single and paired colony *in vitro* experiments were conducted using 2% malt agar media at 2, 10, 15, 20, 25, and 30°C, and pH levels of 5.0, 6.0, 7.0, and 8.0 to compare and contrast growth responses in the absence and presence of competition between the pathogens and their respective biocontrol species and strains.

Results indicate that *A. ostoyae* strain P162-7 (less virulent) grew better at extreme temperatures (2°C and 30°C) compared to strain B249-28 (more virulent) ($p = 0.04311$), while *He. irregulare* showed little growth at 2°C and none at 30°C. Among *Hypholoma* spp., *Hy. fasciculare* strain Pinnel B consistently outgrew others across all temperatures, with the exception of 30°C, where no growth was observed. In paired cultures, *Hy. capnoides* strain TAK 5 maintained consistent growth at 25°C despite competition, while *Hy. sublateritium* strain OKM-6947-SP showed increased growth during competition ($p < 0.001$). *Hypholoma fasciculare* strain RLG-12668-SP emerged as a more efficient antagonist strain compared to Pinnel B at elevated temperatures, showing higher growth in the presence of *A. ostoyae.* Biocontrols *Hy. fasciculare* and *P. gigantea* demonstrated higher growth rates at all tested temperatures (all $p<0.05$), and displayed antagonistic interactions with *A. ostoyae* and *He. irregulare*, respectively.

Armillaria ostoyae showed optimal growth at pH 5, while *He. irregulare* preferred pH 7. Among *Hypholoma* spp., *Hy. fasciculare* (Pinnel B) consistently showed highest growth across all pH levels, while *Hy. capnoides* (TAK 5) showed the lowest. *Phlebiopsis gigantea* showed maximum growth at pH 6. In paired cultures, *Hy. fasciculare* and *Hy. sublateritium* effectively inhibited *A. ostoyae* growth at pH 5 and 6, but *Phlebiopsis gigantea* could not effectively inhibit *He. irregulare* growth at pH 8, suggesting potential challenges in disease control in the event of increased soil pH due to climate change. These findings demonstrate the necessity of accounting for temperature and pH variations in developing biocontrol strategies against *A. ostoyae* and *He. irregulare*.

Key words: Armillaria ostoyae, *Heterobasidion irregulare*, *Hypholoma* spp., *Phlebiopsis gigantea*, abiotic factors, temperature, pH, growth, interactions, climate change.

Lay summary

Lakehead University's Department of Biology mission statement is that "faculty and students in the department of Biology are bound together by a common interest in explaining diversity of life, the fit between form and function, and the distribution and abundance of organisms". In line with this mission, my study investigates how environmental factors, specifically temperature and pH, affect the growth and control of common forest pathogens responsible for root rot diseases and revenue loss: *Armillaria ostoyae* and *Heterobasidion irregulare*, as well as non- pathogenic species and strains of *Hypholoma* spp. and *Phlebiopsis gigantea*, that may be used to control these pathogens.

The main research questions addressed are: (1) do different temperatures and pH impact the growth of various strains of pathogenic *A. ostoyae* and *He. irregulare*, and their biocontrol counterparts? (2) How does the presence of biocontrol species influence pathogen growth? Additionally, the response of biocontrol species to changing environmental conditions is explored. Conducted in controlled laboratory settings, this study reveals that different temperatures have a significant effect on the growth and spread of pathogens and biocontrol species. Higher temperatures, potentially linked to climate change, may elevate the risk of root rot diseases by *Armillaria ostoyae*, while certain biocontrol species, such as *Phlebiopsis gigantea*, show promise in inhibiting the growth of *Heterobasidion irregulare* even under warmer conditions. Furthermore, it was found that soil pH plays a crucial role in influencing the growth of all species under investigation. Interestingly, biocontrol species demonstrated higher growth even at pH levels favored by pathogens, suggesting their potential for controlling disease spread.

This research sheds light on the dynamics between forest pathogens and biocontrol species in response to changes in environmental conditions. The importance of considering

both temperature and pH variability when developing biocontrol strategies against forest pathogens is emphasized.

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Chapter 1 Introduction

1.1 Overview

Pinus resinosa (red pine) conifer plantations are known for generating high-value timber and are extensively planted in southern Ontario (McLaughlin et al., 2010). However, decline in growth and mortality continue to be the major concerns for forest managers (Davis, 2011; McLaughlin et al., 2011). Decay and mortality of these coniferous plantations in southern Ontario are caused by the synergy of biotic and abiotic elements. Biotic factors include pests and fungal root rot pathogens, such as the Pine false webworm and *Armillaria* spp., while abiotic factors involve weather-related challenges like windthrow, droughts, and snow damage, along with anthropogenic pollutants such as smog and road salts, leading to substantial ecological and economical losses (Davis, 2011; McLaughlin et al., 2011; Ontario Ministry of Northern Development, Mines, Natural Resources and Forestry, 2021). Over 20 million m^3 of wood is lost annually in Ontario due to forest decay, and of that 11 million m^3 is specifically attributed to fungal pathogens causing root rot diseases (Dumas, 2013).

Fungal pathogens reduce water and nutrient absorption ability of trees by killing the roots and decaying the lignin and cellulose in the roots (Hammel, 1997; Heinzelmann et al., 2019). The pathogens, once established, become permanent components of the infected site by surviving saprotrophically in the root systems for decades, even after the death of the host, and thus, can have long-term impacts on forest ecosystems (Baumgartner et al., 2011; Lockman and Kearns, 2016).

Most fungal pathogens spread either through root-to-root contact between an infected and a healthy tree or by the production of airborne spores (Lockman and Kearns, 2016). Their establishment and spread are highly influenced by environmental factors and soil

characteristics such as temperature, pH, precipitation, and moisture (Mallet and Maynard, 1998; Kliejunas et al., 2009).

The most common disease-causing pathogens of trees in the circumpolar boreal forest regions are *Armillaria* spp. (causing *Armillaria* root rot) and *Heterobasidion* spp. (causing Annosus root rot) (Cleary et al., 2013). These pathogens are also of concern in southern Ontario, Canada, as *Armillaria* root rot and Annosus root rot cause substantial mortality in conifer plantations throughout the region (McLaughlin et al., 2010; Dumas, 2013).

1.2 Root rot diseases

1.2.1 *Armillaria* root rot

Armillaria root rot is caused by *Armillaria* species, which belong to the group of mushroom-forming fungi known as Agaricomycetes, Division - Basidiomycota (Heinzelmann et al., 2019; Wijayawardene et al., 2022). *Armillaria* is one of the most important fungal genera causing root rot diseases in a wide range of tree and shrub species worldwide, including numerous managed stands of economically important timber species such as *Pinus* and *Abies* spp. (Keca 2009; Oliva et al., 2009; Lushaj et al., 2010; Baumgartner et al., 2011; Tsykun et al., 2011). There are over 40 known species of *Armillaria* present worldwide, with varying degrees of pathogenicity to conifers and hardwoods of all ages (Baumgartner et al., 2011; Heinzelmann et al., 2019). Among these known species, six species of *Armillaria* can be found throughout central and southern Ontario: *A. ostoyae, A. calvescens, A. mellea, A. gallica, A. sinapina, and A. gemina* (McLaughlin, 2001b). Of these, *A. ostoyae* is the most commonly found, aggressive, and virulent species (Rishbeth, 1982; Mallett, 1990; Rizzo and Harrington, 1993). *Armillaria ostoyae* is typically found on sites dominated by conifers (Rishbeth, 1982; McLaughlin, 2001b) and is the most widely distributed species in Ontario, British Columbia, and the prairie provinces of Canada

(Morrison et al., 1985; Dumas, 1988; Mallett, 1990; Shaw and Kile, 1991; Guillaumin et al., 1993; McLaughlin, 2001a, 2001b ; La porta et al., 2008; Hagle, 2010; McLaughlin and Hsiang, 2010).

Armillaria ostoyae grows rapidly through root-to-root contact between healthy and infected trees and spreads infection by root-like structures called rhizomorphs in the upper layer of soil (Rishbeth, 1985; Baumgartner et al., 2011; Kubiak et al., 2017). The transmission of disease by basidiospores, generated as a result of sexual reproduction, is restricted due to their brief viability (Kim et al., 2022). Hence, infection spread is primarily through mycelium and rhizomorph formation (Tsykun et al., 2011; Kim et al., 2022). Rhizomorphs are a network of thick, extensive cords of cylindrical aggregates of hyphae with tough pigmented walls (for its protection), that spread under the soil to find new food sources, ultimately penetrating root tissues of host trees (Guillaumin et al., 1993; Isaac, 1995; Tsykun et al., 2011). Upon coming in contact with the host, these structures form a white-coloured mycelial fan at the base of the host trunk (Heinzelmann et al., 2019), which is a key diagnostic sign of the disease.

1.2.2 Disease cycle of *Armillaria* spp.

Basidiospores germinate on a woody substrate and form a haploid or diploid mycelia. The compatible haploid mycelia mate and form a diploid mycelium that colonizes the substrate. The diploid mycelium spreads further and infects the host, forming reproductive fruiting bodies at the base of the host, which later mature and release basidiospores into the environment (Fig. 1) (Kim et al., 2022).

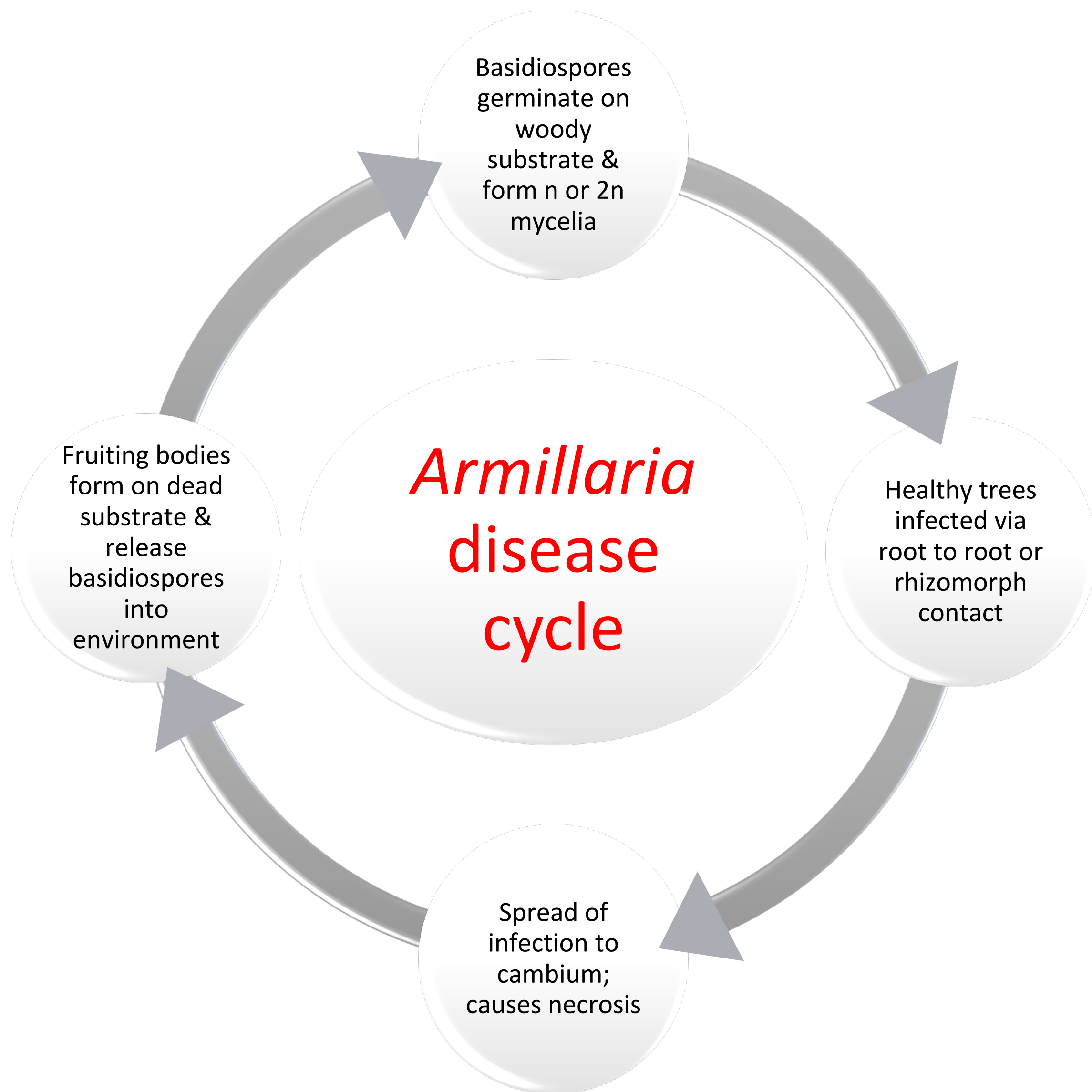


Figure 1. Representation of disease cycle of *Armillaria* species (Kim et al., 2022)

Armillaria spp. are both saprobic and parasitic. If a parasitic species establishes on the roots of a living healthy host, it can infect the cambium and degrade all components of the plant cell wall, including lignin, cellulose, hemicellulose, and pectin, and ultimately cause mortality (Heinzelmann et al., 2019). Some *Armillaria* species act as a primary parasite in healthy plantations or persist as a secondary parasite in plantations already weakened by other abiotic and biotic agents, and are, therefore considered opportunistic pathogens (Gregory et al., 1991; Shaw and Kile, 1991). Note that some species such as *A. cepistipes* and

A. gallica are generally saprotrophic and may be less virulent (Tsykun et al., 2011). These species can coexist with parasitic species such as *A. ostoyae* or *A. mellea* (Heinzelmann et al., 2019) and play an important role in mineral and carbon cycling (Heinzelmann et al., 2019), making them important components of forest ecosystem (Kile et al., 1991). The necrotrophic-saprobic nature of parasitic *Armillaria* species allows the mycelia to survive on the site for decades and serve as inocula for subsequent plantations (Baumgartner and Rizzo, 2001; Ferguson et al., 2003; Baumgartner et al., 2011). One strain of *A. ostoyae* found in mixed coniferous forests in Oregon, USA, for example, is estimated to be at least 1,900 years old (Ferguson et al., 2003).

Symptoms of infection by *Armillaria* spp. may include wood decay, thin foliage, wilting and premature defoliation, lower stem deformation, resinous root lesions, and growth reduction (Baumgartner et al., 2011; Cruickshank and Filipescu, 2012; Heinzelmann et al., 2019). Infection can also be recognized by the formation of white-colored mycelial mats and rhizomorphs that grow below ground, killing the roots and eventually the whole tree (McLaughlin et al., 2010; Baumgartner et al., 2011).

Various methods have been used to control and prevent the spread of *Armillaria* root rot in managed forests. This includes planting resistant tree species, avoiding hazardous sites, chemical treatments, and biological control methods (Shaw and Roth, 1978). To better manage this disease, forest managers need to more closely monitor plantation health and adopt strategies that favour the resistant or more tolerant host plant species of pine during thinning and regeneration operations (Filip et al., 2010). For example, soil pH should be checked before establishing new plantations as it has been found that red pine plantations established in alkaline soils having an average pH of 8 or above (especially in horizon C) are more susceptible to root-rot diseases (McLaughlin et al., 2011). Avoiding these high-risk sites may help prevent infection. In infected stands, since the mycelia can remain viable for a

long time, even in dead plantations, mechanically removing infected stumps can be done to reduce long-term disease spread (Cleary et al., 2013; Morrison et al., 2014). However, this method is not always successful, as the site disturbances caused while removing the infected stumps can increase the aggressiveness of *Armillaria* (Quesnel and Curran, 2000). In general, converting or clearing the sites may cause an increase in the *Armillaria* infection due to soil disturbances (Quesnal and Curran, 2000). Soil disturbances may lead to other soil-related problems such as soil erosion and nutrient loss (Heinzelmann et al., 2019), and any tree wounding during stump removal process or thinning operations can risk the further spread of the infection (Filip et al., 2010). Moreover, the mechanical removal of stumps can be prohibitively expensive. Chemical products such as Armillatox (phenolic compounds) have shown some ability to decrease rhizomorph activity in conifer plantations, but these phenolic compounds may cause severe phytotoxic effects in tree roots (Shaw and Roth, 1978; withdrawn in 2003 in UK). As such, the biocontrol method, using biological agents such as fungi, antagonistic to the pathogen, is suggested to be an effective alternative because of its more environmentally friendly nature (Rönnberg & Cleary, 2012; Sivanandhan et al., 2017). Biological control (or biocontrol) of plant diseases is the application of a biological agent such as a bacteria or fungi to a host plant species to prevent and control the development of a pathogenic disease in the host (Witters, 1985; O' Brien, 2017). The use of a biocontrol can be an effective means of curbing the damage caused by pathogens. Being natural enemies of the disease-causing pathogens, biocontrol agents are likely to be more specific in effect than most chemicals, and less likely to leave a harmful residue in the environment (Collinge et al., 2022).

The development of antagonistic organisms and their inoculation on *Armillaria* infected stumps may be effective where measures such as stump extraction are impractical (Shaw and Roth, 1978). Dowson (1988) suggested that saprotrophic cord-forming fungi such

as *Hypholoma* spp. can be used as biocontrol fungi for *Armillaria* root disease. Fungal biocontrols are specific to their targets, using mechanisms such as (a) competing for resources, such as oxygen and nitrogen, or for space by producing extracellular hydrolytic enzymes and toxic metabolites to restrict pathogen growth, (b) mycoparasitism, or by (c) indirectly inducing host resistance against the pathogen (Pieterse et al., 2014; Jensen et al., 2017; Thambugala et al., 2020, Collinge et al., 2022). Cord formers immobilize the pathogen by inhibiting or destroying the outgrowth of *Armillaria* rhizomorphs (Dowson, 1988). Being saprotrophic, they can persist in wood for a long time (Dowson, 1988). Their rapid growth can prevent or reduce *Armillaria* invasion and colonization by reducing the available wood base through aggressive competition for space and by exuding chemicals that kill or weaken the *Armillaria* (Chapman and Xiao, 2000). Currently, there are no biological control products registered for use against *Armillaria* root rot.

1.2.3 Annosus root rot

Annosus root rot is caused by *Heterobasidion* species. *Heterobasidion* species are present worldwide and can be found in almost every coniferous plantation (Schmitt et al., 2000). *Heterobasidion annosum* sensu lato (s.l.) is a species complex of deadly necrotrophic fungal species that infects conifers in the northern hemisphere (Asiegbu et al., 2005). It comprises five fungal species, three of which are European: *He. annosum s.s., He. parviporum, He. abietinum*, and the remaining two are native to North America: *He. irregulare* and *He. occidentale* (Asiegbu et al., 2005; Dalman et al., 2010). *Heterobasidion* species have a wide range of conifer and broad-leaved tree hosts. One species, *He. irregulare,* is specifically known for infecting commercial species of conifers such as pine (Asiegbu et al., 2005; Otrosina and Garbelotto, 2010). It is the most virulent fungal pathogen of red pine plantations in southern Ontario, Canada (McLaughlin et al., 2010).

The infection spreads mainly by basidiospores, produced through sexual reproduction, which travel through wind or insects such as *Hylobius abietis* (Kadlec et al., 1992; Asiegbu et al., 2005). Stumps from freshly harvested trees serve as a port of entry for the disease (Redfern and Stenlid, 1998). Further infection occurs through vegetative spread of mycelium through the soil that establishes root-to-root contact between infected and healthy hosts (Hodges, 1969; Asiegbu et al., 2005). The disease cycle begins with natural wounds on the trees (Fig. 2). Sexually produced basidiospores arrive and germinate on this wounded wood substrate, forming mycelia that spread and colonize the wood, eventually decomposing the substrate. Fruiting bodies are formed outside the trunk of the tree after obtaining enough nutrition from the decomposition activity.

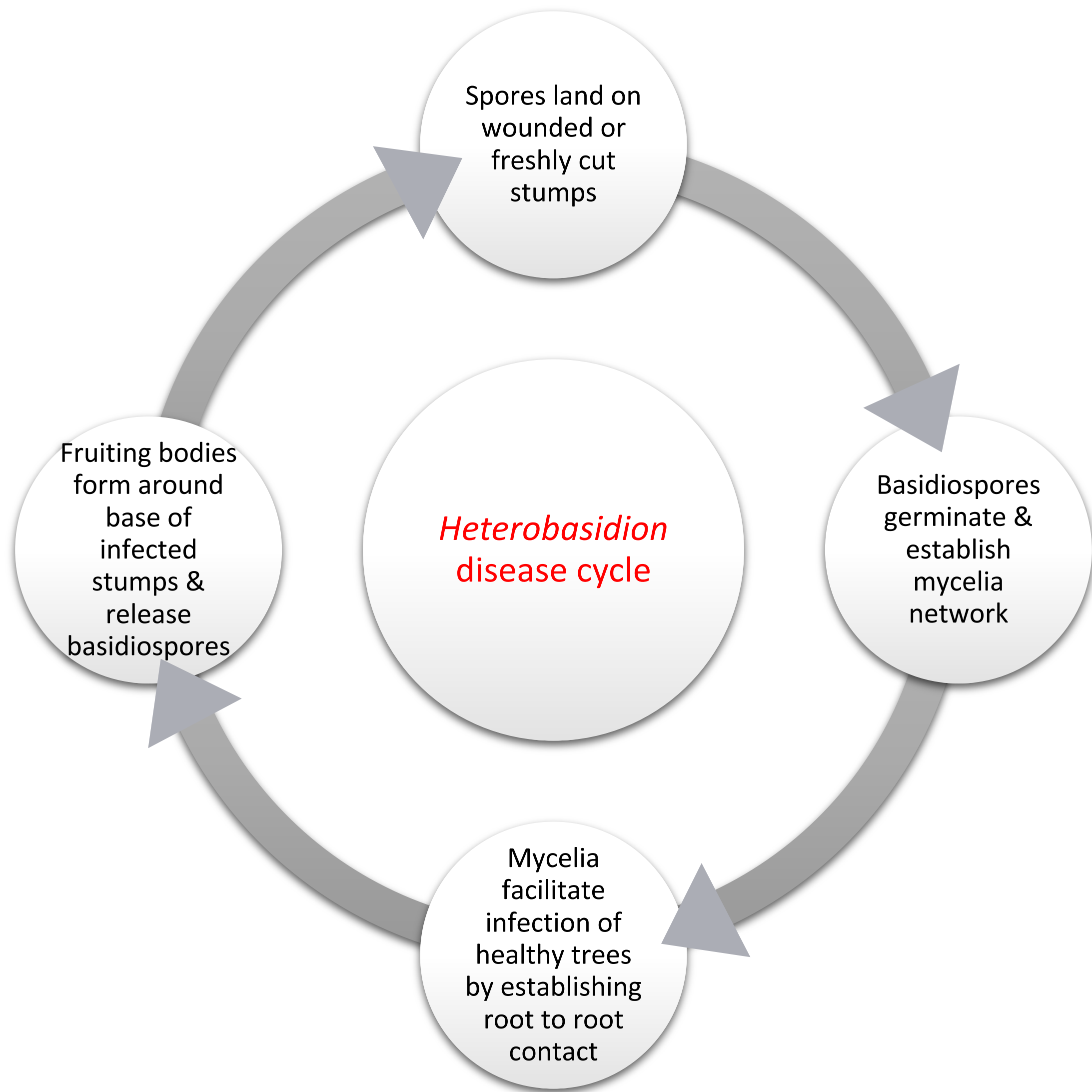


Figure 2. Representation of disease cycle of *Heterobasidion* species (Asiegbu et al., 2005)

The symptoms of infection by *Heterobasidion* spp. are difficult to detect during the initial stages because the host often appears healthy until nearly half of the root system is damaged (Wang et al., 2014). Initial symptoms of rot appear as a pale-yellow stain on the bark. This is followed by a light brown decay and then white pocket rot when in advanced stages (Asiegbu et al., 2005). *Heterobasidion* infection increases plantation susceptibility to windthrow and predisposes the host to other pathogens like bark beetles (Kliejunas et al., 2009). Consequently, it causes chronic ecological and economic loss.

Stump removal after thinning is the primary method used to prevent disease establishment (Asiegbu et al., 2005). Other mechanical methods, such as converting the site to more disease-tolerant plant species, increasing tree spacing to decrease root-to-root contact, and thinning during cold seasons can also help to decrease chance of infection (Alexender, 1989; Asiegbu et al., 2005; Oliva et al., 2010). However, economic constraints and other logistic factors can limit their use.

Chemical treatments have also been proposed such as the use of urea solution or DOT (Disodium Octoborate Tetrahydrate) on the surface of newly cut stumps to prevent basidiospore establishment (Brandtberg et al.,1996; Thor and Stenlid, 2005; Oliva et al., 2008). However, these chemicals can cause severe damage to ground vegetation species and alter soil properties (Westlund and Nohrstedt, 2000).

Due to the potential negative environmental effects of stump removal and chemical control methods, the use of environmentally friendly alternatives such as biocontrol agents are being investigated as an option to control *Armillaria* and Annosus root rot diseases. Recent studies have suggested the use of *Hypholoma* spp. to biologically control the spread of *Armillaria* root rot. *Hypholoma* spp. shows its antagonistic nature against *Armillaria* spp. by considerably decreasing the growth of *Armillaria* spp., when grown together in various lab and field studies (Chapman and Xiao, 2000; Chapman et al., 2004; Keca, 2009; Stevens, 2019). Another antagonistic fungus, *Phlebiopsis gigantea*, is currently used as a biocontrol against *Heterobasidion* (Nicolotti and Gonthier, 2005; Zhao, 2013), and it is considered more environmentally friendly and successful than other control methods (Westlund & Nohrstedt, 2000). It is applied as a solution containing *Phlebiopsis gigantea* spores on freshly cut stump surfaces (Asiegbu et al., 2005, Berglund et al., 2005).

1.3 Biological controls

An ideal biocontrol agent is environmentally safe, sustainable, economically viable, resilient against adverse environmental conditions, and highly specific (Ghorbanpour et al., 2018). It demonstrates faster germination and growth, producing more inoculum than the targeted pathogen (Thambugala et al., 2020).

Biocontrols can provide alternative pathways for disease control where other methods such as stump removal are impractical (Shaw and Roth 1978). Because, despite being partially effective, stump removal has limitations. Drawbacks include soil disturbances and infection spread during the process, and incomplete removal serves as potential source of inoculum (Chapman et al., 2004). Stump removal is not advisable on certain soil types, such as calcareous soils, which can negatively impact the establishment and growth of trees due to alterations in their physical or nutritional properties (Chapman and Xiao, 2000). A more promising approach involves the use of biocontrol such as *Hypholoma* spp., which proved more effective than stump removal, especially considering the limitations and challenges associated with stump removal. Research supports the use of *Hypholoma* and *P. gigantea* to control *Armillaria* and Annosus root rot (Chapman and Xiao, 2000; Chapman et al., 2004; Nicolotti and Gonthier, 2005; Keca, 2009; Zhao, 2013; Stevens, 2019).

1.3.1 *Hypholoma* species as biocontrol for *Armillaria* root rot

Hypholoma is a genus of saprotrophic fungi belonging to the Basidiomycota, naturally occurring in coniferous, mixed, and deciduous forests across North America (Parker, 1933). *Hypholoma* spreads under the soil by forming extensive networks of mycelial cords (Dowson et al, 1988). *Hypholoma* spp. are active wood and litter decomposers, usually found in clusters on dead logs and stumps, and hence play a significant role in forest ecosystem nutrient cycling (Chuluunbaatar, 2021). Various *Hypholoma* species such as *Hy.*

fasciculare, and *Hy. sublateritium* (now *Hy. lateritium* (Schaeff.) P. Kumm), have been found to be effective biocontrol choices against *Armillaria* root rot disease (Stevens, 2019*)*. Most studies have been done using *Hy. fasciculare* as a biocontrol (e.g., Chapman and Xiao, 2000; Stevens, 2019).

Hypholoma fasciculare is a competitive fungus that can overgrow the colonies of *A. mellea*. This results in a decreased inoculum of the pathogen and so, threats to the neighboring and subsequent plantations also decreases (Cox and Scherm, 2006). Most studies on *Hy. fasciculare* have focused on its ability to reduce the growth of pathogenic fungi, colonize the wood effectively, and to exclude the pathogen from host plantations (Chapman and Xiao, 2000; Lakomy et al., 2014). *Hypholoma fasciculare* can grow extremely fast; colonising the wood resource before pathogenic fungi can establish (Dowson, 1988; Chapman and Xiao, 2000). *In vitro* studies have also supported the use *of Hy. fasciculare* and *Hy. capnoides* to control *Armillaria* root rot (Keca, 2009). Hence, there is a possibility that *Hypholoma* species could be used as a potential biocontrol agent against *Armillaria ostoyae* (Dowson et al.,1988; Chapman and Xiao, 2000; Chapman et al., 2004; Stevens et al., 2020)

Studies suggest that the mycelium of the *Hy. fasciculare* shows different growth rates under different temperature conditions (Wells and Boddy, 1995). However, no such studies have been done on other *Hypholoma* species such as *Hy. sublateritium* and *Hy. capnoides*. Since efficacy and target range of biocontrols can be affected by variation in environmental parameters such as the soil temperature and pH (Kredics et al., 2004; Juroszek and Tiedmann, 2011; Simberloff, 2011). *Hypholoma* species need to be assessed under different abiotic conditions (e.g., temperature and pH) to understand their long-term reliability for the disease control. Past literature does not provide much information on the performance of *Hypholoma* species and their different strains under varying temperature and pH regimes.

1.3.2 *Phlebiopsis gigantea* as biocontrol for Annosus root rot

Phlebiopsis gigantea is a commonly found wood decaying basidiomycete of conifer forests (Sun et al., 2011). It has long been used as a biocontrol agent against *Heterobasidion* species (Rishbeth, 1963; Greig, 1976; Sun et al., 2009). The effectiveness of *P. gigantea* as a biocontrol is mainly because of its high competitiveness for nutrients, a higher colonization rate of substrates; and, most importantly, its perceived harmlessness to ground vegetation (Chapman and Xiao, 2000; Westlund and Nohrstedt, 2000; Adomas et al., 2006; Sun et al., 2009). These attributes make *P. gigantea* an ideal biocontrol against *Heterobasidion* species. The control mechanism of *P. gigantea* against *Heterobasidion* spp. probably relies mostly on the rapid growth of *P. gigantea* (Asiegbu et al., 2005). However, it is likely that the control properties, such as rapid growth, may vary among the different stains of *P. gigantea.*

Various commercial *P. gigantea* products like PG suspension, PG IBL, and Rotstop were developed in Europe (Pratt et al., 2000; Ronnberg et al., 2006) using the spores of *P. gigantea*. These products are currently being used on Norway spruce (*Picea abies*) plantations in Sweden after thinning operations every year (Berglund and Ronnberg 2004). Several studies also support the inoculation of Rotstop on wood stumps to decrease *Heterobasidion* biomass (Tubby et al., 2008; Oliva et al., 2010, 2015). *Phlebiopsis gigantea* strain VRA 22 is registered for use against *Heterobasidion* species in Canada and is currently being used in British Columbia for controlling Annosus root rot caused by *He. irregulare* and *He. occidentale* (Ministry of Forests, Lands, Natural Resource Operations and Rural Development, British Columbia, 2018).

Rotstop effectiveness relies on several factors such as the area of its application on the freshly cut stumps, and the concentration of active spores (Berguland and Ronnberg, 2004; Ronnberg et al., 2006; Zhao, 2013). Other factors, such as wood moisture (Redfern, 1993; Bendz -Hellgreen and Stenlid, 1998), and the species of *Heterobasidion* to which it is

applied, also play a key role in determining its effectiveness (Daniel et al., 1998; Vasiliauskas and Stenlid, 1998). Abiotic factors such as temperature and pH may also affect the capacity of *P. gigantea* as a biocontrol by influencing the growth rates and interactions among *P. gigantea* and *Heterobasidion* species (Asiegbu, 2000; Zhao 2013; Oliva et al., 2015).

Studies indicate that the potential increase of infections under warming conditions caused by climate change could expand the current geographical range of *Heterobasidion* species, such as *He. annosum* in Scandinavia (Witzell et al., 2010). Hence, further investigation is needed to understand the influence of abiotic factors such as temperature and pH on the establishment of *Heterobasidion* and its biocontrol *P. gigantea* on forest plantations.

1.4 Abiotic factors associated with climate change that can affect root rot diseases in plantations.

Temperature, precipitation, nutrient deficiencies, soil pH, and soil texture are some of the abiotic environmental factors that affect root rot disease spread in plants (Shield and Hobbs, 1979; Kliejunas et al., 2009, McLaughlin et al., 2011). Abiotic factors not only affect the pathogenicity of root rot causing pathogens (Otrosina and Cobb, 1989) but also influence the efficacy of biocontrols (Burpee, 1990; Mallett and Maynard, 1998). Several studies have linked abiotic factors such as droughts, soil pH, soil temperature, soil moisture, and soil type with the growth and activity of disease-causing fungal pathogens such as *Armillaria* (Rishbeth 1978; Shields and Hobbs, 1979; Singh 1983; Wargo and Carey, 2001). For example, extremely elevated temperatures can increase the host susceptibility and pathogen aggressiveness (Desprez-Loustau et al., 2006). Increased temperatures also have a positive impact on rhizomorph production of some basidiomycetes, for example, *Hy. fasciculare* (Wells and Boddy, 1995). Fungi can live in a broad range of temperatures, but their growth rate and metabolism are different at different temperatures, even when other conditions such

as nutrient and water activity are constant (Li et al., 2009). Hence, understanding the effect of temperature on the growth of the pathogen and their biocontrol is essential to understand their physiology and efficacy of treatment.

Soil pH impacts host susceptibility to root rot diseases (Shield and Hobbs, 1979; Singh, 1983). For example, soil pH above 8 (in horizon C) makes red pine plantations more susceptible to *Armillaria* root rot caused by *A. ostoyae* in southern Ontario (McLaughlin et al., 2011).

The effect of abiotic factors such as temperature and soil pH on the growth of *Armillaria* is not completely understood. Moreover, there is little evidence of how these environmental factors affect the interactions among various plant pathogens and their biocontrol. Given the changing climatic conditions due to global warming, it is essential to understand the interactions of pathogens and biocontrols in varying abiotic conditions to predict their response to altered environmental conditions.

1.4.1 Effect of temperature variation on pathogens (*Armillaria* spp. and *Heterobasidion* spp.) and their biocontrols (*Hypholoma* spp. and *Phlebiopsis gigantea*)

Temperature is one of the most important abiotic factors influencing fungal growth and activity (Li et al., 2009). Temperature impacts the activity rate of the fungi involved in the decomposition (Dang et al., 2009). Temperature plays a key role in the initiation and development of rhizomorphs (Rishbeth, 1978; Singh, 1983). Optimum fungal mycelial growth and wood decay for most fungi occur between 18 to 35 °C (Baker, 1969). However, the rate of decomposition varies at different temperatures for different species of fungi.

Temperature is one of the most important abiotic factors affecting the growth and development of *Armillaria* rhizomorphs (Keca, 2005; Lech et al., 2023). *Armillaria* species grow at temperatures between 10 and 31°C, with an optimum range between 20 and 22°C.

However, the species are sensitive to changes in temperature (Rishbeth, 1968; Keca, 2005). For example, the *in vitro* growth rate of *Armillaria mellea* mycelia varied from no growth at the two extremes 5 °C and 32°C to optimal growth at 25°C (0.75mm/day) (Rishbeth, 1968). Other studies also support that extreme low and high soil temperatures may inhibit the growth of *Armillaria* rhizomorphs (Redfern, 1973; Classen et al., 2015; Lech et al., 2023). The mycelial growth of *Armillaria gallica* and *A. ostoyae* was also found to be greatest at temperatures ranging between 22 and 27°C, lower at 13°C, and lowest at 32°C (Rind and Losel, 2005). However, the effect of temperature on different strains of the same species of *Armillaria* is unknown.

There is concern that the incidence of *Armillaria* root disease may increase if global climate conditions continue to change (Kliejunas et al., 2009; Klopfenstein et al., 2009; Kubiak et al., 2017). For example, increased temperature, droughts, and decreased precipitation may increase host susceptibility while increasing *Armillaria* aggressiveness (La Porta et al., 2008; Kliejunas et al., 2009; Klopfenstein et al., 2009).

Taubert (2008) indicates that *Heterobasidion* species growth shows variability in sensitivity to the changes in temperature. For example, the optimal growth temperature for *He. parviporum* was found to be 27°C, while that of *He. annosum* was between 17 and 22 °C. Moreover, growth rates vary between species exposed to similar temperatures. For example, the growth rate of *He. parviporum* exceeds that of *He. annosum* even under ideal growth conditions for *He. annosum* at 22°C (Taubert, 2008). Note that *He. parviporum* does not tolerate temperatures over 30°C (Müller et al., 2014). However, unlike *Armillaria* species, *Heterobasidion* species can survive and grow (although limited) at temperatures as low as 0 °C (Olson, 1941; Rishbeth, 1951a, b). *In vitro* studies on *He. annosum* and *He. parviporum* show growth of mycelium at 2°C (Taubert, 2008).

The effect of temperature on different strains of the same species of *Heterobasidion* is not known. Since the extension rate of mycelia varies among strains of the same species (Dowson et al., 1989), studies need to be done to understand the effect of temperature on different strains of the same *Heterobasidion* species. Also, past studies on *Heterobasidion* have focussed exclusively on determining the optimal temperature and growth limiting temperatures. Research needs to be done across different temperature ranges to gain a comprehensive understanding of growth patterns of *Heterobasidion* spp. in the context of climate change.

Limited literature is available to explain the influence of temperature on the growth of the biocontrol *Hypholoma* spp. Studies suggest that the growth of the mycelia of *Hy. fasciculare* is different at different temperatures (Dowson et al., 1989; Wells and Boddy, 1995). One strain of *Hy. fasciculare*, native to Canada, could grow underground during winter temperatures as low as 2°C (Stevens et al., 2020). However, there is no information on the effect of varying temperatures on other species of *Hypholoma* such as *Hy. capnoides* (native to Canada), and *Hy. sublateritium*. The temperature effect on various species and strains of *Hypholoma* needs to be understood to predict its biocontrol efficacy against *Armillaria* in varying climate conditions.

Another biocontrol fungus, *P. gigantea*, which is currently used under the name Rotstop against *Heterobasidion* species, can tolerate temperatures as high as 35 to 40°C (Thor et al., 1997). Very limited literature is available that distinctively shows the individual growth pattern of this biocontrol at different temperatures.

Several studies have been done testing *Hypholoma* spp. as a potential biocontrol for *Armillaria*, but most of the studies have been only done at a single temperature setting, e.g., at 22°C (Keca, 2009) and 15°C (Stevens, 2019). No studies have been done at multiple temperatures within the same study. Interaction studies between *Hypholoma* and *Armillaria*

need to be done at various temperatures to understand its biocontrol efficacy at low temperatures representing wintertime and at elevated temperatures to simulate warmer conditions vis-a-vis global warming.

For assessing biocontrol efficacy of *P. gigantea* against *Heterobasidion* species, Zhao (2013) and Oliva et al. (2015) used a range of temperatures from 5 to 25°C *in vitro* that showed the ability of *P. gigantea* to overgrow *He. parviporum* and *He. annosum*. However, there is no such study conducted so far with *He. irregulare*, a species native to Canada.

1.4.2 Effect of pH on pathogens (*Armillaria* spp. and *Heterobasidion* spp.) and their biocontrol (*Hypholoma* spp. and *Phlebiopsis gigantea*)

Past studies on *Armillaria* spp. provide some information on the soil/substrate pH preferences for optimal growth of this pathogen. The optimal range of pH for the growth of rhizomorphs of *Armillaria i*s different for various *Armillaria* species. For example, the growth of the rhizomorphs of *A. ostoyae* and *A. mellea* was higher in acidic soils (Shield and Hobbs, 1979; Singh, 1983 Oliva et al., 2009), whereas *A. gallica* preferred a neutral to alkaline soil pH for optimal growth (Oliva et al., 2009). However, *A. ostoyae* can be destructive on basic soil, depending upon host susceptibility. For example, pine plantations growing on alkaline soil in southern Ontario were found to be more affected by *A. ostoyae* (McLaughlin et al., 2011) than on acidic soil. In the UK, plantations of *Pinus sylvestris* were more prone to diseases by *Armillaria* spp. if soil is alkaline with pH 7 or above (Rishbeth, 1985).

Soil pH not only has a varying effect on growth on different species but also on different strains of the same species. For instance, one isolate of *A. mellea* (Isolate 2) was found to produce more rhizomorphs on acidic soils (4.8 pH) in terms of dry weight, while the other (Isolate Bg) produced more on less acidic soils (6.2 pH) (Morrison, 1974). Therefore, it is essential to examine the effects of soil pH on different strains of *Armillaria* species to

understand the distribution of *Armillaria* strains and species in plantations and their growth variations when exposed to different soil pH.

Another pathogen responsible for tree mortality, *Heterobasidion* spp., can grow in a wide range of soil pH (Majewska et al., 2004). However, the fungus prefers less acidic substrates. *Heterobasidion* can cause infection at a pH as low as 2.6 (Gaitnieks et al., 2016) but is more prolific when soil pH is higher (Gibbs et al., 2002; Bruna et al., 2019). The optimum pH for *He*. *annosum* is known to be between 4.0 and 5.7 (Majewska et al., 2004), but tree mortality caused by *He. annosum* was greater when pH was 6 (Gibbs, 2002; Grieg, 1984). The effect of pH on other commonly found species of *Heterobasidion* like *Heterobasidion irregulare* is not well known.

There is a possibility that soil pH can affect the growth rates of different strains of the same species of *Heterobasidion* differently. This needs to be further investigated to completely understand the role of soil pH in the spread of Annosus root rot.

In the case of the biocontrols *Hypholama* spp. and *P. gigantea*, there is no available information showing the effect of pH on these species.

1.4.3 Climate change and root diseases

Past studies confirm that the growth of the pathogens *Armillaria* spp. and *Heterobasidion* spp. are influenced by abiotic factors such as temperature and pH (Rishbeth, 1968; Shield and Hobbs, 1979; Singh, 1983; Keca, 2005; Taubert, 2008; Oliva et al., 2009; Bruna et al., 2019). Changes in these abiotic factors due to climate change may affect the severity of root rot diseases in plantations (Puddu et al., 2003; Kliejunas et al., 2009; Sturrock et al., 2011).

The global temperature is predicted to rise by 1.5 °C in the next two decades (IPCC, 2022: Climate change 2022), and the average temperature in Ontario may rise by 2.5 to 3.7 °C by 2050 (Ministry of Environment Conservation and Parks, Ontario, 2016). A rise in

temperature and drought conditions may increase the damage caused by *Armillaria* and Annosus root diseases among host plantations (Kliejunas, 2011). The rise in temperature may also increase the spread of disease. For example, in the Pacific Northwest, the annual mean temperature is below the optimum temperature for the growth of *Armillaria* (Kleijunas et al., 2009). An increase in temperature in these areas might boost the growth and spread of *Armillaria* root rot (Kliejunas et al., 2009; Klopfenstein et al., 2009). Changes in climatic conditions will also influence edaphic properties such as soil pH (Singh et al., 2013; Kubiak et al., 2017).

Hence, it is imperative to examine the growth and interaction patterns of pathogens and biocontrol agents under variable abiotic conditions like temperature and pH to better understand the potential impacts of climate change on root rot diseases in plantations.

1.5 Research questions

This study focuses on determining the effect of a range of temperatures and pH on the growth *in vitro* of pathogens *A. ostoyae* and *He. irregulare* and their potential biocontrol agents *Hypholoma fasciculare*, *Hy. capnoides*, *Hy. sublateritium,* and *P. gigantea* in control (single culture setting with no competitor) and paired culture settings. The results will help to understand the growth and interactions of these biocontrol agents under different temperature and pH conditions in presence and absence of the pathogen.

Specifically, I will address the following questions:

1. Do different temperature and pH conditions impact the growth of different strains of *A. ostoyae* and their biocontrol species *Hy. fasciculare, Hy. capnoides,* and *Hy. sublateritium*?
2. Is the growth of pathogen species impacted by the presence of a biocontrol species in paired culture settings?
3. Are impacts consistent across a range of temperatures and pH conditions?

Chapter 2 Effect of different temperatures on the growth and interactions of *Hypholoma* species and *Phlebiopsis gigantea* with fungal pathogens *Armillaria ostoyae* and *Heterobasidion irregulare*

2.1 Abstract

Armillaria ostoyae and *Heterobasidion irregulare*, are aggressive fungal pathogens that cause substantial root and butt rot diseases in conifers, posing significant management challenges. *Hypholoma* species and *Phlebiopsis gigantea* are biocontrol agents that show promise in managing these pathogens. However, their effectiveness may be sensitive to temperature, an aspect not fully explored. Experiments in controlled lab environments using 2% malt agar media across a range of temperatures: 2, 10, 15, 20, 25, and 30°C were conducted in single and paired culture settings. The single culture study focused on temperature- dependent responses of *Armillaria ostoyae (A. ostoyae*), *Heterobasidion irregulare (He. irregulare*) and selected strains of *Hypholoma* spp. (*Hypholoma fasciculare, Hy. sublateritium, Hy. capnoides*) and *P. gigantea*, the respective biocontrols. The paired culture study focused on investigating the interactions of species by observing the temperature-dependent variations in their growth rates in the presence and absence of pathogens. Significant temperature impacts were observed, with *A. ostoyae* strain P162 -7 showing better growth at extreme temperatures (2°C and 30°C) compared to strain B249-28. *Heterobasidion irregulare* showed no growth at 30°C. *Hypholoma fasciculare* strain Pinnel B consistently outgrew others across all temperatures, although no growth was observed at 30°C. In paired cultures, *Hy. capnoides* strain TAK 5 maintained a consistent growth at 25°C despite competition, while *Hy. sublateritium* strain OKM-6947-SP showed increased growth during competition. *Hypholoma fasciculare* strain RLG-12668-SP grew better compared to Pinnel B at higher temperatures, suggesting its potential utility as a biocontrol in future warming scenarios. Biocontrols *Hy. fasciculare* and *P. gigantea* demonstrated efficacy against *A. ostoyae* and *He. irregulare* by exhibiting higher growth in competition and decreasing the growth of the pathogens across the range of temperatures tested.

2.2 Introduction

Commercially important conifer plantations, such as red pine, are extensively grown in the regions of North America because of their rapid growth and potential for producing high-value timber products, including saw logs, cabin logs, and utility poles (Gilmore and Palik, 2006). However, these conifer plantations are facing a decline due to the spread of root and butt rot diseases caused by pathogenic fungal species (Wargo and Kile, 1992; McLaughlin, 2001a, b; McLaughlin and Hsiang, 2010; Garbelotto and Gonthier, 2013). *Armillaria ostoyae* and *Heterobasidion irregulare*, for example, are among the most destructive agents of root diseases in eastern United States and Canada (Mallett, 1990; Morrison et al., 1991; McLaughlin, 2001b; McLaughlin and Hsiang, 2010; Dumas and Laflamme, 2013; Otto et al., 2021). Controlling these pathogens is extremely difficult due to the hidden underground growth of mycelium, the long- distance dispersal capabilities of basidiospores (>160 km), and the saprophytic persistence of disease for decades in deadwood (Goheen and Otrosina, 1998; Lygis et al., 2004a; Heinzelmann et al., 2019). Various disease control strategies, such as mechanical removal of infected stumps (Shaw and Roth, 1978; Thor and Stenlid, 2005) and pesticides, have been implemented in the past to remove fungal inoculum of these pathogens from the soil. Mechanical methods, however, are very costly and can result in substantial loss of nutrients and increased susceptibility to soil erosion due to soil disturbance (Chapman and Xiao, 2000; Morrison et al., 2014; Achat et al., 2015), while chemical interventions have toxic effects on non-target species and can disrupt soil processes (Westlund and Nohrstedt, 2000; Baumgartner et al., 2011). For example, fungicides such as methyl bromide and carbon disulphides have been used to control *Armillaria* spp. (Filip, 1976; Shaw and Roth; 1978a; Baumgartner et al., 2011), while urea was used for *Heterobasidion* spp. (Oliva et al., 2008). However, these methods, while effective, raise concern due to their potential to cause harm to the environment. Therefore, development and

use of more innocuous methods, such as biocontrols that restrict the establishment and/or growth of pathogenic fungi (Dumas, 1992; Chapman and Xiao, 2000; Lakomy et al., 2014) without causing damage to critical soil processes and ground vegetation, are being encouraged (Hunt et al., 1971; Shaw and Roth, 1978a; Thomas and Willis, 1998).

2.2.1 Past studies on potential biocontrols

Several *in vitro* and field studies have assessed biocontrol species such as *Hypholoma* spp. for controlling diseases caused by *Armillaria* spp. (Pearce et al., 1995; Chapman and Xiao, 2000; Chapman et al., 2004; Keca, 2009; Stevens 2019). *Hypholoma* spp. are naturally occurring fungi that show antagonistic activity against *Armillaria* spp. (Chapman and Xiao, 2000; Chapman et al., 2004). For example, *Hy. fasciculare* was naturally robust in areas where *A. ostoyae* was absent, and vice versa, but when paired against each other, *Hy. fasciculare* outcompeted and disrupted the growth of *A. ostoyae* (Chapman and Xiao, 2000). *Hypholoma fasciculare* can control the spread of *Armillaria* spp. by outgrowing the pathogen and disrupting the infectious rhizomorph production (Chapman and Xiao, 2000; Chapman et al., 2004; Keca, 2009; Stevens, 2019). Stevens (2019) observed a 70% growth reduction in *A. ostoyae* when grown in competition with *Hy. fasciculare in-vitro.* Similar results were observed when *A. ostoyae* was paired with other *Hypholoma* species such as *Hy. sublateritium* (Stevens, 2019) and *Hy. capnoides* (Keca, 2009). *Hypholoma capnoides*, for example, restricted rhizomorph development by up to 52% in several *Armillaria* species., including *A. mellea, A. gallica, A. ostoyae, A. cepistipes and A. tabescens* (Keca, 2009). Several field studies performed using *Hy. fasciculare* against *Armillaria ostoyae* have supported the in vitro results (Lakomy, 1998; Chapman et al., 2004). Note that the effectiveness of *Hypholoma* species as a biocontrol agent against all root rot-causing fungi is not universal. For example, *Hy. fasciculare* when grown in the presence of *Heterobasidion*

annosum, another important root rot fungal pathogen, but did not suppress its growth (Nicolotti et al., 2008; Lakomy et al., 2014).

In addition to resource competition, which leads to higher growth of *Hypholoma* spp. (biocontrol), it may also employ hyphal interference as an antagonistic mechanism to limit the pathogen's growth (Cox and Scherm, 2006). For example, when paired together *invitro*, *Hy. fasciculare* was observed to induce swelling and eventual bursting (lysis) of *Armillaria's* hyphae (Cox and scherm, 2006). These interactions represent a form of antagonism that results in cessation of growth and leads to hyphal lysis even before physical contact (Boddy, 2000). The inhibitory effects of *Hy. fasciculare* on *Armillaria* spp. may involve the production of diffusible antifungal compounds or toxic metabolites produced by it, such as amylase, laccase, alkaloids, and phenolic compounds (Dennis and webster, 1971).

Phlebiopsis gigantea, another naturally occurring saprotrophic fungus found in conifer plantations, is currently being used commercially as an effective biocontrol against *Heterobasidion annosum* and *Heterobasidion irregulare* (Korhonen et al., 1994; Government of Canada, 2014). This is due to its ability to suppress the growth of *Heterobasidion* spp. and/or significantly reduce pathogen infection in treated stumps (Korhonen et al., 1994; Nicolotti and Gonthier, 2005; Sun et al., 2009; Ronnberg and Cleary, 2012; Dumas and Laflamme, 2013). The primary mode of action of *P. gigantea* against *Heterobasidion* spp. involves rapid colonization of the substrate thereby rendering it unavailable to the pathogen (Kalvo et al., 2018). However, release of antifungal compounds such as o-orsellinaldehyde may also facilitate pathogen inhibition (Kavlo et al., 2018). Although *P. gigantea* is an ideal biocontrol species for *Heterobasidion* spp., it is ineffective against other fungal pathogens such as *Armillaria* spp. (Keca, 2009; Stevens, 2019).

In this study, *Hypholoma* spp. *(Hy. fasciculare, Hy. capnoides*, and *Hy. sublateritium*) and *P. gigantea* (VRA 22) will be tested for their antagonistic abilities against *Armillaria ostoyae* and *Heterobasidion irregulare*, respectively.

2.2.2 Temperature and fungal growth

One of the most important features contributing to the efficacy of these biocontrol species in reducing pathogen growth is their fast growth rate. For example, *Hypholoma fasciculare*, being a cord- forming fungus, can grow extremely fast individually and even faster when paired against the pathogen, *Armillaria* spp., enabling it to successfully overgrow and control the pathogen growth (Dowson, 1988; Chapman and Xiao, 2000; Stevens, 2019). Similarly, higher growth rate of another biocontrol fungus, *P. gigantea* enables it to successfully overgrow *Heterobasidion* species (Oliva et al., 2015; Kalvo et al., 2018). The growth rates of antagonistic fungi play important role in influencing their interactions with other fungi (Goldfarb et al., 1989). Interactions like hyphal interference, induced by the production of secondary metabolites, involving rapid cessation of growth, granulation, swelling of hyphal tips, are also temperature dependent (Dennis and Webster, 1971). The growth rates and fungi activity are highly dependent on temperature (Rishbeth, 1978; Li et al., 2009). Generally, biological activity such as respiration and nutrient uptake, of fungi increases with temperature until an optimum is reached; beyond which, activity declines due to the impacts on enzyme-catalyzed reactions (Wells and Boddy, 2005; Muller et al., 2014; Boddy, 2016). Optimum temperature may also lead to higher production of metabolites having antifungal properties, ultimately influencing the growth and interactions among species (Dennis and Webster, 1971; Schoeman et al., 1996). Since fungi cannot control their internal temperature and rely upon the favourable climatic conditions for their growth, changing environmental conditions can have significant effects on the occurrence and development of fungi on host species and interspecific interactions (Colhoun, 1973).

2.2.2.1 *Armillaria* spp.

Studies on disease-causing pathogens, such as *Armillaria* spp., suggest that they are susceptible to changes in temperature conditions (Rishbeth, 1968; Keca, 2005; Rind and Losel, 2005). For example, optimal mycelial and rhizomorphic growth of *Armillaria mellea* and *A. gallica* occurred between 20°C-22°C (Rishbeth, 1978; Rind and Losel, 2005; Keca, 2009) while little to no growth is observed below 10 and above 26°C (Rishbeth, 1978). *Armillaria ostoyae*, being the most destructive pathogen in Ontario (Dumas, 1988) needs to be studied under variable environmental conditions to fully understand its physiology. This may help improve understanding of its current and future geographical distribution, as well as support the development of effective control strategies against this pathogen. Pearce & Malakzuk (1990) concluded that the annual growth of *A. luteobubalina* fluctuated at different temperature conditions occurring in the field and that the abundance and scarcity of this pathogen is mainly dependent upon the seasonal patterns of temperature and rainfall. Temperature changes also affect the sporulation and dispersal of the fungal spores (Tubby and Webber, 2010). It is probable that the regular rate of spread of *Armillaria* spp. may increase in a few decades due to the potential increase in temperature caused by global warming (Klopfenstein et al., 2009; Kliejunas et al., 2011; Kubiak et al., 2017). Change in climatic conditions, such as increase in temperature, may result in milder winters that will favour the survival and infection caused by several pathogens such as *Armillaria* spp. Such conditions will further increase the intensity and frequency of fungal disease outbreaks (Tubby and Webber, 2010; Kubiak et al., 2017). Hence, knowledge of the effect of range of temperatures similar to ambient climate conditions on species such as *A. ostoyae* growth rates may help predict the future of *A. ostoyae* pathogenesis under current and future climate scenarios in Canada.

2.2.2.2 *Heterobasidion* spp.

In past studies, the impact of temperature on other disease-causing pathogens in *Heterobasidion*, including, *He. annosum* and *He. parviporum,* has been explored, including information on the effect of temperature on the viability of fungal spores, growth patterns and colonization rates of the mycelia. Zhao (2013) demonstrated that the colonization rate of the mycelia of *Heterobasidion* spp. varied with the range of temperature (5°C - 25°C) *in vitro*, with *He. annosum* showing higher colonization rates as the temperature increased. However, different *Heterobasidion* species show varying growth rates when exposed to different temperatures. For example, the growth rate of *He. annosum* increased with temperature, reaching its optimum at 22°C, while the growth rate of *He. parviporum* continued to increase until reaching its optimum at 27°C (Taubert, 2008). *Heterobasidion* spp. appear to tolerate extreme temperatures as low as -8°C and -20°C (Gooding et al., 1966; Myers et al., 2018a), while temperatures exceeding 35°C can kill the mycelia (Taubert, 2008). The incidence of the infection depends upon the viability and successful establishment of the basidiospores on the host species, and it has been found that the spores of *Heterobasidion* spp. can survive and germinate at temperatures as low as -25°C (Schwantes et al., 1976; Taubert, 2008; Myers et al., 2018a). Hence, lower temperatures are unlikely to eliminate or affect the survival of *Heterobasidion* spp. (Myers et al., 2018a). However, higher temperature may be an advantage to the antagonistic fungi such as *Phlebiopsis gigantea,* given that this antagonist can survive and grow well at higher temperatures such as 30°C (Thor et al., 1997). Moreover, growth tends to slow for *Heterobasidion* spp. at temperatures above 30°C due to enzyme inactivation (Gooding et al., 1966; Taubert, 2008). In the case of *He. parviporum*, which produces more inocula during summer (Gonthier et al., 2005), higher temperatures attributed to climate change may not impact its competing and infecting ability (Gooding et al., 1966). No such predictions can be made with respect to other *Heterobasidion* species, such as *He.*

irregulare, since very limited literature is available showing higher temperature effects on this species (Oliva et al., 2015).

2.2.2.3 *Hypholoma* spp.

Temperature also affects the mycelial growth of potential biocontrols such as *Hypholoma fasciculare* (Dowson et al., 1989). However, information on the effect of temperature on other species of *Hypholoma* such as *Hy. capnoides* and *Hy. sublateritium,* which have shown effectiveness against *Armillaria* spp. in various studies (Keca, 2009; Stevens, 2019), is not available. Previous studies on the effectiveness of *Hypholoma* spp. against *Armillaria* spp. have often been conducted by pairing them at optimum or suboptimum temperatures such as 20°C, 22°C and 15°C *in vitro* (Chapman and Xiao, 2000; Keca, 2009; Stevens, 2019). This does not provide conclusive information on *Hypholoma* spp. effectiveness against *Armillaria* spp. (*A. ostoyae* in our case) given that responses at more extreme temperature conditions can vary for both the pathogen and biocontrol species as discussed above.

2.2.2.4 *Phlebiopsis gigantea*

Temperature studies on the biocontrol species, *Phlebiopsis gigantea,* show that the growth rate slowed as temperatures decreased from optimal conditions of 25°C. Substantial declines were observed at 20 °C, 15°C, and 5°C as well as above 30°C (Thor et al., 1997; Swanwick, 2007; Zhao, 2013).

2.2.3 Climate change and impact on fungal growth

Average temperature in Ontario is predicted to increase 3 to 8°C over the next century (Gough et al., 2016), which together with the associated changes in soil conditions, will impact the relative survival, growth, and competitive ability of fungus species. These changes may lead to increased forest damage by predisposing the host plantations to the fungal diseases (La Porta et al., 2008; Kubiak et al., 2017). Researchers such as Yeo et al. (2003), Dagno et al. (2010), Zhao, (2013), and Carro-Huerga et al. (2021) have discussed the variable effects of temperature changes on spore germination, hyphal growth, and the antagonistic activity of fungal biocontrols against fungal pathogens.

Climate change favours organisms better adapted to higher temperatures with faster growth rates (Petterson and Baath, 2003). *Armillaria* spp. and *Heterobasidion* spp., for example, increase sporulation and spore dispersal at elevated soil temperatures (Tubby and Webber, 2010). Furthermore, the pathogenicity of *Armillaria* spp. will likely increase given that its geographical range is expected to expand, as low soil temperatures in winter may no longer restrict the growth of its rhizomorphs (Kubiak et al., 2017). Although species like *Hypholoma* spp. and *P. gigantea* have been thoroughly studied as biocontrol agents, their efficacy against fungal pathogens across a range of temperature conditions has not been extensively explored. *Hypholoma fasciculare* can grow at temperatures as low as 2°C in winters in Ontario (Stevens et al., 2020), but its interaction and patterns of growth rate in the presence of *Armillaria* spp. was not tested. It is important to note that the mere presence or rise in the population of antagonistic fungi with changes in temperature conditions should not be interpreted as conclusive evidence of enhanced biocontrol activity against pathogens (Burpee, 1990). Specific activities such as interactions with antagonistic fungi are probably more important than the population proliferation (Chapman and Xiao, 2000; Burpee, 1990).

Thus, fungal pathogens and biocontrols should be paired across a range of temperature conditions to note any changes in growth rates, patterns of growth, and signs of inhibition.

A better understanding of the effects of elevated temperatures on *He. irregulare* in the presence of different strains of *P. gigantea* is needed so that appropriate control strategies can be developed. This is because different strains may respond to competition differently under different temperature conditions. The focus of this study is on *P. gigantea* VRA22 as it is currently registered and used in Canada as a biocontrol against *He. irregulare.* Its competitive ability against *He. irregulare* will be examined across a range of temperatures under controlled lab conditions. This will help improve understanding of how the seasonal variation in Canadian soil conditions can impact growth. Unlike *Heterobasidion* spp. and *P. gigantea* spp., no such study to understand the impacts of temperature variation has been done on *Armillaria* spp. and *Hypholoma* spp. (Oliva et al., 2015).

2.2.4 Objectives

Examining the growth patterns of the pathogens and their biocontrols in individual and paired settings across a range of temperatures will provide insight on the potential responses and interactions between pathogens and their biocontrol agents as climate conditions change due to global warming. The results will help to shape strategies associated with biocontrol use in a changed climate scenario (Simberloff, 2011) to make them more efficient and economically viable.

The main objective of this study was to investigate the *in vitro* growth of the pathogens: *Armillaria ostoyae* and *Heterobasidion irregulare* and their potential biocontrols: *Hypholoma fasciculare, Hy. sublateritium, Hy. Capnoides*, and *Phlebiopsis gigantea* in isolation and in the presence of a competitor. This will be conducted across a range of temperatures typically observed in south-central Ontario, Canada.

I hypothesised that *He. irregulare* will show a higher resistance against *P. gigantea* at lower temperatures. This expectation is based on the assumption that the growth rates of *P. gigantea* will be lower at low temperatures (Zhao, 2013). As the temperature increases, the growth rates of *P. gigantea* may increase, given its optimum growth temperature of 28℃ (Govt. of Canada, 2014). This means that potential rise in temperature, associated with climate change, may not necessarily favour the disease-causing pathogen such as *He. irregulare* in presence of biocontrol *P. gigantea* (VRA). Therefore, it will be interesting to study the growth pattern of *He. irregulare* in presence of *P. gigantea* under different temperature conditions.

Secondly, I hypothesised that the species and strains of *Hypholoma fasciculare* and *Hy. sublateritium* may be able to grow better at all temperatures as compared to *Hy. capnoides.* This assumption is based on the observation that *Hy. fasciculare* demonstrates growth even at low temperatures, as evidenced by its ability to grow at 2℃ (Stevens et al., 2020).

2.3 Materials and methods

2.3.1 Fungal isolates and experimental design

This study involved monitoring and comparing the growth of pathogens *Armillaria ostoyae* (2 strains) and *Heterobasidion irregulare,* and their potential biocontrols, *Hypholoma* species: *Hy. fasciculare* (2 strains), *Hy. capnoides* (2 strains), *Hy. sublateritium* (2 strains), and *Phlebiopsis. gigantea* (VRA 1992) at different temperatures individually and in pairs (Table 1). These fungal species and strains were selected because they had previously shown antagonistic activity against *A. ostoyae* strains (Stevens 2019). Approximately 20 mL of 2% malt agar (MA) media was dispensed in (100 x 15 mm) 5 plastic petri plates, in both single and paired culture settings, at 6 different temperatures: 2, 10, 15, 20, 25, and 30℃. The 2% MA was prepared using 7.5 g agar and 10 g malt extract per 500 ml of distilled water. Cultures were maintained by transferring them to fresh 2% MA media as required and incubating at room temperature in the dark. Malt agar media has been widely used in previous studies for carrying out *in vitro* studies on fungal growth (Goldfarb et al., 1989; Lakomy, 1998; Chapman and Xiao, 2000; Keca, 2009; Nikolajeva et al., 2012; Stevens, 2019). The temperature settings were chosen to represent variation of the ambient temperatures in Simcoe County, Ontario, Canada, throughout the year. Fungal species and strains were generously shared by Ryan Stevens (Lakehead University grad. student) who obtained this collection from BioForest Technologies Inc. and the United States Forest Service (Stevens 2019) (Table 1).

Table 1. Fungal species, strains, region, and country of original collection for the present study.

Species	Strain	Region	Country
Armillaria ostoyae	B249-28	Ontario	CANADA
Armillaria ostoyae	P162-7	Ontario	CANADA
Heterobasidion irregulare	unknown	Ontario	CANADA
Hypholoma fasciculare	RLG-12668-Sp	Arizona	USA
Hypholoma fasciculare	Pinnel B	British Columbia	CANADA
Hypholoma sublateritium	HHB-11948-Sp	Michigan	USA
Hypholoma sublateritium	OKM-6947-Sp	Maryland	USA
Hypholoma capnoides	TAK 2	Ontario	CANADA
Hypholoma capnoides	TAK 5	Ontario	CANADA
Phlebiopsis gigantea	VRA 1992	Quebec	CANADA

2.3.2 Experiment 1: Impact of temperature on growth of fungal pathogen and biocontrol isolates

Pure cultures of all the species and stains were grown in the laboratory for 1-3 weeks on 2% MA media in petri dishes at 22°C. The pure inoculum, 7mm agar disc with mycelium, was cut with a sterile cork-borer from the leading edge of the 1–3-week-old culture growing at room temperature and transferred in aseptic conditions in a Biosafety hood. The plugs were taken approximately 4 mm inside from the perimeter of the growing colony. Each strain was inoculated onto one edge of five Petri plates containing approximately 20 mL of 2% MA and incubated at different temperatures. Three lines were drawn through the inoculum plug, one through the centre (perpendicular) of the plug and the others at 45-degree angles on either

side of the centre line (Fig. 3) The extent of mycelial growth was measured along these lines at 48- hour intervals, starting 5 days after inoculation, and an average value was recorded. Five replicates for each fungal species/strain were established at each temperature in the dark. All 50 petri plates were inoculated with one agar disc placed at the edge of the petri plate. Growth measurements were taken in mm using a ruler under a dissecting microscope and inverted microscope at 48-hour intervals, starting from day 5 until day 71 (approx. 2 months; nb., the duration the cultures took to reach the other end of the Petri plates) for *A. ostoyae* and *Hypholoma* spp. across all temperatures. In the case of *He. irregulare* and *P. gigantea,* similar measurements at 48-hour intervals were taken starting from day 3 to day 21. These days were chosen to start the measurements to avoid the initial lag phase of the species. Three measurements (along the three lines as explained above) were averaged to get an average radial growth for each strain per replicate until the mycelium reached the opposite edge of the plate (71 days in case of *A. ostoyae* and *Hypholoma* spp. and 21 days in case of *He. irregulare* and *P. gigantea).* The number of days taken by each strain at different temperatures to reach the edge of the plate was also determined. This study was done to determine the differences in radial growth rate of each species and strain at different temperatures.

2.3.3 Experiment 2: Impact of temperature on growth of competing (paired) fungal culture

In this study, two strains of *A. ostoyae* were paired with two strains of each of 3 *Hypholoma* species: *Hy. fasciculare, Hy. sublateritium, Hy. capnoides*. Another pathogen, *He. irregulare* was paired with its biocontrol *P. gigantea* (n=5). This study was done to determine the effect of competition on the radial growth of biocontrol species and strains under different temperature conditions. Five replicates of each strain of *A. ostoyae* were paired with each strain of all three *Hypholoma* spp. (Fig. 4). The fungi used in pairing

experiments were inoculated on 2% MA + Ethanol and incubated at temperatures 2, 10, 15, 20, 25 and 30°C in the dark. 3mL/L of ethanol was added to stimulate the growth of rhizomorphs in *Armillaria* (Weinhold, 1963; Chapman and Xiao, 2000; Stevens, 2019). Biocontrols (*Hypholoma* spp.) were introduced 2 weeks after *A. ostoyae* inoculation in case of higher temperatures: 20°C, 25°C and 30°C (Chapman and Xiao, 2000; Keca, 2009), 3 weeks later for 15°C (Stevens 2019), and 4 weeks later for 10°C (Nikolajeva et al., 2012). This was because (1) *A. ostoyae* took more time to establish at lower temperatures than the higher temperatures and (2) to have significant growth that can be measured. The growth of strains of *A. ostoyae* was not included in this study since the production of rhizomorphs (due to the addition of ethanol) in the media made it difficult to take accurate measurements along the 3 lines. However, in the case of *He. irregulare* and *P. gigantea*, the growth measurements were taken 2 days after their inoculation at all temperatures. Only 2% MA was used (no ethanol) in the case of *He. irregulare* and *P. gigantea* interactions. For the measurement of growth, three lines were drawn, and growth was measured along those lines on a 48-hour interval. All pairings were performed in 5 replicates where pathogens and biocontrols were plated 2 cm away from the opposite edges of the plate (Fig. 3; Rahman et al., 2009). The controls were prepared similarly with same species and strains growing solo at the respective temperatures. The radial growth of strains in solo and paired cultures was compared on day 11 in case of *A. ostoyae* and *Hypholoma* spp. while day 5 was chosen to compare growth in case of *He. irregulare* and *P. gigantea* interactions. These days were chosen carefully for the growth comparisons as it represents growth just before the intermingling of the mycelium of antagonistic species and strains occurred. The efficiency of *P. gigantea* in inhibiting the growth of the *He. irregulare* at different temperatures was also calculated using R1-R2/R1 * 100, where R2 is the growth of *He. irregulare* in direction of biocontrol while R1 is the growth of *He. irregulare* in single culture on day 5 (Rahman et al., 2009). This study was

done to determine the ability of different biocontrol species and strains to grow at different temperatures in presence of pathogen, which may help in determining their biocontrol efficiency at different temperatures.

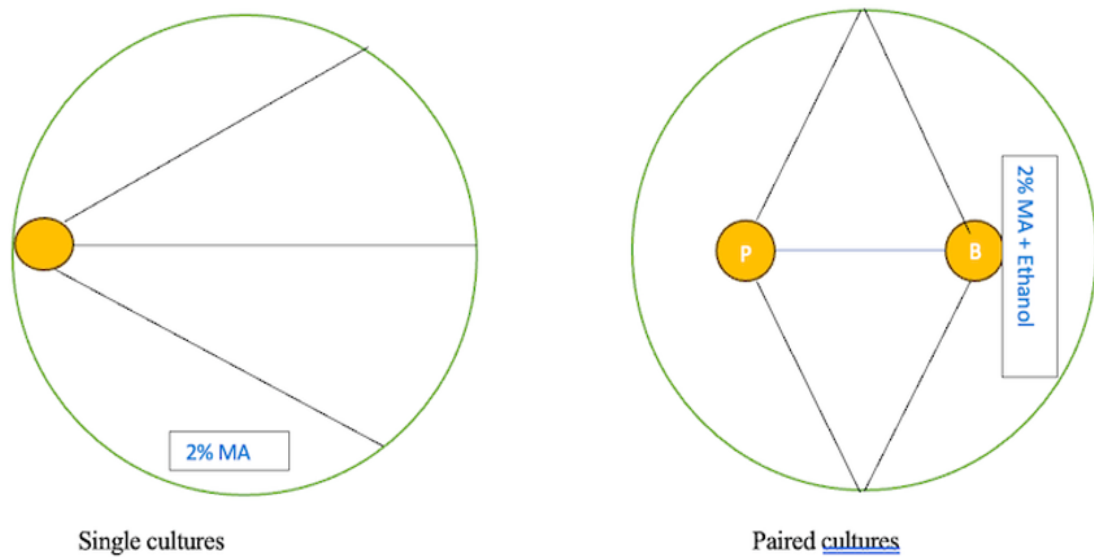


Figure 3. Representation of single and paired cultures. Black lines indicate locations of radial growth measurements.

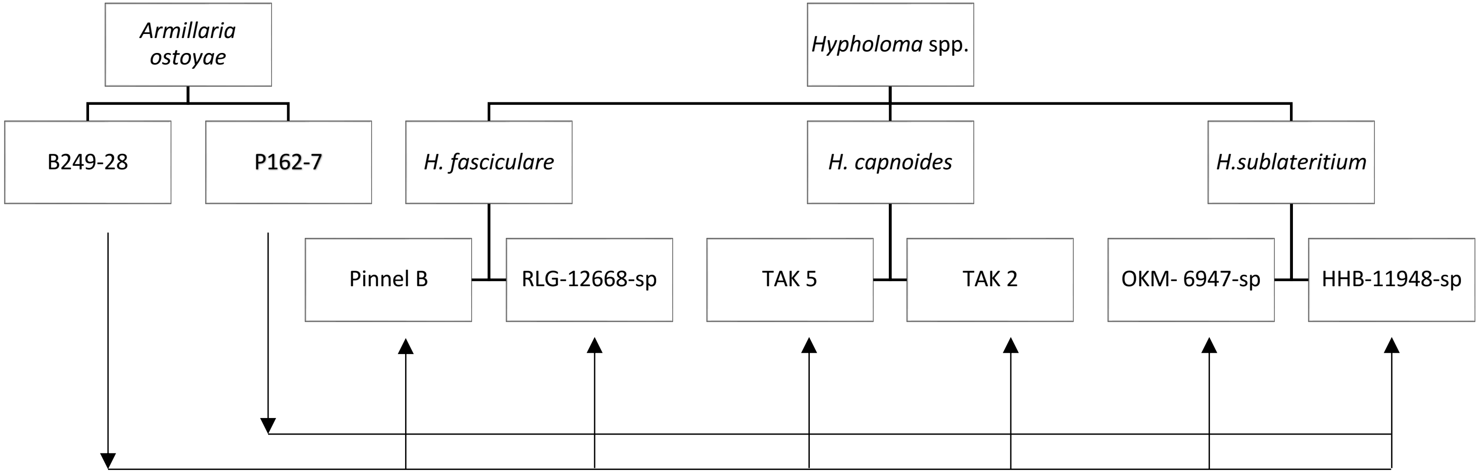


Figure 4. Representation of paired culture studies of *Armillaria ostoyae* and *Hypholoma* spp.

2.3.4 Statistical analyses

The growth of pathogen species, *A. ostoyae* and *He. irregulare*, and their biocontrol species, *Hypholoma* spp. and *P. gigantea,* was determined under different temperature conditions: 2, 10, 15, 20, 25 and 30°C in single and paired cultures (dual cultures). The difference in mean single culture growth of *A. ostoyae* strains and biocontrol *Hypholoma* spp. (mean of 5 replicates) at different temperatures over different days was analysed using a three-way repeated measures ANOVA. Post hoc analyses was performed to compare the increase in growth between strains on each day to the previous day with the help of an independent sample t-test. Bonferroni correction was applied to the p-value to adjust the inflated alpha error due to multiple comparisons. The difference in mean growth of pathogen *He. irregulare* and its biocontrol *P. gigantea* over the days at different temperatures was analyzed using a one-way repeated measures ANOVA. Post hoc analyses were carried out using paired t-test to compare the change in growth between any two days for these species. The difference in mean growth of biocontrol species and its strains between single and paired cultures was analyzed using three-way ANOVA on a particular day. Post hoc analyses using Tukey's test was used to compare the differences in the mean growth of these strains in single and paired cultures. Assumptions of homogeneity of variance and normality in the data were checked using Levene's test and Shapiro-Wilk test, respectively. Data were log-transformed whenever the assumption of normality was not met. The P-value was considered significant at a 5% level of significance for all the analyses. Trend plots were used to depict the mean growth with their SE values. Post hoc analysis using Tukey's test was employed to compare these differences. Data were analyzed using R software version 4.1.2. R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/.

2.4 Results and discussion

2.4.1 Single culture mean growth of pathogen *A. ostoyae* and biocontrol *Hypholoma* spp. at different temperatures over time:

2.4.1.1 Single culture mean growth of pathogen *A. ostoyae* strains (B249-28 and P162-7) at different temperatures over time (days):

The mean growth of two strains of *A. ostoyae* – B249-28 and P162-7 was observed at temperatures 2°C, 10°C, 15°C, 20°C, 25°C and 30°C from day 5 till day 71. The overall mean growth of B249-28 at the end of 71 days was significantly higher than the mean growth of P162-7 ($F(1) = 59.009$, $p < 0.0001$). The mean growth of two strains at different temperatures also varied significantly ($F(5) = 7153.357$, $p<0.001$). Moreover, the interaction of strains, temperature and day was also statistically significant (Table 2).

Table 2. Three-way repeated measure ANOVA table: Single culture mean growth of pathogen *A. ostoyae* strains (B249-28 and P162-7) at different temperatures over time (days), n=5.

Effect	df	F	P
Strain	1	59.009	<0.001
Temperature	5	7153.357	<0.001
Day	11	1226.871	<0.001
Strain x Temperature	5	28.957	<0.001
Strain x Day	11	7.219	<0.001
Temperature x Day	55	66.19	<0.001
Strain x Temperature x Day	55	3.486	<0.001

At 2 °C, initially similar mean growth was observed between B249-28 and P162-7, but by the end of the experiment, P162-7 surpassed B249-28. Similarly, at 10°C and 15°C, B249-28 consistently outpaced P162-7. while at 20°C and 25°C, B249-28 showed superior growth overall till the end of the experiment. Interestingly, at 30°C, strain P162-7 growth exceeded that of B249-28. Overall, linear growth was observed for both strains across different temperatures.

Over time, the mean growth of the more virulent B249 -28 strain remained higher than that of the less virulent P162-7 strain across different temperatures, except at 2 °C and 30 °C (Fig. 5). This suggests that P162 -7 may perform better at extreme low or high temperatures compared to B249-28, emphasizing strain specific responses.

In terms of overall growth, the highest growth was recorded at 20°C and 25°C, while the lowest at 2°C and 30 °C (Fig. 5), suggesting an optimal range for *A. ostoyae* growth. This aligns with both Rishbeths's (1978) and Keca's (2005) observations. Rishbeth (1978) observed minimal growth of *Armillaria* spp. rhizomorphs at extreme temperatures, specifically at 10°C and 26 °C in his study. In our study, both strains of *A. ostoyae* showed optimal growth at 25°C. Keca (2005) recorded the highest growth of *A. ostoyae* on malt agar between temperatures 22 -27°C, lower at 13°C and lowest at 32°C. Very low growths observed at 2°C and 30°C might be due to the metabolic changes such as enzyme inactivation occurring in these strains (Pearce and Malakzuk, 1990). Note that at 30°C, the growth of both strains was recorded only until day 17 due to the incubator's inability to maintain the set temperature (temperature dropped down to room temperature after day 17). Both strains showed resilience at extreme temperatures, suggesting that higher temperatures might restrict growth but not eliminate the pathogen.

During the study, rhizomorph growth was not detected, but mycelial appearance varied with temperature. Denser mycelia observed at 20°C and 25°C suggest potential

variations in mycelia growth at different temperatures, indicating variable mycelium growth in different seasons in the field and this necessitates furthers field study. The low mycelium production of this pathogen at extreme temperatures may also imply that it can be easily controlled by biocontrol species. Another reason for further investigation of this pathogen in the field or natural substrates (wood panels in the lab) is that the potential difference in the growth of this pathogen observed on MA and in the wood panels in the field. For example, Rishbeth (1968) noticed difference in growth of another species of *Armillaria* - *A. mellea* in malt agar (rhizomorphs growth was observed at 30 °C) and natural substrate – wood (no rhizomorph growth was observed at 30°C).

This research sheds light on temperature-dependent growth dynamics of *A. ostoyae* emphasizing strain-specific responses and potential implications for biocontrol strategies.

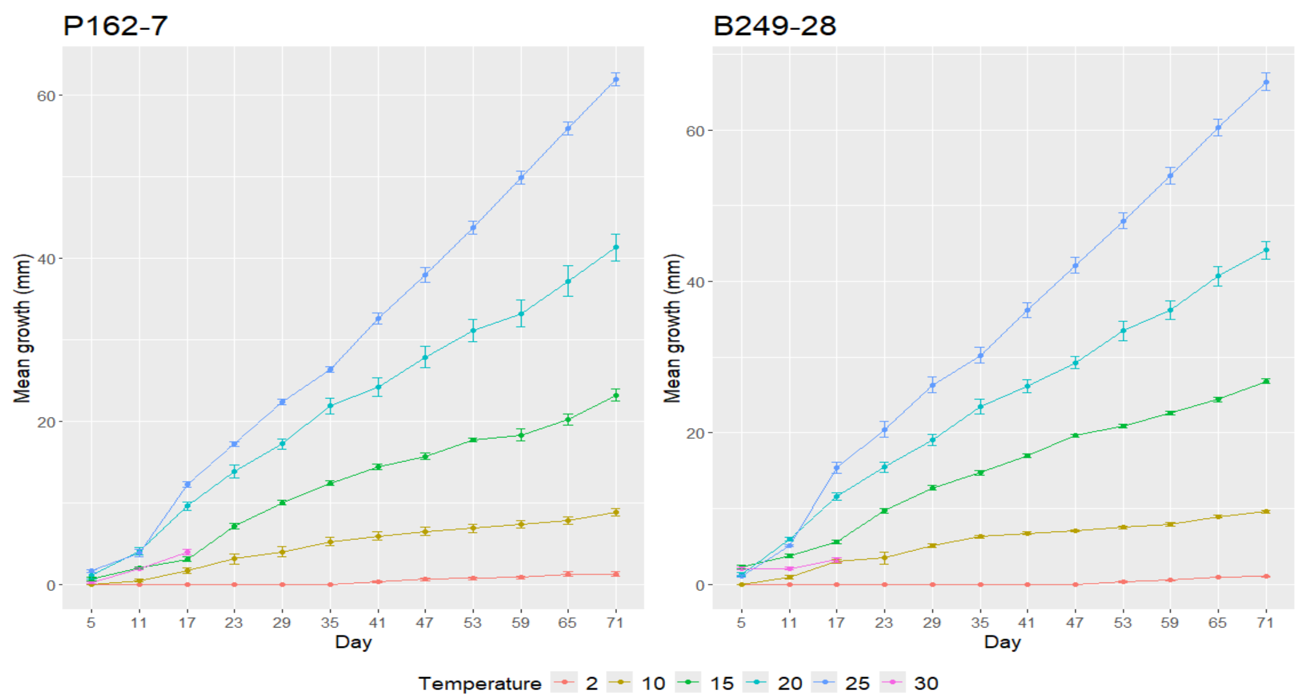


Figure 5. Mean growth of *A. ostoyae* strains (P162-7 and B249-28) over time at different temperatures 2, 10, 15, 20, 25 and 30°C (*growth was recorded till day 17). Bars above and below each mean indicate ±1 SE.

2.4.1.2 Single culture mean growth of biocontrol *Hypholoma* spp. – *Hy. fasciculare*, *Hy. capnoides* and *Hy. sublateritium* at different temperatures over time (days):

The mean growth of species and strains of *Hy. fasciculare* (Pinnel B, RLG-12668-SP), *Hy. capnoides* (TAK 2 and TAK 5) and *Hy. sublateritium* (HHB-11948-SP, OKM-6947-SP) was observed at temperatures 2, 10, 15, 20, 25 and 30 °C over a maximum period of 71 days (Fig. 8, 9, and 10). The mean growth of Pinnel B was significantly higher than other strains of *Hypholoma* spp. under study, $F(1) = 968.503$, $p < 0.0001$. For each strain, the mean growth at each temperature was also significantly different, $F(4) = 7783.412$, $p<0.001$ (Table 3).

Table 3. Three-way repeated measure ANOVA table: Single culture mean growth of biocontrol *Hypholoma* spp. – *Hy. fasciculare*, *Hy. capnoides* and *Hy. sublateritium* at different temperatures over time, n=5.

Effect	df	F	P
Strain	5	968.503	<0.001
Temperature	4	7783.412	<0.001
Day	11	2707.338	<0.001
Strain x Temperature	20	78.349	<0.001
Strain x Day	55	17.159	<0.001
Temperature x Day	44	27.577	<0.001
Strain x Temperature x Day	220	6.426	<0.001

The optimum growth for *Hy. fasciculare* occurred at 20 °C, with strain Pinnel B displaying higher growth than RLG-12668-SP at this temperature. Although the mean growth at 25°C was lower than at 20°C, it remained significantly higher than at 15°C and 10 °C. In

contrast to Wells and Boddy's (1995) observations, this study showed differences in growth rates, particularly at 25°C. Unlike Hiscox et al., (2016) findings, this study demonstrated no growth at 30°C. Hiscox et al., (2016) also noticed a higher growth rate at 12°C as compared to growth at 25°C, which is not like our observation. Differences in growth rates might be due to the differences in strains used. Similar to A'Bear et al.'s (2014) findings, this study also indicated an increase in growth with an increase in temperature up to 20°C. However, the growth declined at 25°C.

For *Hy. capnoides* and *Hy. sublateritium,* there are no prior studies which explored their growth at various temperatures. Both species failed to grow at 30°C, potentially due to mycelium viability loss or enzyme inactivation. *Hypholoma sublateritium* showed the highest growth at 20°C, with a decrease at 25°C*,* while *Hy. capnoides* thrived better at lower temperatures. This shows that this species may adapt and grow well at lower temperatures (2°C and 10 °C). The optimal temperature for *Hy. capnoides* was 25°C.

Over time, the strain Pinnel B consistently showed a significantly higher growth than other species and strains at all the temperatures. At the end of the experiment, Pinnel B showed the highest growth among all the strains of *Hypholoma* at all temperatures (Fig. 6, 7, and 8), showcasing its ability to outperform other species and strains even at extreme temperatures. *Hypholoma sublateritium* strain OKM 6947-SP had the lower mean growth at 2°C, 10°C, and 15°C. When the temperatures increased to 20°C and 25°C, *Hy. capnoides* showed minimal mean growth. At higher temperatures (20°C and 25 °C), *Hy. sublateritium* strains showed higher growth than *Hy. capnoides* strains but lower than *Hy. fasciculare* (Fig. 6, 7, and 8). All three *Hypholoma* species and their strains displayed the lowest growth at 2°C and the highest growth at 20°C and 25°C. Growth rate decreased from 20°C to 25°C for all the strains and species, except for *Hy. capnoides* (Fig. 6, 7, and 8), which continued to grow at the same rate and maintained growth at 25°C. The study highlights *Hy. capnoides* capacity

to survive and thrive at extremely high temperatures, suggesting potential to use as biocontrol agent in warming conditions. The observed results do not fully support the hypothesis that *Hy. fasciculare* and *Hy. sublateritium* may outperform *Hy. capnoides* at all temperatures in terms of growth.

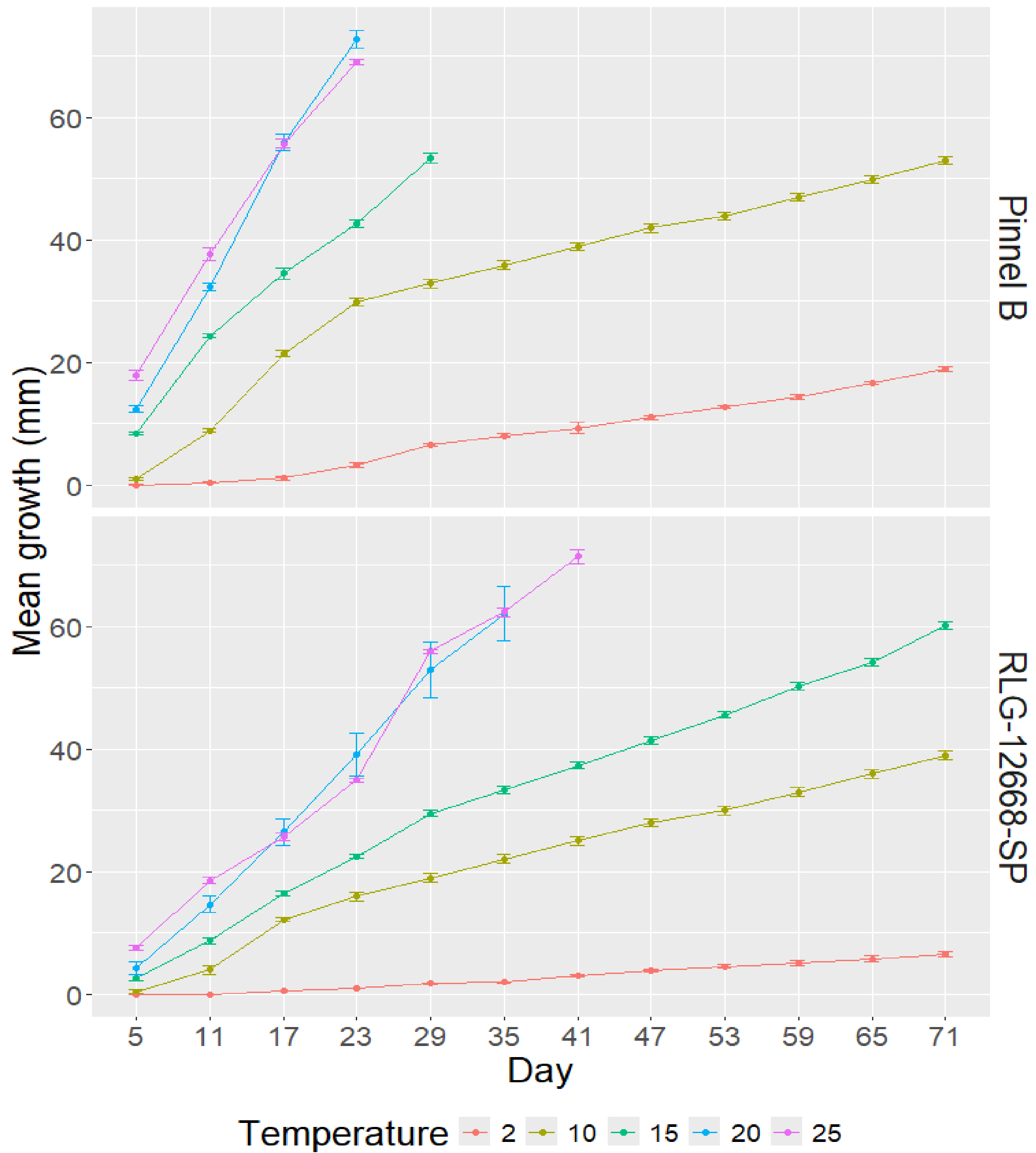


Figure 6. Mean growth of *Hy. fasciculare* strains at temperatures 2, 10, 15, 20 and 25°C over time. No growth was observed at 30°C. Graph lines ended as the strains reached the edge of the plate, indicating maximal growth before the experiment's end. Bars above and below each mean indicate ±1 SE

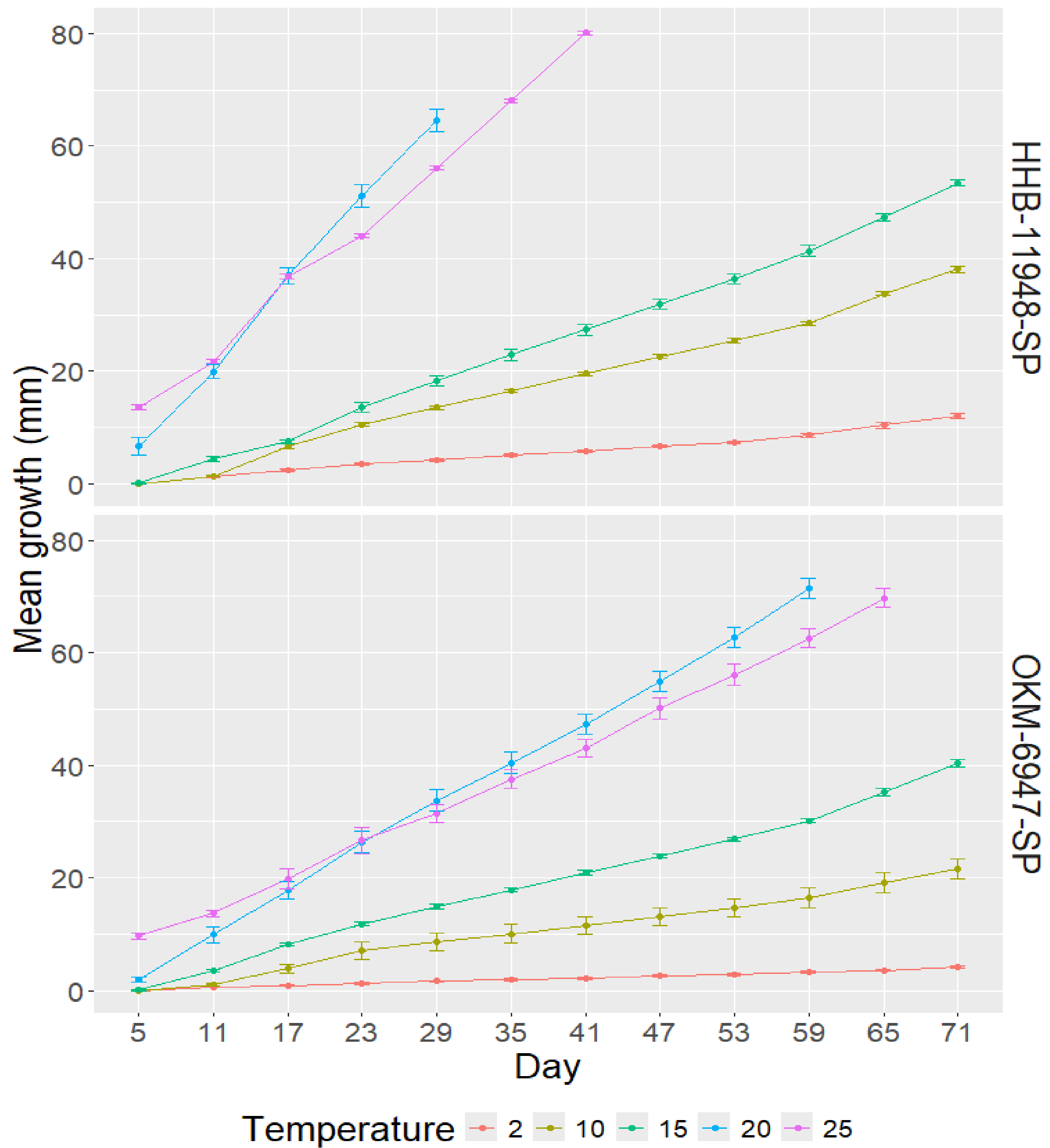


Figure 7. Mean growth of *Hy. sublateritium* strains at temperatures 2, 10, 15, 20 and 25°C over time. No growth was observed at 30°C. Graph lines ended as the strains reached the edge of the plate, indicating maximal growth before the experiment's end. Bars above and below each mean indicate ±1 SE.

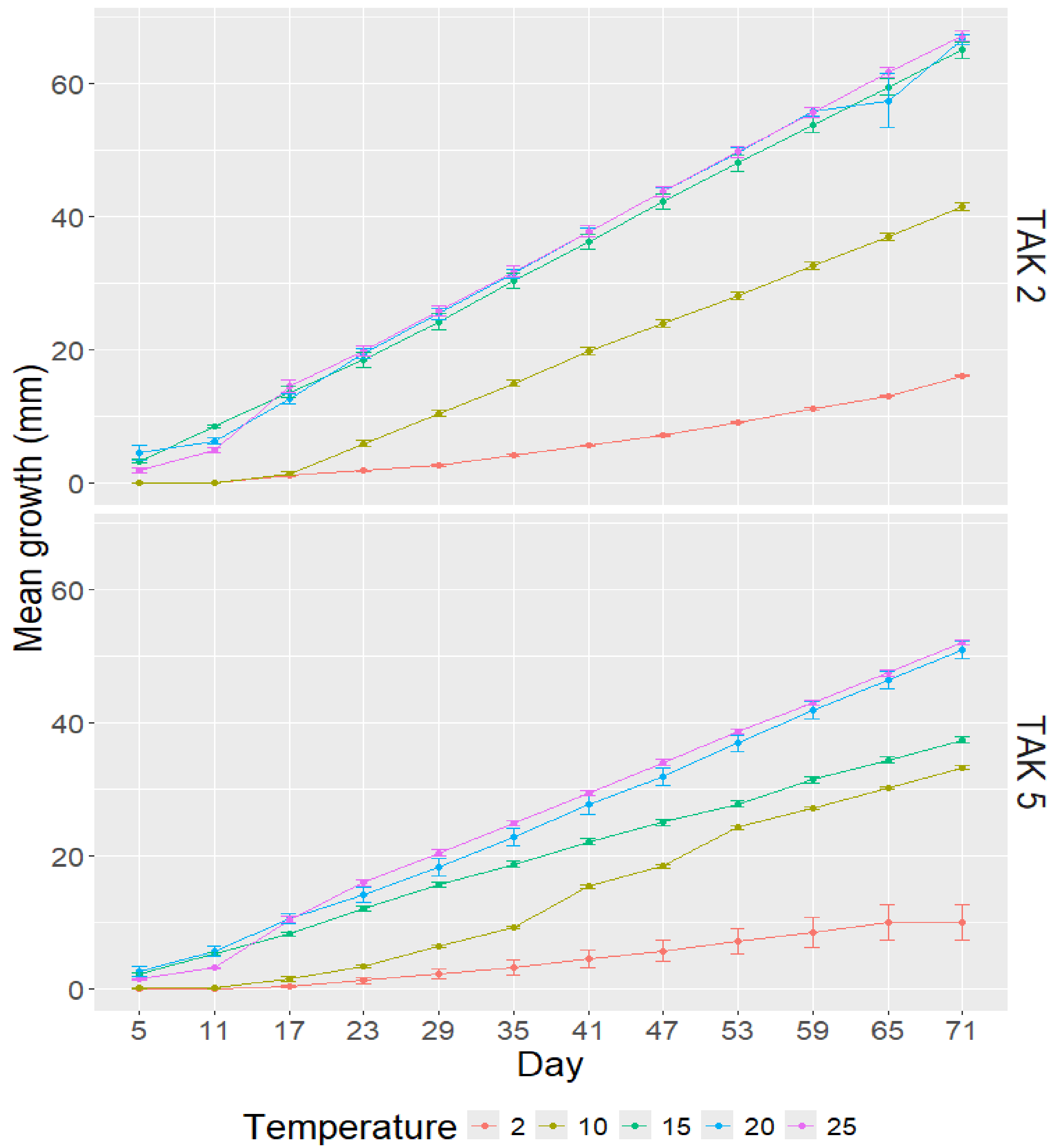


Figure 8. Mean growth of *Hy. capnoides* strains at temperatures 2, 10, 15, 20 and 25°C over time. No growth was observed at 30°C. Bars above and below each mean indicate ±1 SE.

2.4.2 Single culture mean growth of the pathogen *He. irregulare* and biocontrol *P. gigantea* at different temperatures over time

2.4.2.1 Single culture mean growth of pathogen *He. irregulare* at different temperatures over time (days)

The single culture mean growth of pathogen species *He. irregulare* was observed at temperatures 2, 10,15, 20, 25, and 30°C (Fig. 9). The mean growth of this pathogen increased significantly over the study period of 21 days at 2°C ($F (11) = 35.489$, $p < 0.0001$). The post hoc analyses revealed that the mean growth between each sampling interval significantly increased until day 19, with the maximum increase observed between day 17 and 19. At 10°C, the mean growth significantly increased over the 21-day study ($F (11) = 932.49$, $p < 0.0001$). Post hoc analyses indicated a significant increase in mean growth over each study interval at this temperature, with the maximum increase observed between day 5 and 7.

Similarly, at temperature 15°C, there was a significant increase in mean growth over the study period ($F (11) = 669.243$, $p < 0.0001$). Post hoc analyses revealed the significant increase observed until day 15, with the maximum increase recorded between day 5 and 7. At 20°C, the mean growth significantly increased over the period of study ($F (11) = 237.46$, $p < 0.0001$). Post hoc analyses indicated a significant increase until day 9, with the maximum increase observed between day 7 and 9. Finally, at 25 °C, the mean growth increased significantly over days ($F (11) = 26.251$, $p < 0.0001$). The post hoc analyses revealed a significant increase over every study interval, with the maximum increase observed between day 5 and 7.

Similarly, at temperature 15°C, there was a significant increase in mean growth over the study period ($F (11) = 669.243$, $p < 0.0001$). Post hoc analyses revealed the significant increase observed until day 15, with the maximum increase recorded between day 5 and 7. At

20°C, the mean growth significantly increased over the period of study ($F(11) = 237.46$, $p < 0.0001$). Post hoc analyses indicated a significant increase until day 9, with the maximum increase observed between day 7 and 9. Finally, at 25 °C, the mean growth increased significantly over days ($F(11) = 26.251$, $p < 0.0001$). The post hoc analyses revealed a significant increase over every study interval, with the maximum increase observed between day 5 and 7.

The highest mean growth by the end of the experiment was observed at 20 °C, followed by 15, 25, 10, and 2°C. The mean growth significantly increased from 2°C to 20°C and declined considerably at 25°C, with no growth observed at 30°C. This growth pattern of *He. irregulare* aligns with findings by Taubert (2008) and Oliva et al. (2015). Taubert (2008) observed a growth rate increase in *He. annosum* until 22°C, followed by a plateau until 27°C. Our study supports Myers et al. (2018a) and Taubert (2008), suggesting that lower temperatures such as 2°C cannot eliminate *Heterobasidion* spp., while higher temperatures such as 35°C, leaves the mycelium non-viable.

In this study, the absence of growth at 30°C, was possibly due to enzyme inactivation occurring at higher temperatures (Gooding, 1996; Taubert, 2008). Higher growth rates with increasing temperature up to 20°C may be due to the increased metabolic activities of this pathogen (Muller et al., 2014). In the event of climate change, our study suggests a potential decrease in *He. irregulare* growth with rising temperatures exceeding 25°C. During hot seasons exceeding 30°C, a decrease in *He. irregulare* infection may occur, as also suggested by Cooding et al. (1966). Rishbeth (1951b) observed *He. annosum* at various temperatures including 30°C, but our study did not detect growth at 30°C. Interspecific differences could be the reason for these differences in observations. This study provides valuable information on the growth patterns of this North American pathogen, native to Simcoe County, Ontario, Canada.

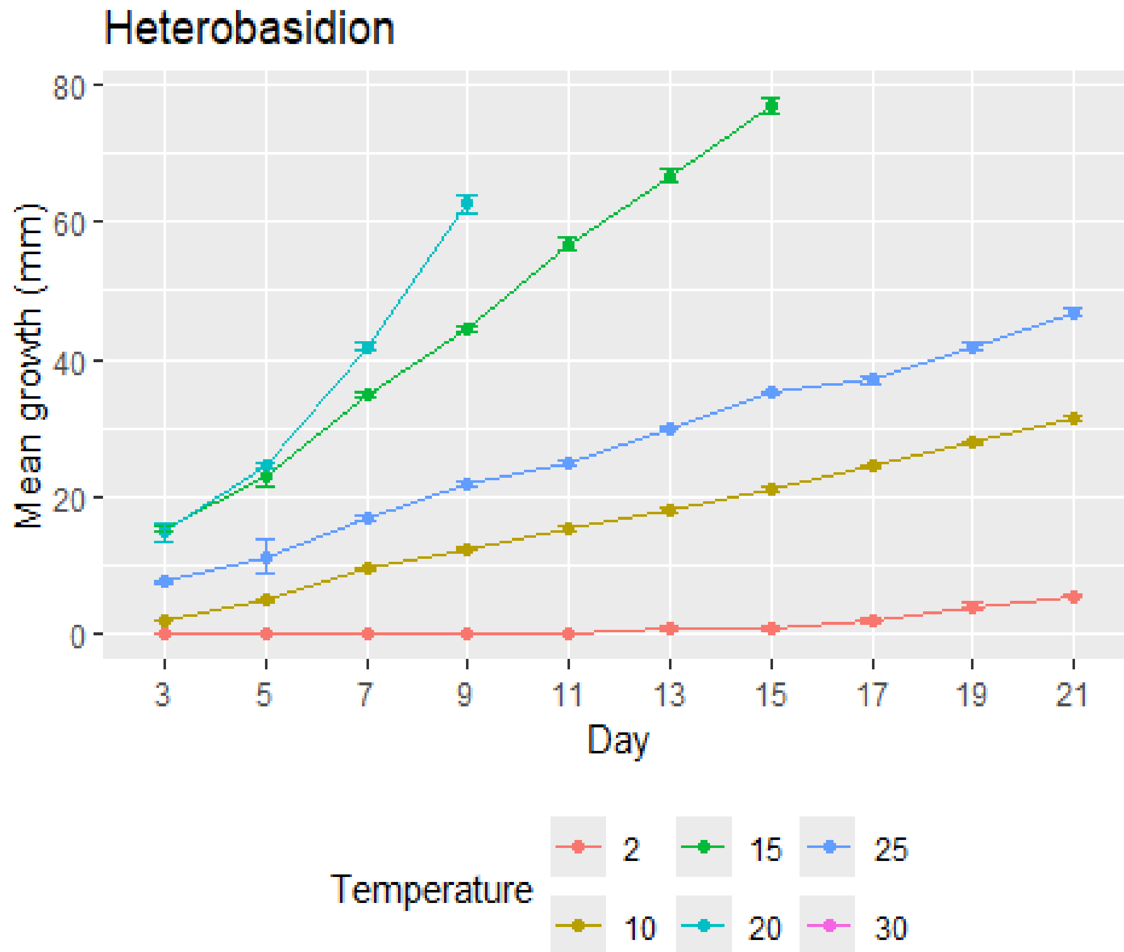


Figure 9. Mean growth of *Heterobasidion* spp. at different temperature 2, 10, 15, 20, and 25°C over time. No growth was observed at temperature 30°C. Graph lines ended as the species reached the edge of the plate, indicating maximal growth before the experiment's end. Bars above and below each mean indicate ±1 SE.

2.4.2.2 Single culture mean growth of *P. gigantea* at different temperatures over time (days)

The single culture mean growth of the *P. gigantea* was studied at temperatures 2, 10, 15, 20, 25, and 30°C (Fig. 10). At 2°C, a significant increase in mean growth was observed over 21 days, the period of study ($F(11) = 33.223$, $p < 0.0001$). Post hoc analyses revealed a significant increase after day 11, with the maximum growth observed between day 11 and 13. At temperature 10°C, mean growth significantly increased over the 21 days ($F(11) =$

1489.018, $p < 0.0001$). The post hoc analyses revealed significant increase in mean growth over all study intervals. The maximum difference ocuured between day 5 and 7. Similarly, at 15 °C, mean growth significantly increased over the study period ($F(11) = 1143.451$, $p < 0.0001$). The post hoc analyses revealed significant increase in growth until day 17, after which the species reached the edge of the plate. At 20°C, mean growth significantly increased over 21 days ($F(11) = 4004.579$, $p < 0.0001$). Post hoc analyses revealed a significant increase on each day, with the maximum increase between day 9 and 11. At 25°C, the mean growth significantly increased over days ($F(11) = 1895.04$, $p < 0.0001$). The post hoc analyses revealed significant increase till day 11, with the maximum increase between day 5 and 7. At 30°C, mean growth increased significantly over days, with the maximum increase between day 7 and 9. Our study revealed that the optimal mean growth of *P. gigantea* was at 25°C, while it was lowest at extreme temperatures of 2 °C and 30°C. The growth did not vary much at temperature 10 °C and 15°C at the end of the experiment.

As reported earlier, the mycelia production of this species decreased with decrease in temperature and viability of spores decreased at temperatures above 30°C (Thor et al., 1997; Swanwick, 2007; Zhao, 2013). For example, the growth rate of *P. gigantea* decreased with lower temperatures., Compared to the grwoth at 25°C a decrease was seen at 20 °C, with a further decrease at 15°C (Swanwick, 2007). Zhao (2013) had also noticed a low colonization rate in *P. gigantea* at lower temperatures, such as 5°C. However, spores of this species can germinate at temperature as high as 30°C (Thor et al., 1997). We also noticed growth at this high temperature.

Temperature influences fungal growth by influencing the metabolic rates and nutrient uptake, as mentioned before (Gavito et al., 2005). Higher growth rates observed with increased temperature in this study may be because of the elevated metabolic rates and

nutrient uptake. Conversely, decreased growth at temperatures higher than 20 – 25 °C may be a result of enzymes inactivation and reduced metabolic rates. In this study, the optimal temperature for *P. gigantea* (VRA 1992) was 25°C. The ability of this biocontrol species to thrive and grow at temperatures as high as 30°C suggests its potential as an efficient biocontrol agent against *H. irregulare* in the event of average temeprature hike due to global warming.

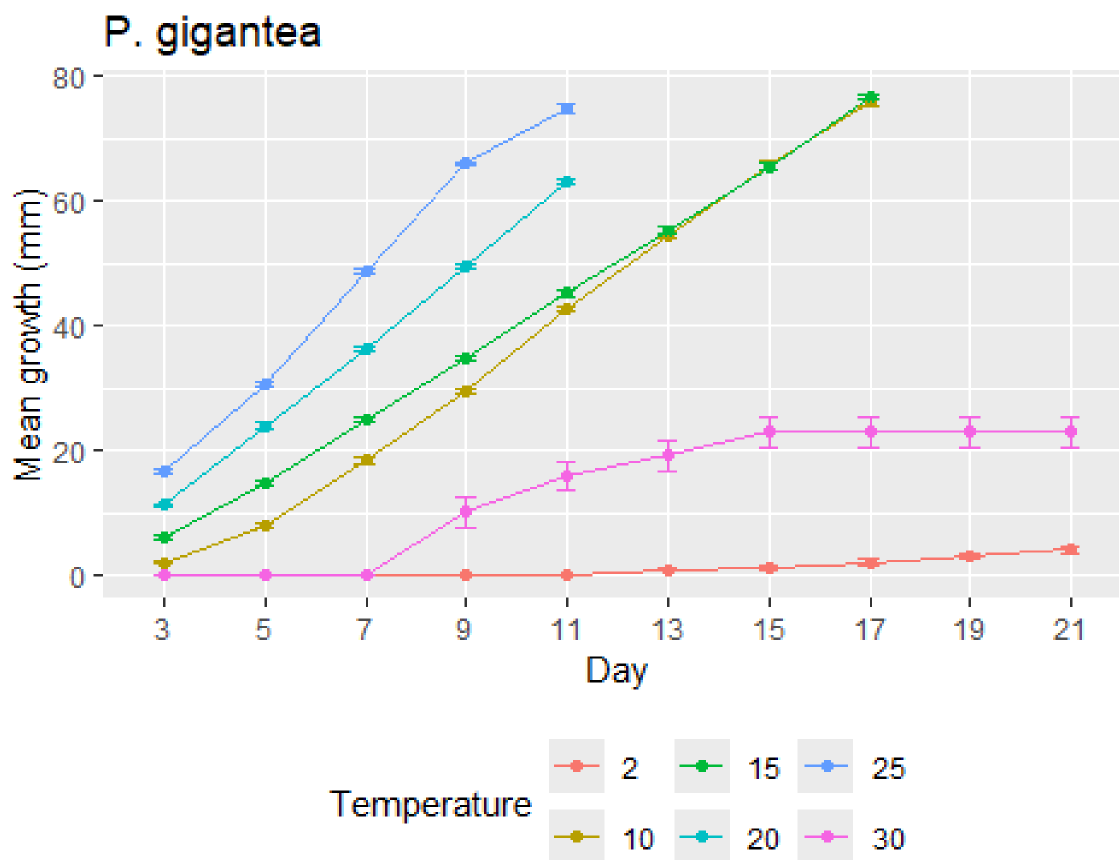


Figure 10. Mean growth of *P. gigantea* (VRA 1992) at temperature 2, 10, 15, 20, 25, and 30°C over time. Graph lines ended as the species reached the edge of the plate, indicating maximal growth before the experiment's end. Bars above and below each mean indicate ±1 SE.

2.4.3 Growth of biocontrols *Hypholoma* spp. and *P. gigantea* when paired with pathogens *A. ostoyae* and *He. irregulare* at different temperatures

2.4.3.1 *Hypholoma* spp. paired with *A. ostoyae* at different temperatures on day 11

The growth of *Hypholoma* species and strains was compared in single colony cultures and when paired with both strains of *A. ostoyae* (B249-28 and P162-7) at temperatures 10, 15, 20, and 25°C on Day 11 (Table 4). Temperatures 30°C and 2°C were not included in this pairing study because no growth was observed in *Hypholoma* spp. at 30°C and *A. ostoyae* growth was extremely low at 2°C. Note that single colony cultures were treated as controls. Day 11 was chosen for this comparison study since the biocontrol and pathogen strains started physically interacting with each other by this day at temperatures 20°C and 25°C. The mean growth of *Hypholoma* spp. in paired cultures varied significantly from their growth in single cultures for all *Hypholoma* strains (HHB-11948-SP, OKM-6947-SP, Pinnel B, RLG-12668-SP, TAK 2 and TAK 5), with Pinnel B showing higher growth than all strains in both single and paired cultures ($F(5) = 1443.94$, $p < 0.0001$). The mean radial growth also varied among all the biocontrol strains in paired cultures on day 11 ($F(2) = 145.59$, $p < 0.0001$). The mean growth of each strain of *Hypholoma* spp. also varied at each temperature on day 11 ($F(4) = 2173.32$, $p < 0.001$).

Table 4. Three-way ANOVA Table: Growth of *Hypholoma* spp. paired with *A. ostoyae* at different temperatures on day 11, n=5.

Effect	df	F	P
Strain	5	1443.94	<0.001
Temperature	3	2173.32	<0.001
Pairing	2	145.59	<0.001
Strain x Temperature	15	103.01	<0.001
Strain x Pairing	10	63.62	<0.001
Temperature x Pairing	6	57.12	<0.001
Strain x Temperature x Pairing	30	20.75	<0.001

The mean growth of *Hy. fasciculare* strain Pinnel B was highest when in single cultures and lowest when paired with B249-28 strain of *A. ostoyae* at all temperatures (Fig. 11). However, the growth of strain RLG-12668-SP increased when paired with *A. ostoyae* at temperatures 15°C, 20°C, and 25°C. This study suggests that RLG-12668-SP strain might be a more efficient biocontrol compared to Pinnel B in the event of a temperature rise in the future. The decrease or increase in growth observed in *Hypholoma* species and its strains when paired with *A. ostoyae* strains might be due to internal chemical changes occurring the in the presence of the pathogen, as discussed in 2. 2.1 (Dennis and Webster, 1997; Chapman and Xiao, 2000; Cox and Scherm, 2006; Stevens, 2019). Further investigation is needed to get more clarity of these interaction results.

A potential shortcoming of this study is that an increase or decrease in growth of biocontrol species and strains in the presence of pathogen may not be the best indicator of their efficiency in controlling the pathogen. For example, there was a decrease in the growth of Pinnel B in the presence of *A. ostoyae,* but Pinnel B was still able to overgrow the *A. ostoyae* colonies at all temperatures (macroscopic observations, not included). Pinnel B was

also found to control and significantly decrease the growth of *A. ostoyae* in macroscopic studies done by Stevens (2019).

This study demonstrates that temperature not only affects the growth of different species but also influences the interspecific interactions between the pathogens and biocontrols. These interactions can determine the ability of biocontrol agents to inhibit *A. ostoyae* at different temperatures, as increased growth of the biocontrol in the presence of the pathogen demonstrates its antagonism towards the pathogen. Chapman and Xiao (2000) observed swelling of hyphal tips and exudate release from the hyphae of *Armillaria* spp. and *Hy. fasciculare* in their microscopic studies by pairing these species *invitro.* This occurred before *Hy. fasciculare* overgrew *Armillaria* spp. We observed similar interaction where hyphae of *Hy. fasciculare* strains formed globular structures at hyphae tips upon coming in close proximity of *A. ostoyae*. (results not included), which might explain the efficacy of this biocontrol against *Armillaria* spp. Further investigation is needed to determine the mechanism behind these interactions.

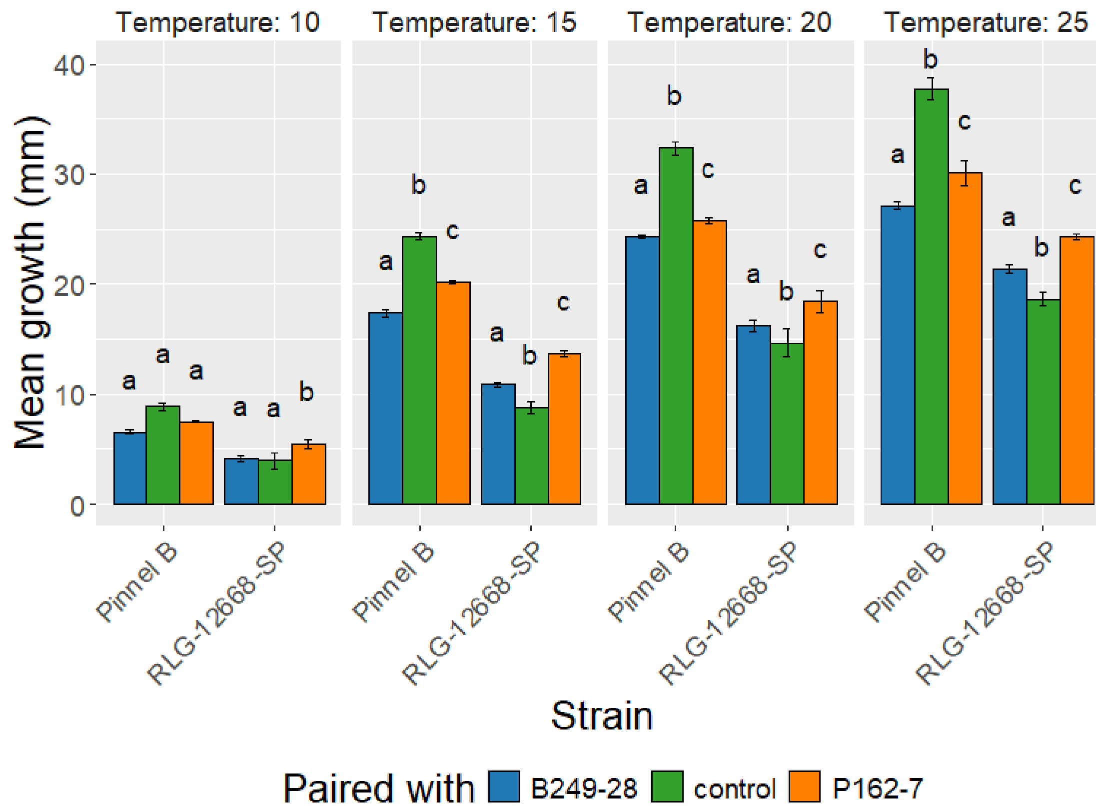


Figure 11. Mean growth of *Hy. fasciculare* strains (Pinnel B and RLG-12668-SP) in single (Control) cultures and when paired with strains of *A. ostoyae* (B249-28 and P162-7) at temperatures 10, 15, 20 and 25°C on day 11. Bars above and below each mean indicate ±1. Same letters above the bars indicate no significant difference between the mean growth of strains while different letters indicate significant differences at p = 0.05 (Tukey's test).

This study also examined the mean growth of *Hy. sublateritium* in the presence and absence of *A. ostoyae* strains B249-28 and P162-7 at temperatures – 10, 15, 20 and 25°C on day 11 (Fig. 12). The mean growth of *Hy. sublateritium* strain HHB-11948-SP was higher than control when paired with both the strains of *A. ostoyae* (B249-28 and P162-7) at all temperatures, except at the higher temperature of 25°C (Fig. 12), indicating that the higher temperature may reduce the biocontrol efficacy of this strain to control the pathogen. However, in the case of OKM-6947-SP strain, the mean growth increased when paired with

both the strains of *A. ostoyae* at temperatures 15°C, 20°C and even at higher temperature, 25°C, with the highest increase observed when paired with pathogen strain P162-7. This outcome supports the studies done by Schoeman et al. (1996) and Crowther et al. (2012b), who observed that different responses of strains to different temperature can reverse the outcome of the interspecific interactions by stimulating the antagonistic activity of slower-growing strain to the extent that it grows faster in presence of an antagonist.

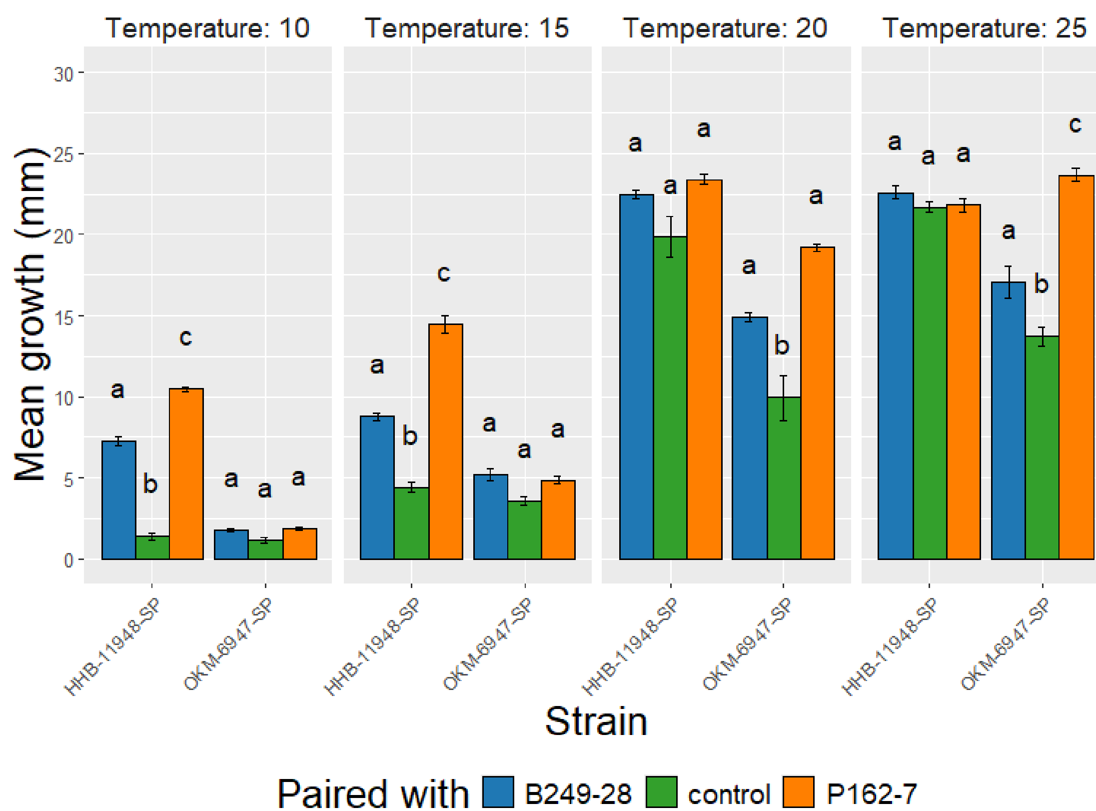


Figure 12. Mean growth of *Hy. sublateritium* strains (HHB-11948-SP and OKM-6947-SP) in single (Control) cultures and when paired with strains of *A. ostoyae* (B249-28 and P162-7) at temperatures 10, 15, 20 and 25°C. on day 11. Bars above and below each mean indicate ±1. Same letters above the bars indicate no significant difference between the mean growth of strains while different letters indicate significant differences at p = 0.05 (Tukey's test).

In the case of *Hy. capnoides,* the mean single culture growth of both strains (TAK 2 and TAK 5) was higher compared to when paired with the strains of *A. ostoyae* (B249-28 and P162-7) (Fig. 13) at 10°C, while there were no significant differences at 25°C. At 15°C, the growth of TAK 2 was higher in single cultures (controls), implying that the presence of *A. ostoyae* significantly decreased its growth. On the other hand, TAK 5 showed a higher growth when paired with B249-28 but did not decrease significantly when paired with P162-7. This suggests that at 15°C, TAK 5 may outperform B249-28. At 20 °C, the growth of TAK 5 showed significant increase in presence of B249-28 and P162-7 as compared to their single colony growth. The highest growth rate of this strain in the presence of competition at 20°C suggests that different temperatures result in different growth rates in this strain by stimulating certain physiological or chemical responses. This study supports the findings of Keca (2009), who noticed a considerable decrease in the growth of *A. ostoyae* in the presence of *Hy. capnoide*s, suggesting that higher growth rates of this biocontrol species in presence of a pathogen may inhibit the growth of the pathogen. However, further investigation is needed to determine the growth of *A. ostoyae* in presence of *Hy. capnoides* strain TAK 5 at high temperatures, such as 25 °C. This will help us to understand how this strain behaves in the event of climate change resulting in a higher atmospheric temperature.

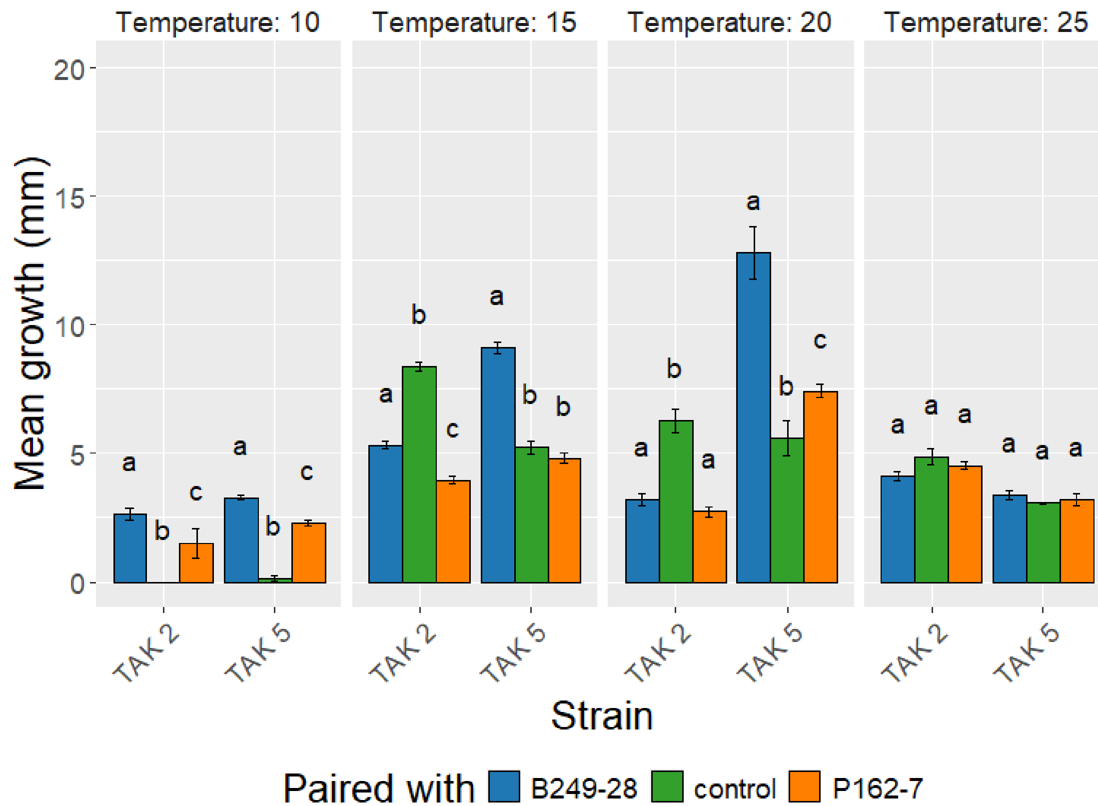


Figure 13. Mean growth of *Hy. capnoides* strains (TAK 2 and TAK 5) in single (Control) cultures and when paired with strains of *A. ostoyae* (B249-28 and P162-7) at temperatures 10, 15, 20 and 25°C on day 11. Bars above and below each mean indicate ±1. Same letters above the bars indicate no significant difference between the mean growth of strains while different letters indicate significant differences at p = 0.05 (Tukey's test).

2.4.3.2 *Phlebiopsis gigantea* paired with *He. irregulare* at different temperatures on day 5

The mean growth significantly varied between species *P. gigantea* and *He. irregulare* on day 5 (Table 5) ($F(1) = 1012.06$, $p < 0.0001$). Day 5 was chosen for interaction studies because after this day, the biocontrol and pathogen mycelium started physically interacting, which would have compromised radial growth measurement accuracy. The mean growth also varied significantly when the above species were paired together ($F(2) = 224.10$, $p < 0.0001$) at all temperatures ($F(3) = 1224.19$, $p < 0.001$).

Table 5. Three-way ANOVA Table: *P. gigantea* paired with *He. irregulare* at different temperatures on day 5, n=5.

Effect	df	F	P
Strain	1	1012.06	<0.001
Temperature	3	1224.19	<0.001
Pairing	2	224.1	<0.001
Strain x Temperature	3	226.06	<0.001
Temperature x Pairing	6	21.44	<0.001

The mean growth of *He. irregulare* and *P. gigantea* was studied at 10, 15, 20 and 25°C on day 5. Post hoc analyses revealed a significant decrease in the mean growth of *He. irregulare* at all temperatures when paired with *P. gigantea*, compared to its single culture growth at all temperatures, except at 25°C, where it remained unchanged (Fig. 14). This suggests that at temperature higher than optimal, the growth of *He. irregulare* may not be inhibited significantly by *P. gigantea*.

There was a significant increase in the growth of *P. gigantea* when paired with *He. irregulare* at all different temperatures (Fig. 15), indicating its antagonistic nature against *He. irregulare* at all temperatures. Increase in growth and rapid colonization of the substrate is the primary mode of action of *P. gigantea* against *Heterobasidion* spp. However, despite having the highest growth at 25°C, *P. gigantea* could only inhibit the growth of *He. irregulare* by 9.4% (Table 6). Hence, this study does not support our hypothesis that *He. irregulare* will show higher resistance against *P. gigantea* at lower temperatures than at higher temperature. A potential increase in temperature due to possible changing climate conditions may favour the disease-causing pathogen *He. irregulare* even in the presence of *P. gigantea*.

However, this needs to be further investigated through macroscopic, microscopic, and field studies, as with time, *P. gigantea* may still be able to overgrow *He. irregulare* at temperature of 25°C (Oliva et al., 2015).

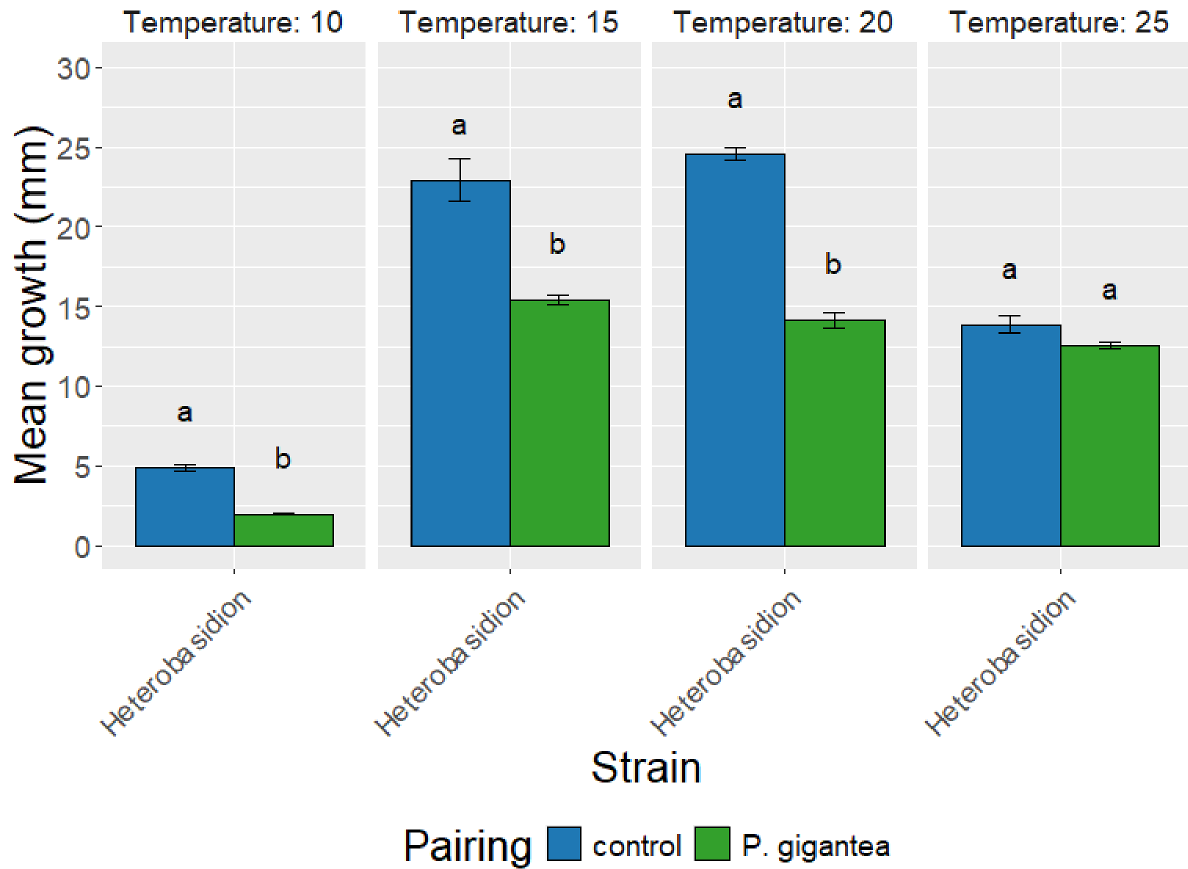


Figure 14. Mean growth of *He. irregulare* in single culture and when paired with *P. gigantea* at different temperatures on day 5. Bars above and below each mean indicate ±1 SE. Same letters above the bars indicate no significant difference between the mean growth of strains while different letters indicate significant differences at p = 0.05 (Tukey's test).

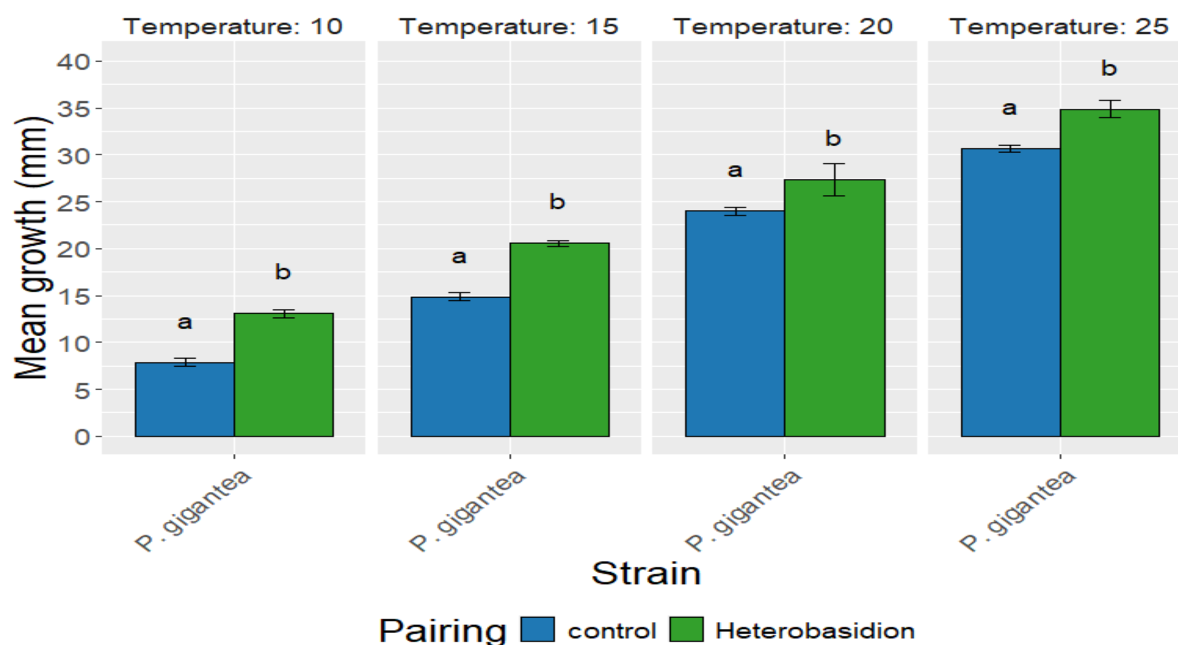


Figure 15. Mean growth of *P. gigantea* in single cultures and when paired with *He. irregulare* at different temperatures on day 5. Bars above and below each mean indicate ±1 SE. Same letters above the bars indicate no significant difference between the mean growth of strains while different letters indicate significant differences at p = 0.05 (Tukey's test).

Table 6. Percentage inhibition of *He. irregulare* at different temperatures by *P. gigantea* on day 5, when *He. irregulare* was paired with *P. gigantea*.

Temperature	**Mean growth of *H. irregulare* in control (mm)**	**Mean growth (mm) of *H. irregulare* when paired with *P. gigantea***	**Inhibition %**
10°C	4.9	1.96	60
15°C	22.9	14.1	32.83
20°C	24.5	15.4	42.44
25°C	13.8	12.5	9.4

2.5 Conclusions

Significant differences in the growth and interactions among potential biocontrols, *Hypholoma* spp. and *P. gigantea*, and the fungal pathogens *A. ostoyae* and *He. irregulare,* particularly at extreme temperatures of 2°C and 25°C, were observed. Growth rate disparity was evident not only between different species but also among different strains of the same species at all temperatures. While *Hy. capnoides* showed better single colony growth at lower temperatures, *Hy. fasciculare* and *Hy. sublateritium* showed comparatively faster growth in paired cultures across all temperatures. The results suggest that at higher temperatures, *Hy. fasciculare* and *Hy. sublateritium* may be effective in controlling the growth of *A. ostoyae* due to their faster growth and antagonistic interaction with *A. ostoyae*. This requires further investigation to confirm competition outcomes and to determine the mechanisms involved once the species intermingle. Once confirmed, these biocontrol species could be developed to provide year-round protection against *A. ostoyae* in southern Ontario, especially the *Hy fasciculare* strain Pinnel B. Further, understanding the impact of these biocontrol species on pathogen growth at different temperatures is crucial for implementing effective biocontrol measures.

Given the variability among different strains of the same biocontrol species when paired with different pathogen strains at different temperatures, future studies should focus on native strains of *Hypholoma* spp. to identify the optimal biocontrol option. The ability of the pathogen *A. ostoyae* to grow at high temperatures, 30°C, and the inability of biocontrol *Hypholoma* spp. to thrive at such high temperature, raises concerns about the biocontrol efficacy of this species in the face of potential temperature increases associated with climate change.

Conversely, *P. gigantea's* ability to grow more rapidly at higher temperatures (25°C and 30°C) compared to *He. irregulare,* along with the inability of *He. irregulare* to grow at

30°C, suggested that the biocontrol *P. gigantea* might be highly effective at extremely high temperatures. However, when paired together, *P. gigantea* inhibited the growth of *He. irregulare* by only 9.4% at 25°C, while 60 % growth inhibition was observed at 10°C. These comparisons, conducted on day 5 for *He. irregulare* and *P. gigantea*, may provide different results with longer pairing periods. There is a possibility that *P. gigantea* may overgrow *He. irregulare* at 25°C, given its faster growth at this temperature.

Overall, our study confirmed the potential for year-round efficacy of *P. gigantea* against *He. irregulare,* but further investigation using various native strains of *He. irregulare* in the natural environment is necessary. Additionally, exploring the impact of different temperatures on red pine growth is essential to understand the host defense mechanism in response to climate change, influencing the virulence and disease-causing ability of these pathogens and their interactions with biocontrols.

Chapter 3 Effect of different pH on growth and interactions of *Hypholoma* spp. and *Phlebiopsis gigantea* with *Armillaria ostoyae* and *Heterobasidion irregulare* pathogens

3.1 Abstract

Hypholoma species and *Phlebiopsis gigantea* are potential biocontrol agents against the pathogenic fungi *Armillaria ostoyae* and *Heterobasidion irregulare* in conifer plantations. The growth and virulence of these pathogens and their interactions with the biocontrols are likely influenced by soil pH, which is not fully explored. Controlled *in vitro* experiments were conducted using 2% buffered malt agar media at pHs of 5.0, 6.0, 7.0, and 8.0. Radial growth of single colony cultures of the pathogens *A. ostoyae* and *He. irregulare* along with two strains each from three *Hypholoma* spp. (*Hypholoma fasciculare, Hy. sublateritium, Hy. capnoides*), and one strain of *P. gigantea*, the respective biocontrols was quantified and compared. Paired cultures were established to examine interactions between *A. ostoyae* and *Hypholoma* spp. and between *He. irregulare* and *P. gigantea*, ultimately, to compare and contrast biocontrol potential against their respective pathogens. In single colony cultures, optimal growth for the pathogen *A. ostoyae* occurred at pH 5 while *He. irregulare* showed the highest growth at pH 7, followed by pH 6, 5, and 8. Among biocontrol species of *Hypholoma*, *Hy. fasciculare* (Pinnel B) showed consistently higher growth than other species and strains, while *Hy. capnoide*s (TAK 5) showed comparatively lower growth at all pH levels. *Phlebiopsis gigantea* showed maximum growth at pH 6, followed by pH 7, 5, and the least at pH 8. In paired cultures, *Hy. fasciculare* and *Hy. sublateritium* inhibited both strains of *A. ostoyae,* as growth of *A. ostoyae* decreased in their presence, at pH 5 and 6, which are optimal conditions for *A. ostoyae. Phlebiopsis gigantea* could not to effectively inhibit *He. irregulare* growth at higher pH level (8), suggesting potential challenges in biocontrol efficacy under changing soil pH conditions.

3.2 Introduction

Armillaria and *Heterobasidion* spp. are important pathogenic fungi that cause root and butt rot diseases in timber-producing conifer plantations across Canada (Rudolf, 1990; McLaughlin, 2001; McLaughlin and Hsiang, 2010), the virulence and spread of which, are contingent on complex interactions between various biotic and abiotic factors in the environment (Holusa et al., 2018). These pathogens affect the economic value of these plantations by weakening root systems, subsequently causing reduced height and diameter growth of plantation trees, and ultimately resulting in complete canopy dieback (McLaughlin, 2001; Holusa et al., 2018).

3.2.1 Soil pH and fungal pathogens

Various abiotic factors affect the spread and pathogenicity of these pathogens (Otrosina and Cobb, 1989). One critical factor affecting the development and spread of root and butt rot diseases in conifer plantations is soil pH (Redfern, 1978; Wargo and Harrington, 1991; Hietala et al., 2016). Soil pH impacts the growth of plantation trees and their ability to defend against pathogens (Shield and Hobbs, 1979; Singh, 1983; McLaughlin, 2001). For example, McLaughlin (2001a) suggested that pines growing on mildly acidic soils were healthier than those in alkaline soils given their reduced susceptibility to root diseases caused by *Armillaria* and *Heterobasidion* spp. However, conifer trees in moderately acidic sandy soils were more susceptible to root rot by *Armillaria* spp. such as *A. mellea* than those in alkaline clay soils (Redfern, 1978). Shield and Hobbs (1979) observed similar effects of low soil pH on Douglas fir (*Pseudotsuga menziesii*) plantations. The influence of low soil pH on disease incidence is species- specific. Soil pH also affects *Heterobasidion* butt rot incidence (Hietala et al., 2016), where the infection rate of Norway spruce plantation trees in pH 4.0 soils was approximately half of that observed in pH 5.0 soils (Hietala et al., 2016).

However, the mechanism by which soil pH affects tree growth is not fully understood (Termorshiuzen, 2000). McLaughlin and Hsiang (2010) suggested that soil pH might affect host access to soil nutrients such as iron. Soil alkalinity in upper soil horizons may lead to shallow root systems and low iron solubility, making it unavailable to red pine (McLaughlin and Hsiang, 2010). These unfavourable site conditions make the host more prone to infections by disease-causing pathogens such as *Armillaria* and *Heterobasidion* spp*.,* as a stressed host is less capable of defending itself against these pathogens (McLaughlin, 2001a; Gori et al., 2013). Moreover, Armillaria root rot is considered a disease of weakened trees (Wargo and Harrington, 1991). *Armillaria* species, such as *A. mellea*, act as facultative parasites in already weakened plantations, suggesting they may continue their life cycles on the same host even after its death (Shield and Hobbs, 1979).

3.2.2 *Armillaria* spp.

The spread of pathogens, such as *Armillaria* and *Heterobasidion* spp., occurs either through mycelial formation or via basidiospores. Soil pH significantly affects basidiospore germination and mycelium development and growth (Wargo and Carey, 2001; Bruna et al., 2019). Predicting fungal species' development requires understanding soil pH's influence on their growth rates and virulence. Several studies have linked substrate pH to pathogen growth and virulence (Rishbeth, 1982; Singh 1983; Gibbs et al., 2002). They suggest that *Armillaria* spp. are more pathogenic in low pH conditions (Rishbeth, 1982; Singh, 1983), while *Heterobasidion* spp. are more pathogenic at high pH (Gibbs et al., 2002). However, there is considerable variation among different pathogen species with respect to their distribution, host preferences, damage by infection, and response to substrate pH (Guillaumin et al., 1993). For example, *A. mellea* is very destructive in broadleaf forests, orchards, and fruit and ornamental trees while *A. ostoyae* is more damaging to conifer plantations (Oliva et al., 2009; Anselmi et al., 2021). *Armillaria* species also differ in their growth response to different soil

pH conditions (Oliva et al., 2009). For example, *A. ostoyae*, was found to be growing best in acidic soil (pH 4.82), while other species such as *A. cepistipes* and *A. gallica* preferred weakly acidic conditions (pH 5.14 and 6.15, respectively) (Oliva et al., 2009). The spread and survival of *Armillaria* in soil are facilitated by the production of highly differentiated filamentous aggregations, i.e., rhizomorphs (Garraway et al., 1991). During infection, rhizomorphs penetrate the bark of the host to form a mycelial fan that eventually infects the cambium (Kwasna et al., 2014). Rhizomorph length also differs at different pH levels (Rishbeth, 1985; Tsykun et al., 2011). For example, *A. mellea* produced approximately 50% longer rhizomorphs at pH 5.5 as compared to pH 7, while *A. obscura* and *A. bulbosa* produced comparatively more extensive rhizomorphs in similar soil pH conditions (Rishbeth, 1985). Studies on *A. ostoyae* and *A. cepistipes* also point towards more rhizomorph growth and virulence at relatively low pH, but with differences in pH preferences among these two species as well (Singh 1983; Redfern and Filip, 1991; Browning and Edmonds, 1993; Oliva et al., 2009). For example, *A. ostoyae* rhizomorphs were found in acidic soils having pH 3-4 but absent in soil with a pH above 5.4 (Tsykun et al., 2011). Conversely, *A. cepistipes* rhizomorphs were more frequent in areas with soil pH ranging from 3.5-5.5 (Tsykun et al., 2011). Moreover, low soil pH, accompanied by low nutrient content, not only affects the mycelium growth of pathogens but also the onset of disease symptoms in plantations (Singh, 1983). For example, Singh (1983) found that the development of *Armillaria* spp. mycelia was greater in infected plantations growing at low pH and low nutrient content as compared to higher pH. The disease symptoms were detected 2 to 6 months earlier at lower pH with a greater annual height reduction (about 30%) than infected plantations growing at higher pH and better nutrient content.

Just as there is variability among different *Armillaria* spp. in response to pH, as mentioned above (Redfern and Filip, 1991; Holusa et al., 2018), similar variability may be

observed among different strains of the same species (Morrison, 1974). Some strains of same species of *Armillaria,* like *A. mellea,* prefer alkaline pH over acidic (Morrison, 1974). Similarly, Redfern (1970), noted higher *A. mellea* rhizomorph growth in alkaline soils than in acidic soils. The growth and pathogenicity of the most common Ontario *Armillaria* species, *A. ostoyae* (Dumas, 1988), vary among Ontario conifers and could be associated with low pH (Redfern and Filip, 1991; Browning and Edmonds, 1993; Oliva et al., 2009; Holusa et al., 2018). However, little information is available on different *A. ostoyae* strains' growth at varying pH levels. In this study, growth of two native strains of *A. ostoyae* will be compared across a range of pH conditions to improve understanding of their responses to different pH conditions.

3.2.3 *Heterobasidion* spp.

Previous studies have associated *Heterobasidion* root rot disease growth and development with soil pH (Rishbeth, 1951b; Pratt and Grieg, 1988; Korhonen and Stenlid, 1998; Bruna et al., 2019). However, limited studies have examined how pH variation affects *Heterobasidion* growth. Although *Heterobasidion annosum* mycelium can grow in a wide pH range, the severity of the disease and the volume of trees killed were higher on substrate with pH >7 (Rishbeth, 1951a; Rishbeth, 1951b; Gibbs et al., 2002). In contrast to *Armillaria* spp., low soil pH negatively affects the growth and development of infection by *Heterobasidion* spp., such as *He. parviporum* (Hietala et al., 2016; Bruna et al., 2019). For example, low soil pH significantly restricted *He. parviporum* growth in Northern Finland, reducing *Heterobasidion* butt rot incidence (Hietala et al., 2016). However, *Heterobasidion* species also vary in their distribution, host preferences, pathogenicity, and response to edaphic factors such as soil pH. The *Heterobasidion annosum sensu lato* complex is widely distributed (Garbelotto and Gonthier, 2013) and includes five species, three of which occur in Europe (*He. annosum* (P isolate), *He. parviporum* (S isolate) and *He. abietinum* (F isolate))

and two are widespread in North America (*He. irregulare* and *He. occidentale*) (Dalman et al., 2010; Gaitniek et al., 2016; Lushaj, 2016). The North American species, *He. irregulare* and *He. occidentale*, have different host preferences. *Heterobasidion irregulare* mainly infects conifers such as pine (*Pinus* spp.), while *He. occidentale* has a broader host range and occurs on hemlock (*Tsuga* spp.) and fir (*Abies* spp.) (Lushaj, 2016). *Heterobasidion irregulare* is widespread in British Columbia, Canada and is believed to be the most aggressive pathogen in the entire species complex (Lushaj, 2016; Shamoun et al., 2019). Genetically different isolates may have differences in their growth and pathogenicity in response to different substrate pH (Johansson et al., 2002; Majewska et al., 2004; Lakomy et al., 2014). For example, different strains of P isolate, had varied responses to different pH conditions, such as pH 3.5 (Majewska et al., 2004). Johansson et al. (2002) tested two strains of S isolate and found that their growth was restricted at pH>7. However, there is no such information available for *He. irregulare* strains native to Simcoe County, Ontario.

3.2.4 Antagonistic soil organisms

Soil pH not only influences *Armillaria* and *Heterobasidion* pathogenicity by affecting their growth (as described above) but also by affecting the growth and interactions of organisms antagonistic to these pathogens that are being used as biocontrol agents (Rishbeth 1951b; Korhonen and Stenlid, 1998; Boddy, 2000). Several authors correlated the low level of *Heterobasidion* infection in soils with low pH and the presence of an antagonistic organism (Korhonen and Stenlid, 1998). For example, the growth and spread of *Heterobasidion* infection was greater on alkaline soils where its antagonist, *Trichoderma viride*, was absent as it prefers acidic substrate (Rishbeth, 1951a; Rishbeth, 1951b; Korhonen and Stenlid, 1998).

Information on substrate pH effects on biocontrol fungi species *Hypholoma* spp. and *Phlebiopsis gigantea,* which are potential biocontrols of *A. ostoyae* and *He. irregulare,*

remains scarce (described in 2.1). *Phlebiopsis gigantea* is currently used as a successful biocontrol against *Heterobasidion* spp. (Pratt et al., 2000). However, no studies have been done to determine the effect of pH on the growth of *P. gigantea*, if any. It has been suggested that urea, a chemical used for controlling infection by *Heterobasidion annosum*, controls the growth of this pathogen by increasing the substrate pH, until it is unsuitable for growth of this pathogen (Johansson et al., 2002). Thus, understanding the impact of substrate pH on the growth of *Hypholoma* spp. and *P. gigantea,* both in presence and absence of pathogens, is crucial for determining their biocontrol efficacy.

3.2.5 Objectives

The objective of this study is to determine the radial growth of pathogens *A. ostoyae* (2 strains) and *Heterobasidion irregulare* and their respective potential biocontrols *Hypholoma species – Hy. fasciculare* (2 strains), *Hy. sublateritium* (2 strains) and *Hy. capnoides* (2 strains) and *P. gigantea* (VRA22) at 4 different substrate pH levels (representing soil pH variation observed in Simcoe County, Ontario) in single and paired culture in-vitro settings.

This study will help determine the growth potential and competitive ability of the fungal pathogens and their prospective biocontrols to various pH conditions. Since the effects of soil pH differ among different species of the pathogens, it would be interesting to know the effect of different pH levels on different species of *Hypholoma* and their strains.

Testing the growth of these fungal species and strains will not only provide information concerning pH levels most suitable for their growth, but also indicate the tolerance of the biocontrols to different substrate pH in presence and absence of a competitor (pathogen).

I hypothesised that both *A. ostoyae* strains may show better growth at relatively low pH, given that both occur in similar field conditions in Ontario, Canada. Due to variation in

pH preferences and growth among different species and strains, it is likely that various species and strains of biocontrol *Hypholoma* will show distinct pH preferences. *Hypholoma species* such as *Hy. fasciculare* may grow well and show higher growth at relatively low pH because of its acidophilic nature. Secondly, I hypothesised that *He. irregulare* may show greater radial growth in neutral to slightly alkaline conditions, owing to its preference for higher pH (Gibbs et al., 2002).

3.3 Materials and methods

3.3.1 Fungal isolates and experimental design

The growth of pathogens *A. ostoyae* (2 strains) and *He. irregulare* and their potential biocontrols: *Hypholoma fasciculare* (2 strains), *Hy. capnoides* (2 strains), *Hy. sublateritium* (2 strains), and *Phlebiopsis gigantea* (VRA 1992) were monitored and compared *in-vitro* at different substrate pH levels. The cultures, used as inoculum in the study, were grown and inoculated on fresh 2% MA media under aseptic conditions. The initial pH of the unadjusted medium was 6.15, and the cultures were incubated at room temperature (22°C) in the dark. Inoculum of the isolates was then grown on approximately 20 ml of buffered 2% malt agar (MA) media dispensed in 100 x 15 mm plastic Petri plates in single cultures (experiment 1) and paired culture settings (experiment 2) at pH 5.0, 6.0, 7.0 and 8.0. This pH range was chosen to represent the pH range found in Simcoe County soils, Ontario, Canada (Hoffman et al., 1962). Since *Hypholoma* species are capable of lowering pH (Johansson et al., 2002; Makela et al., 2002; Magnusan and Lasure, 2004; de Boer et al., 2010), buffers were used to ensure that pH levels remained constant (Browning and Edmonds, 1993). The buffering systems were as described for a study of *Serpula lacrymans* (Maurice et al., 2011) (Table 7). Buffer systems were tested for their efficacy, and no significant changes in pH were found after a week of incubation. The pH of each buffered medium was determined using pH probes after a week of incubation for all the species and strains (Maurice et al., 2011). The efficacy of the buffer systems was tested by taking 1 g of 7-day old fungal inoculum in 1 ml of distilled water and measuring the pH after homogenising this mixture. (Johansson et al., 2002). No significant differences were found in the pH was found, suggesting that the buffer system was efficient.

Radial growth of all the fungi isolates was measured in (mm) using a ruler under a dissecting microscope and inverted microscope every 48 hours until day 20 for *A. ostoyae*

and *Hypholoma* spp., and until day 12 for *He. irregulare* and *P. gigantea*. Detailed protocols for each objective are shown below.

Note that although some of the cultures developed rhizomorphs, they were not included in the measurements. Rhizomorphs are important in disease spread under field conditions (Tsykun et al., 2011).

3.3.2 Experiment 1: Impact of pH on growth of fungal pathogen and biocontrol isolates

All the species and stains were initially grown on 2% malt agar (MA) media in Petri dishes under aseptic conditions. The plates were inoculated with a 7 mm agar disc with mycelium, cut with a sterile cork borer from approximately 4 mm inside the leading edge of 10-day-old colonies growing at room temperature (22 °C). Each strain was inoculated onto one edge of 50 petri plates containing approximately 20 ml of 2% buffered MA (Table 1). Five replicates for each fungal species/ strain were established at each pH in the dark (Maurice et al., 2011) and three observations were recorded per replicate (please refer to chapter 2 and Fig. 3 for more details). Measurements were taken at 48-hour intervals, starting from day 4 and continuing until strains reached the edge of the plate. This occurred on day 20 for *A. ostoyae* and *Hypholoma* spp. For *He. irregulare* and *P. gigantea* the measurements started from day 2 until day 12.

Table 7. Buffer systems from pH 5.0 to 8.0 (*x*ml A + *y* ml B) were used and diluted to 200 ml with sterile distilled water (Maurice et al., 2011). The pH of the medium was also adjusted after autoclaving with 0.1N HCL or 0.1N NaOH.

pH	Buffer A	(*x* ml)	Buffer B	(*y* ml)
5	0.1 M sodium acetate	74	0.1 M acetic acid	100
6	0.25 M KH_2PO_4	100	0.25 M NaOH	24
7	0.2 M Na_2HPO_4	16.5	0.1 M citric acid	3.5
8	0.1 M Tris(hydroxymethyl) aminomethane	100	0.1 M HCl	58.4

3.3.3 Experiment 2: Impact of pH on the growth of competing fungal colonies.

Two strains of *A. ostoyae* were paired with two strains of each of three *Hypholoma* species: *Hy. fasciculare*, *Hy. sublateritium*, *Hy. capnoides*. Another pathogen, *Heterobasidion irregulare,* was paired with its biocontrol *P. gigantea*. Five replicates of each strain of *A. ostoyae* were paired with each strain of all three *Hypholoma* spp. The fungi used in interaction experiments were inoculated on 2% MA + buffer systems (Table 7) and incubated at room temperature 22°C in the dark. Biocontrol (*Hypholoma* spp.) were plated two weeks after *A. ostoyae* inoculation. However, in the case of *He. irregulare* and *P. gigantea* interactions, both the pathogen and the biocontrol were plated on the same day. Growth measurements started after both biocontrol and pathogens were inoculated onto the same plate. Measurements were taken every 48 hours over a period of 10 days for *A. ostoyae* and *Hypholoma* spp., and over 6 days for *He. irregulare* and *P. gigantea*. The controls were the same species and strains growing in experiment 1, as both the experiments were carried out together under similar conditions. Growth comparisons between single and paired cultures of *A. ostoyae* and *Hypholoma* spp. were done on day 10, while for interactions

involving *He. irregulare* and *P. gigantea*, day 6 was chosen for analysis. These specific days were chosen because beyond these days, the paired cultures initiated physical interactions, making it difficult to accurately measure the mycelium extensions. The efficiency of biocontrol in inhibiting the growth of the pathogen at different pH was also determined as described in Chapter 2.

Note: Species and strain placement in this study was different from the methodology outlined in chapter 2. *Armillaria ostoyae* and *Hypholoma* spp. were plated 1 cm from the edge of the plate (Dugassa et al., 2021) rather than 2 cm due to *A. ostoyae* being plated 2 weeks earlier than *Hypholoma* spp. *Heterobasidion irregulare* and *P. gigantea* remained at a 2 cm distance from the edge since they were plated simultaneously.

3.3.4 Statistical analyses

The growth of pathogen species, *A. ostoyae* and *He. irregulare,* and biocontrol species, *Hypholoma* spp. and *P. gigantea,* was observed on different substrate pH 5, 6, 7, and 8. Mean growth was recorded for all species in single colony cultures and paired cultures. The difference in mean growth between strains of the pathogen *A. ostoyae* and biocontrol *Hypholoma* spp. on different pH over multiple days was analyzed using a three-way repeated -measures ANOVA. Post hoc analyses compared the increase in growth between strains at each measurement day from the previous day of measurement by using independent sample t-test, with Bonferroni correction applied to adjust the p- value for the inflated alpha error due to multiple comparisons. The difference in mean growth of pathogen *He. irregulare* and biocontrol *P. gigantea* over the days at different pH was analyzed using a one-way repeated-measures ANOVA. Post hoc analyses, using paired t-tests, were carried out to compare the change in growth between successive days for these species. The mean difference in growth of species and strains in single and paired cultures was analyzed using a three-way ANOVA. The assumptions of homogeneity and normality were checked using Levene's test and the Shapiro-Wilk test. When the assumptions were not met, log transformation was applied to the data. For all comparisons, a p-value was considered significant at a 5% level of significance. The trend plots were used to plot the mean growth with their SE values. Post hoc Tukey's test was used to compare the difference of any two pairs in paired cultures. Data was analyzed using R software version 4.1.2. R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/.

3.4 Results and discussion

3.4.1 Single culture mean growth of the pathogen *A. ostoyae* and its biocontrols *Hypholoma* spp. at different pH levels over time

3.4.1.1 Single culture mean growth of pathogen *A. ostoyae* strains on different pH over time (days)

The mean growth of two strains of *A. ostoyae*, B249-28 and P162-7, was observed on pH 5, 6, 7, and 8. B249-28 showed significantly higher radial growth than P162-7 over days ($F(1) = 606.893$, $p < 0.0001$) (Fig. 23). The difference in radial growth between strains at different pH is also significant ($F(3) = 3582.010$, $p < 0.001$).

Table 8. Three- way repeated measures ANOVA Table: Single culture mean growth of pathogen *A. ostoyae* strains at different pH over time, n=5

Effect	df	F	P
Strain	1	606.893	<0.001
pH	3	3582.01	<0.001
Day	8	469.263	<0.001
Strain x pH	3	64.835	<0.001
Strain x Day	8	16.953	<0.001
pH x Day	24	34.161	<0.001
Strain x pH x Day	24	2.711	<0.001

The analyses revealed substantial variation in the growth of both *A. ostoyae* strains with varying pH (Fig. 16). B249- 28 consistently showed higher growth than P162-7 across all pH values tested, and the growth of both strains at pH 5 and pH 6 exceeded that at pH 7 and pH 8. At lower pH levels, the initial growth of both strains did not vary significantly, but

over time, B249-28 showed a higher growth than P162-7. Growth of B249- 28 decreased considerably at higher pH (7 and 8), while the growth of P162-7 was almost negligible at pH 8 (Fig. 16).

The findings suggested that B249-28 thrives at higher pH than P162-7, although growth is considerably lower than at lower pH. Differences in growth between B249-28 and P162-7 on pH 5 and 6 after day 10 could be attributed to the variations in strain adaptation to the media or different catabolic activities. *Armillaria* spp. is known for lowering the pH by producing chemicals such as oxalic acids (Dumas et al., 1989). Buffer system was used to prevent any changes in the pH of media. The pH was measured every week in separate plates kept for monitoring pH changes during the experiment and no changes in media pH were noticed till the end of the experiment. Some rhizomorph growth observed on pH 5 and 6 for both strains could be due to the media acidity.

These findings align with previous observations by researchers such as Rishbeth (1982) and Singh (1983), who observed more rhizomorph growth at lower pH. Unlike some other *Armillaria* spp., such as *A. mellea* (Morrison, 1974), our study demonstrated similar responses of both strains at higher pH. Interspecific differences might explain these differences. Contrary to Tsykun et al.'s (2011) observations of no *A. ostoyae* rhizomorphs growth in soil pH above 5.4, our study found rhizomorphs in both strains growing at pH 6. The absence of rhizomorphs at higher pH (7 and 8) in our study suggests the possibility of lower disease-spread at higher pH, as rhizomorphs are the primary mode of disease spread in this species. However, this could be influenced by host species; for example, pine plantations, which prefer acidic pH, can still be susceptible to *A. ostoyae* on alkaline soils (higher pH) (McLaughlin, 2001a). Hence, careful site selection, soil pH determination, planting resistant host species, and use of biocontrol measures are essential in preventing *A. ostoyae* damage. It is important to note that variations in strains used for studies may account for differences, and

field conditions may reveal varied results, necessitating further investigation in the field to support our findings here.

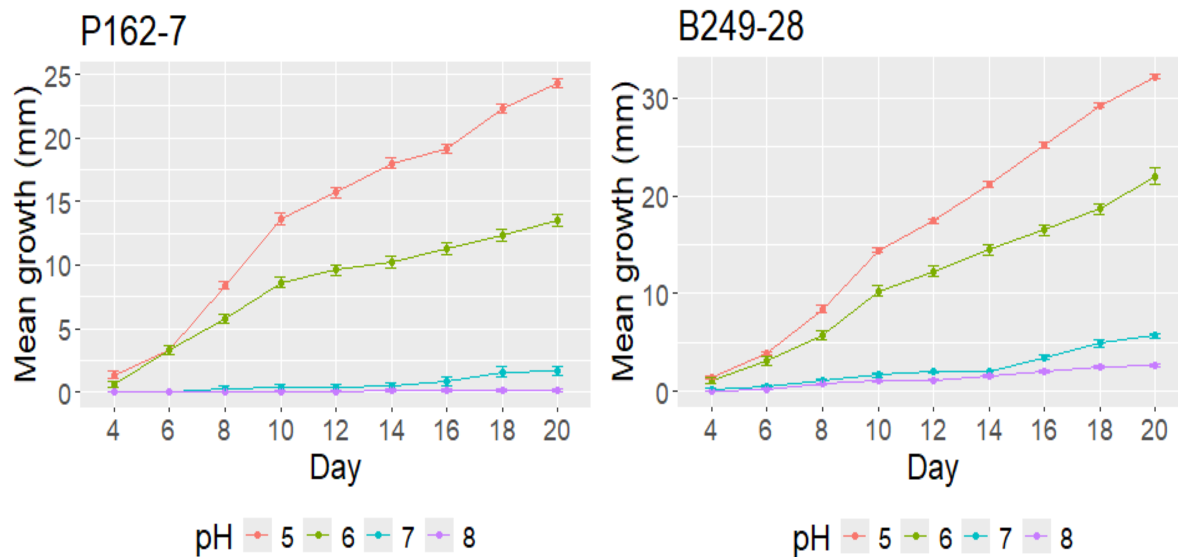


Figure 16. Mean growth of *A. ostoyae* strains (P162-7 and B249-28) over time on pH 5, 6, 7, and 8. Bars above and below each mean indicate ±1 SE.

3.4.1.2 Single culture mean growth of *Hypholoma* spp.: *Hy. fasciculare, Hy. capnoide,* and *Hy. sublateritium* on different pH over time (days)

The single culture mean growth of *Hypholoma* species and their strains – *Hy. fasciculare* (Pinnel B, RLG-12668-SP), *Hy. capnoides* (TAK 2 and TAK 5), and *Hy. sublateritium* (HHB-11948-SP, OKM-6947-SP) varied significantly, with Pinnel B showing a higher growth than all others ($F(5) = 972.681$, $p < 0.0001$). Differences between the mean growth of each strain at different pH were significant ($F(3) = 851.756$, $p < 0.001$).

Table 9. Three- way repeated measures ANOVA Table: Single culture mean growth of *Hypholoma* spp.: *Hy. fasciculare, Hy. capnoides,* and *Hy. sublateritium* at different pH over time, n=5.

Effect	df	F	P
Strain	5	972.681	<0.001
pH	3	851.756	<0.001
Day	8	1259.692	<0.001
Strain x pH	15	7.648	<0.001
Strain x Day	40	1.534	<0.001
pH x Day	24	4.296	<0.001
Strain x pH x Day	119	1.087	<0.001

The mean increase in growth of *Hy. fasciculare* strain Pinnel B was significantly higher than strain RLG-12668-SP at all pH levels and days. Similarly, *Hy. capnoides* strain TAK 2 showed a significantly higher mean increase in growth than TAK 5 at all pH levels. The mean increase in growth of *Hy. sublateritium* strain HHB-11948 was significantly higher than OKM-6947 at all pH and days. Pinnel B showed higher growth on all pH levels (5, 6, 7, and 8) (Fig. 17, 18, and 19) at the end of the experiment. Conversely, *Hy. capnoides* showed lower on all pH levels, with higher growth on pH 5 and 6 compared to pH 7 and 8 (least growth on pH 8).

Mean growth of *Hypholoma* spp. was significantly affected by the pH (5, 6, 7, and 8). *Hy. fasciculare* strains had higher growth on pH 5, decreasing as the pH increased. Pinnel B showed almost double the growth of RLG -12668-SP on pH 7 and 8, indicating its better tolerance to higher pH. Our observations aligned with past studies (Verhagen et al., 1998; Makela et al., 2002; Magnusan and Lasure, 2004, Valaskova et al., 2009), confirming *Hy. fasciculare*'s preference for acidic pH and its ability to grow on alkaline pH, such as pH 8

Observations were only taken till day 20, and in control plates (kept separately for measuring pH changes every week starting from the day of inoculation), pH dropped from 8 to 7.2 on day 21 in Pinnel B, possibly due to chemical production. Growth patterns for other *Hypholoma* species were similar, with the lowest growth at pH 8 and the highest at pH 5. *Hy. capnoides* strains did not show significant differences in growth on pH 6, 7, and pH 8, suggesting similar behaviour on higher pH (lowest growth). At pH 5, TAK 2 had a better growth than TAK 5. From a substrate pH perspective, *Hy. capnoides* might not be suitable for controlling *Armillaria* spp. at pH higher than 6. *Hypholoma sublateritium* strains showed significant difference in growth on all pH, HHB-11948-SP showing a better growth than OKM-6947. Further investigation is needed to explore the potential of *Hy. sublateritium* as a biocontrol for *A. ostoyae*, considering its survival and growth even at higher pH. Also, pH-lowering abilities of this species needs to be examined for insights into its growth rates and response to substrate pH in natural environments.

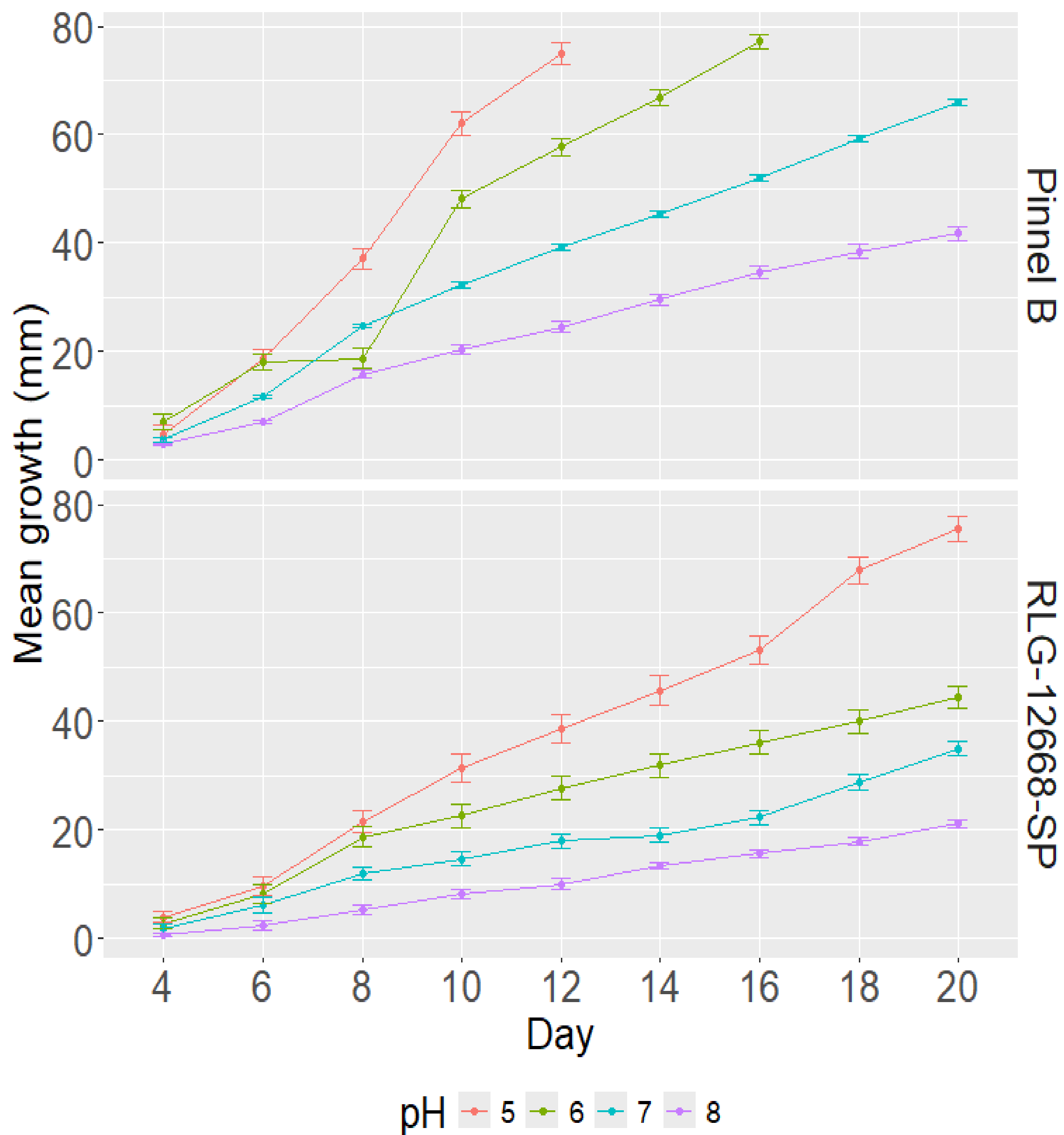


Figure 17. Mean growth of *Hy. fasciculare* at pH 5, 6, 7, and 8 over time. Graph lines ended as the strains reached the edge of the plate, indicating maximal growth before the experiment's end. Bars above and below each mean indicate ±1 SE.

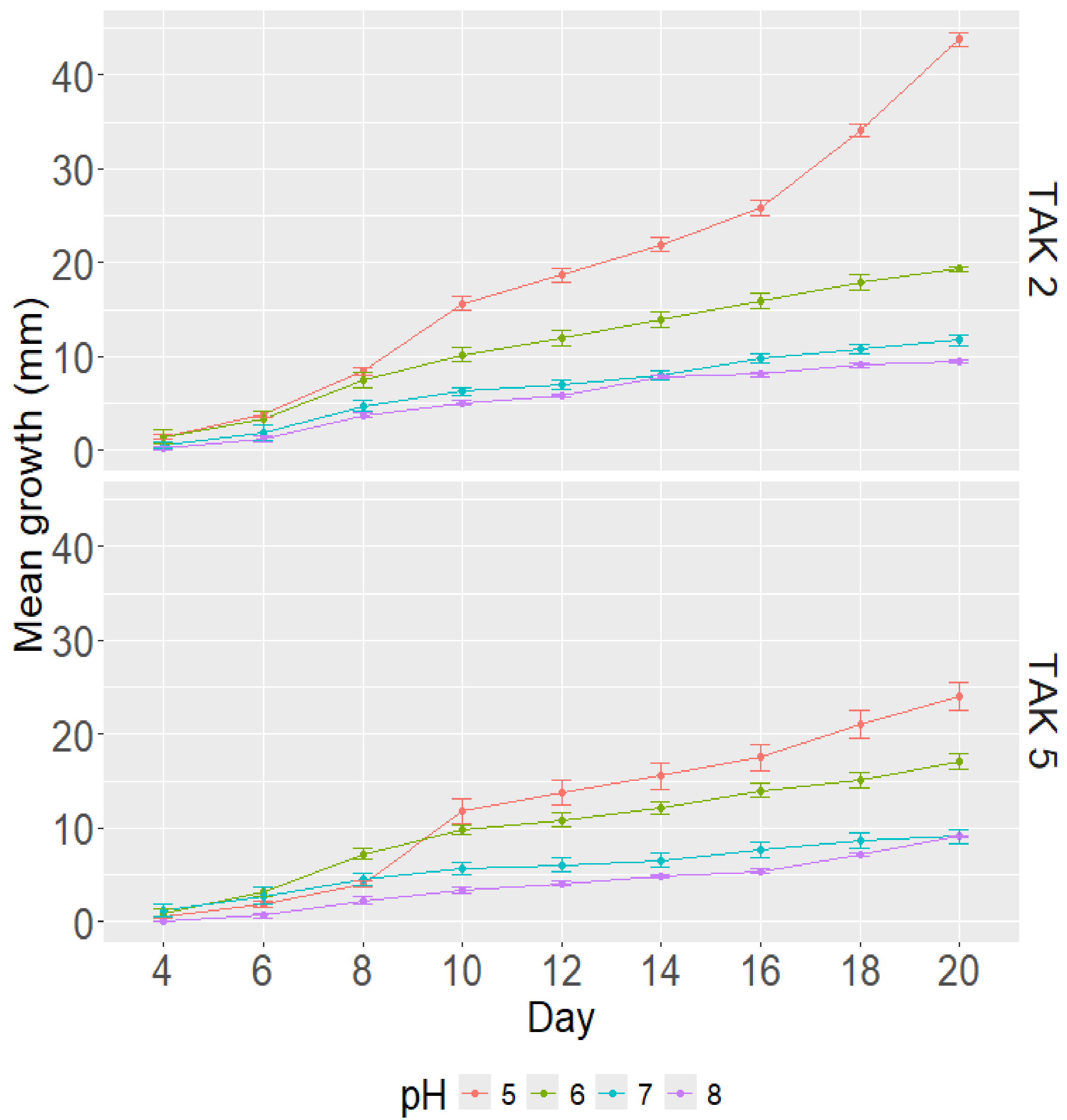


Figure 18. Mean growth of *Hy. capnoides* at pH 5, 6, 7, and 8 over time. Bars above and below each mean indicate ±1 SE.

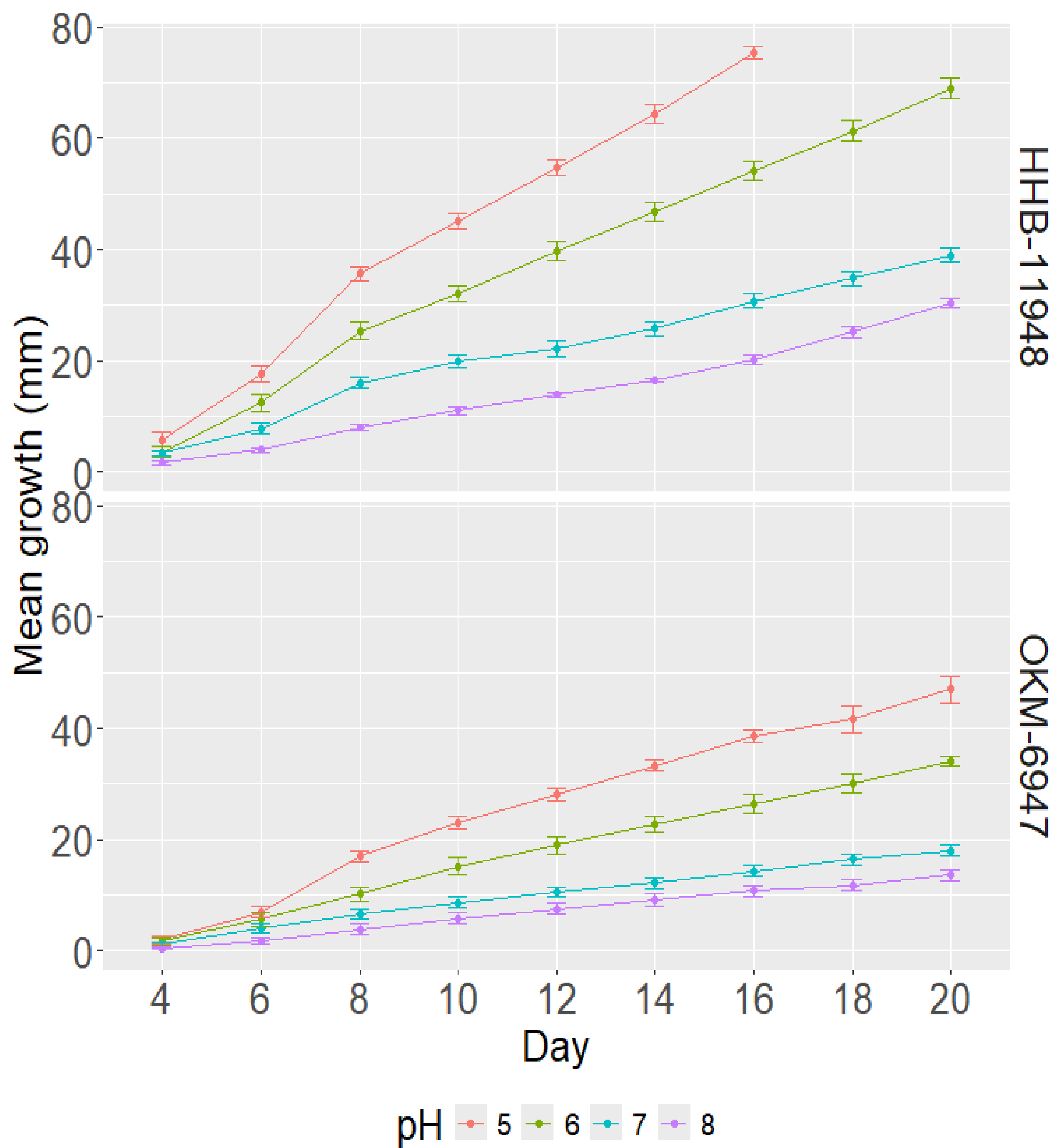


Figure 19. Mean growth of *Hy. sublateritium* at pH 5, 6, 7, and 8 over time. Graph lines ended as the strains reached the edge of the plate, indicating maximal growth before the experiment's end. Bars above and below each mean indicate ±1 SE.

3.4.2 Single culture mean growth of pathogen *He. irregulare* and biocontrol *P. gigantea* at different pH over time

3.4.2.1 Single culture mean growth of *He. irregulare* on different pH over time (days)

Post hoc analyses, using paired t-tests, compared the change in growth between successive days, revealing a significant increase in mean growth at pH 5. Similar patterns were observed for pH 6 ($F (11) = 1545.568$, $p < 0.0001$), pH 7 ($F (11) = 119.868$, $p < 0.0001$), and pH 8 ($F (11) = 71.235$, $p < 0.0001$), with significant increases in mean growth between successive days.

By the end of the experiment, growth of *He. irregulare* was highest on pH 7, followed by pH 6, 5, and 8 (Fig. 20). On pH 7, it reached the edge of the plate by day 10, while on pH 6 it reached the edge of the plate by day 12. Growth was lowest on pH 8 and lower at pH 5. This indicates that *He. irregulare* prefers neutral pH or weakly acidic pH rather than alkaline pH or extreme acidic pH. The observed preference might be due to the inactivation of enzymes of this pathogen on extreme acidic or alkaline pH. Our study agrees well with the observations of Johansson et al. (2002) and Bruna et al. (2019) supporting higher growth of *Heterobasidion* spp. on higher pH.

Heterobasidion spp. is known for its pH lowering abilities by the production of oxalic acid (Nagy et al., 2012). We noted a decrease in substrate pH for this species on day 14, prompting the termination of experiment on day 12 (pH decreased from 7 to 6.3 and 6 to 5.7).

Rishbeth (1951a) found a higher percentage of Scots pine infection by *He. annosum* on alkaline pH sites (greater than 7) than on acidic pH sites. Also, pH higher than 8 decreased the *He. annosum* growth (Johansson et al., 2002). In event of climate change and possible dry

conditions, soil alkalization may occur, (Jia et al., 2021), the growth of *He. irregulare* at pH 8 may be controlled with the biocontrol species that is performing well at higher pH. Also, if the biocontrol is thriving in acidic conditions, growth of *He. irregulare* might be controlled under such conditions. This study focused on one strain of *He. irregulare*, and further research with multiple strains is necessary to account for potential variations of different species in responses to different pH levels.

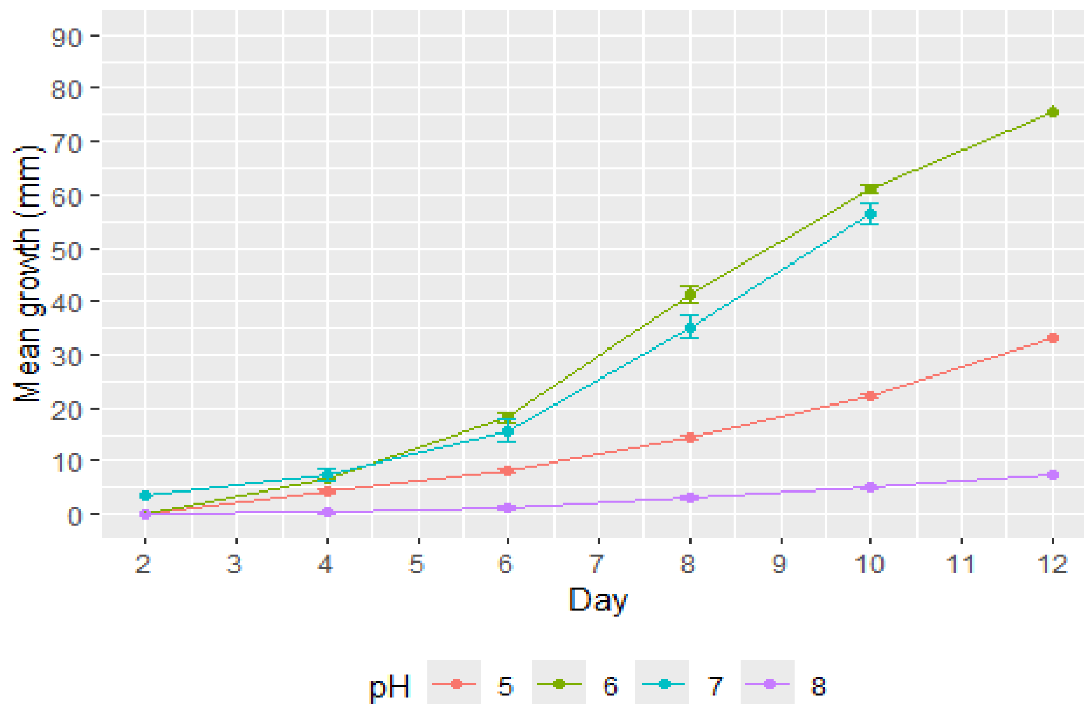


Figure 20. Mean growth of *He. irregulare* on pH 5, 6, 7, and 8 over time. Graph lines ended as the species reached the edge of the plate, indicating maximal growth before the experiment's end. Bars above and below each mean indicate ±1 SE.

3.4.2.2 Single culture mean growth of *P. gigantea* on different pH over time (days)

Post hoc analyses, carried out using paired t-tests, indicated a significant increase in growth between successive days at pH 5. Similar patterns were observed for pH 6 (F (11) =

180.294, $p < 0.0001$), pH 7 ($F (11) = 147.12$, $p < 0.0001$), and pH 8 ($F (11) = 79.387$, $p < 0.0001$), with significant growth increases between successive days.

The mean growth of *P. gigantea* significantly varied at different pH by the end of the experiment, with the highest growth at pH 6, followed by pH 7, 5, and the least at pH 8 (Fig. 21). Even though growth at pH 8 was low, it still indicated the ability of the species to control *He. irregulare* growth. Our study revealed that the species is tolerant to a wide range of pH and can adapt well, unlike the pathogen *He. irregulare*. While studies have been done on this species regarding its growth patterns on different substrate pH, our study adds valuable knowledge to the existing understanding of the effect of soil pH on this species. This is of great significance, considering that this strain is currently registered as a biocontrol against *Heterobasidion* spp. in Canada.

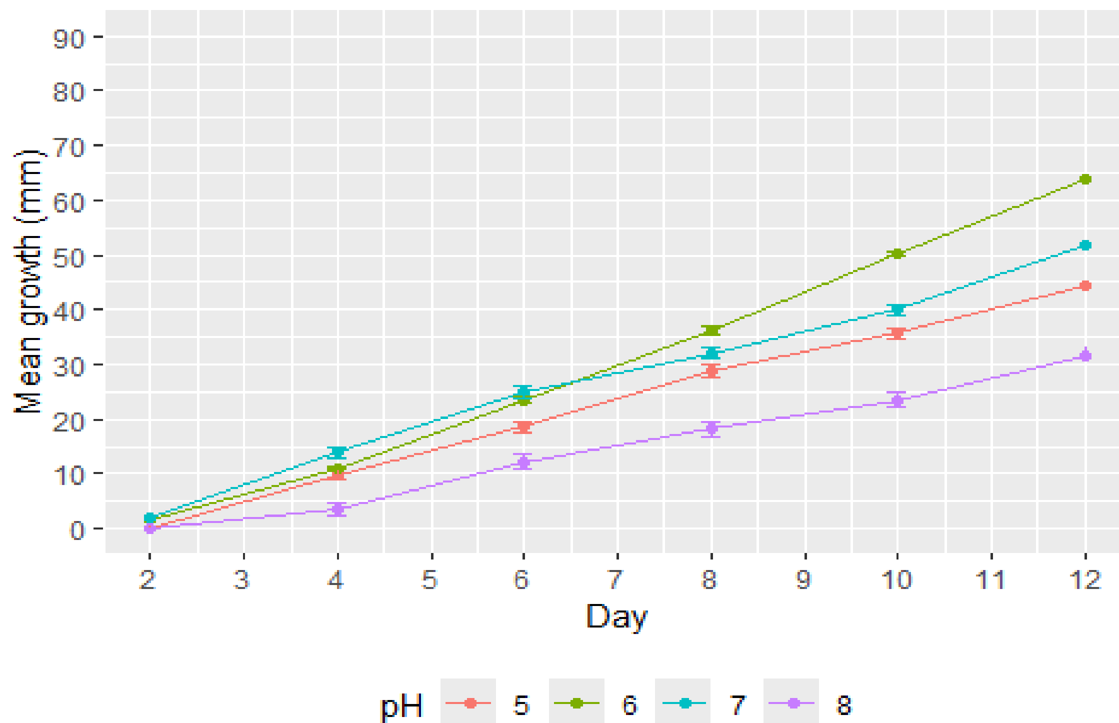


Figure 21. Mean growth of *P. gigantea* on pH 5, 6, 7, and 8 over time. Bars above and below each mean indicate ±1 SE.

3.4.3 Mean paired culture growth of biocontrols *Hypholoma* spp. and *P. gigantea* and pathogens *A. ostoyae* and *He. irregulare* across a range of pH

3.4.3.1 Mean growth of *Hypholoma* spp. when paired with *A. ostoyae* on pH 5 & 6

The mean growth differed significantly between strains of *Hy. fasciculare*, *Hy. capnoides,* and *Hy. sublateritium* (HHB-11948-SP, OKM-6947-SP, Pinnel B, RLG-12668-SP, TAK 2 and TAK 5). Pinnel B showed significantly higher growth than all other strains and species ($F(7) = 759.317$, $p < 0.0001$). The radial growth of all species at pH 5 was significantly higher than pH 6 ($F(1) = 256.230$, $p < 0.001$). This study was conducted until day 10, avoiding interaction between pathogen *A. ostoyae* and biocontrol strains, ensuring accurate measurements. The interaction of strain, pH, and their corresponding pairing strain were also statistically significant (Table 10). Post hoc analyses used Tukey's test to compare single culture growth and growth of biocontrol species and strains when paired with the strains of *A. ostoyae* at different pH levels. Note that single colony cultures were treated as controls for comparisons with the paired colony cultures examined here.

Table 10. Three-way ANOVA Table: Mean growth of *Hypholoma* spp. when paired with *A. ostoyae* on pH 5 and 6 on day 10, n=5.

Effect	df	F	P
Strain	7	759.317	<0.001
pH	1	256.23	<0.001
Pairing	8	92.855	<0.001
Strain x pH	7	108.153	<0.001
Strain x Pairing	16	18.158	<0.001
pH x Pairing	8	27.837	<0.001
Strain x pH x Pairing	16	8.094	<0.001

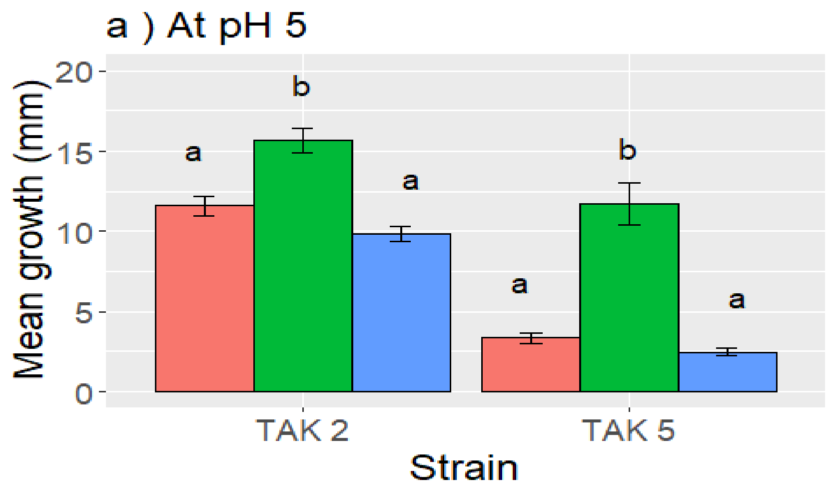
Figures 22, 23, and 24 show that the mean growth of all three *Hypholoma* spp. was higher at pH 5 and 6 in single colony cultures (controls) compared to their growth in paired cultures. This suggests that at both pH levels, the growth of *Hypholoma* spp. decreased in the presence of pathogen *A. ostoyae,* except for the strain OKM-6947-SP of *Hy. sublateritium* and TAK 5 of *Hy. capnoides*. The substrate pH significantly affected the interactions of *Hypholoma* spp., influencing biocontrol growth when paired with *A. ostoyae*.

The growth of *A. ostoyae* strains was notably low on pH 7 and 8 during the initial two weeks, preventing co- inoculation with *Hypholoma* spp. *Hypholoma capnoides* strains TAK 2 and TAK 5 showed significantly higher growth in controls (single cultures) compared to growth when paired with B249-28 and P162-7. The growth of both *Hy. capnoides* strains decreased significantly when paired with *A. ostoyae* strains. However, at pH 6, the growth of TAK 5 was higher in presence of B249-28 and P162-7 than control, suggesting an antagonistic response in these strains in presence of the pathogen. The results indicate that the biocontrol growth was considerably reduced in presence of the pathogen, except for TAK 5 at pH 6 (Fig. 22). *Hypholoma sublateritium* strains showed a significant decrease in growth

when paired with the pathogen on both pH levels, except for OKM- 6947 strain when paired with B249-28 and P162-7 (Fig. 23). This suggests the ability of this strain to resist changes in growth when paired with the pathogen. *Hypholoma fasciculare* strain growth also decreased in presence of the pathogen on pH 5 and 6, except for RLG-12668-SP strain, whose growth did not change much in the presence of B249-28 and P162-7 (Fig. 24).

At pH 5, the growth of *A. ostoyae* strain B249-28 was measured in the presence of *Hypholoma* spp. The mean growth did not significantly vary in presence of *Hypholoma* spp., except for B249-28 when paired against Pinnel B and P162-7 when paired with RLG-12668-SP at pH 5. Growth of P162-7 also decreased in the presence of TAK 2 but increased in presence of TAK 5 (Fig. 25). *Hypholoma fascicular*e emerged as the best candidate for biocontrol against *A. ostoyae* at lower pH, such as 5. At pH 6, the growth of B249-28 decreased, but it was decreased in the presence of almost all strains of *Hypholoma* spp. For P162-7, there was a decrease in growth by all strains but not by *Hy. capnoides* strain TAK 5 (Fig. 25). This study shows that the best candidate for inhibiting the growth of *A. ostoyae* at pH 5 and 6 is *Hy. fasciculare* species, while *Hy. capnoides* may not be a suitable candidate, as its presence increased the growth of the pathogens at both pH levels. Despite being considered less virulent, P162-7 showed higher survival abilities than B249-28 in different pH and presence of competitors.

Hypholoma fasciculare proves capable of inhibiting the growth of this pathogen at different pH levels and might do so even at higher pH, considering the observed growth of the pathogen at pH 7 and 8. This needs further investigation in the field in presence of forest microflora. *Hypholoma sublateritium* may also limit *A. ostoyae* growth as it was able to inhibit the growth of both the strains at pH 6.



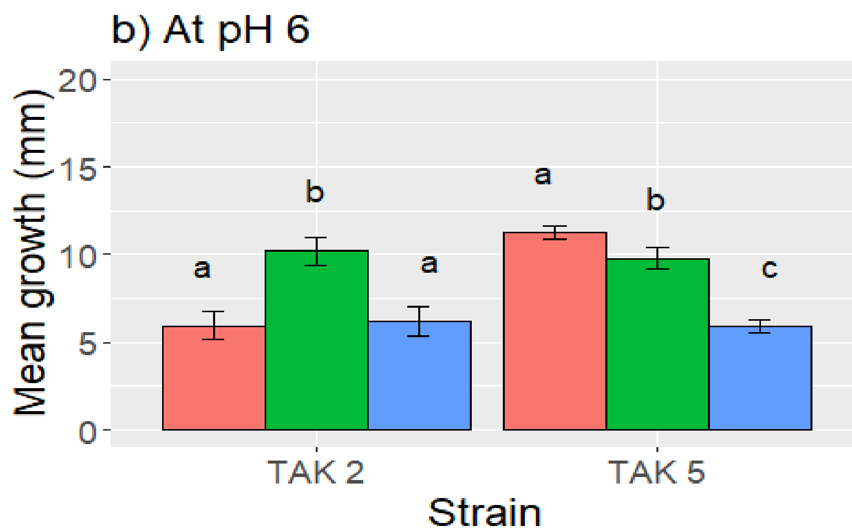
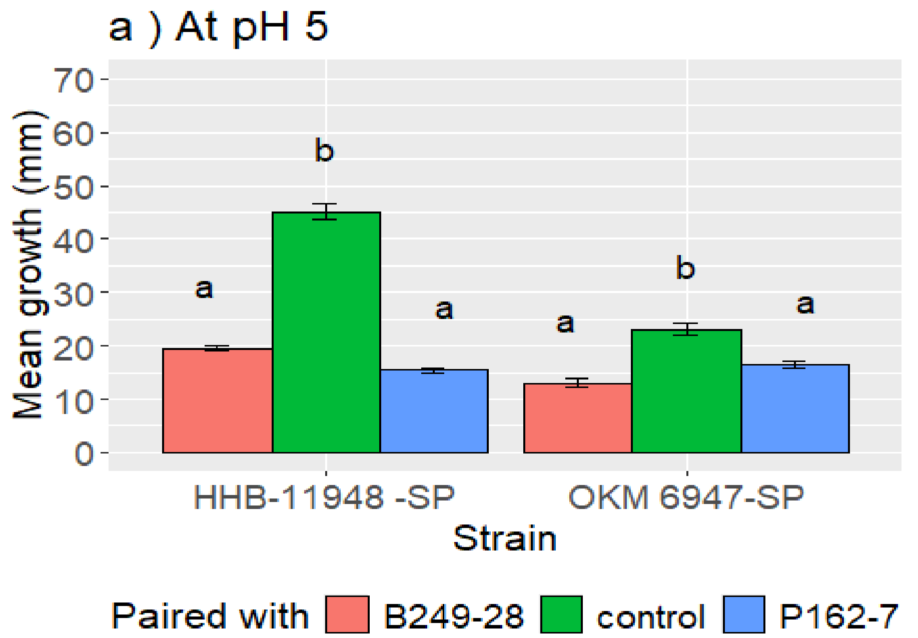


Figure 22. Mean growth of *Hy. capnoides* in single and paired colony cultures with 2 strains of *A. ostoyae* (B249-28 and P162-7) at pH 5 and pH 6 on day 10. Bars above and below each mean indicate ±1 SE. Same letters above the bars indicate no significant difference between the mean growth of strains while different letters indicate significant differences at $p = 0.05$ (Tukey's test).



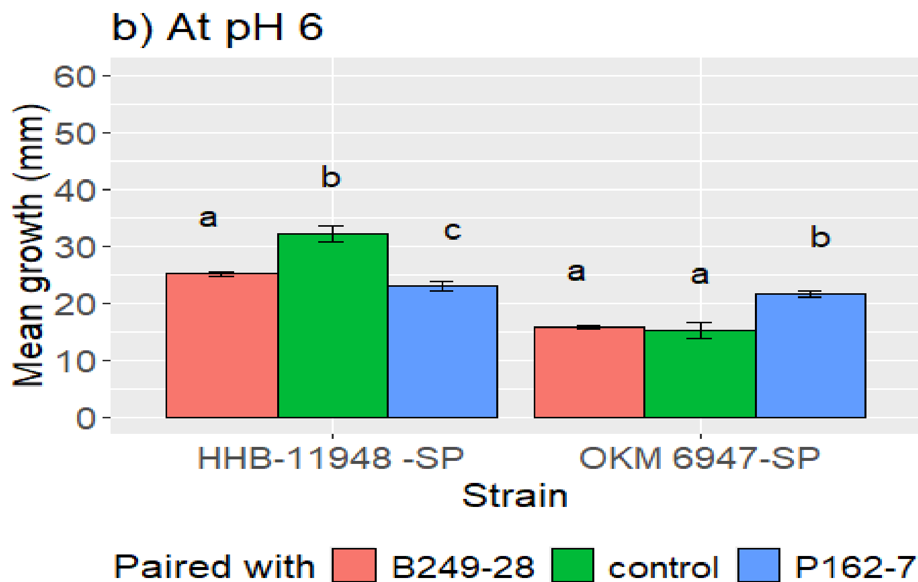
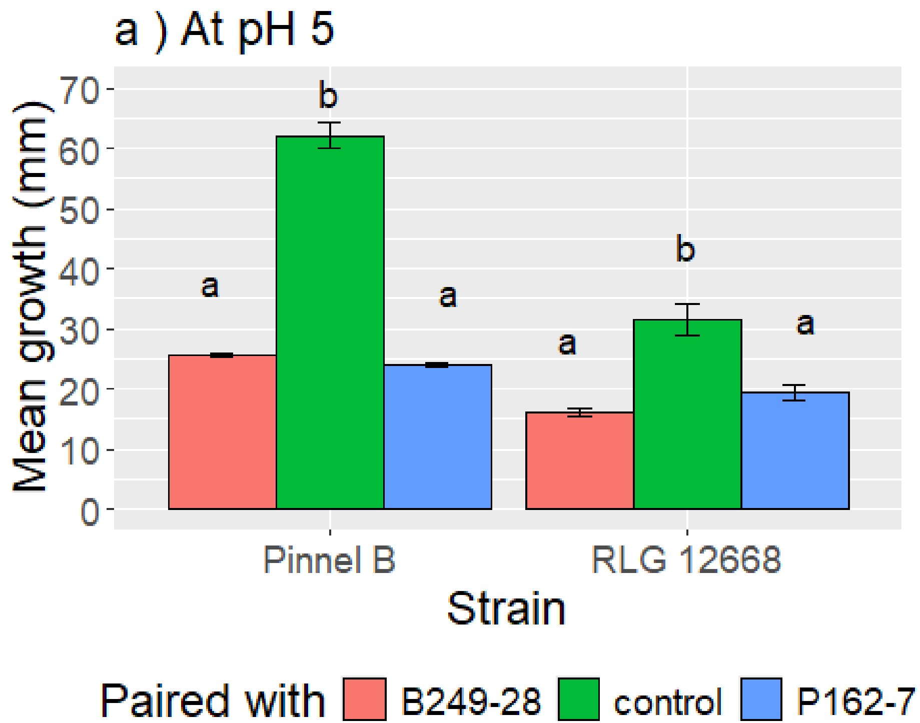


Figure 23. Mean growth of *Hy. sublateritium* in single and paired colony cultures with 2 strains of *A. ostoyae* (B249-28 and P162-7) at pH 5 and pH 6 on day 11. Bars above and below each mean indicate ±1 SE. Same letters above the bars indicate no significant difference between the mean growth of strains while different letters indicate significant differences at $p = 0.05$ (Tukey's test).



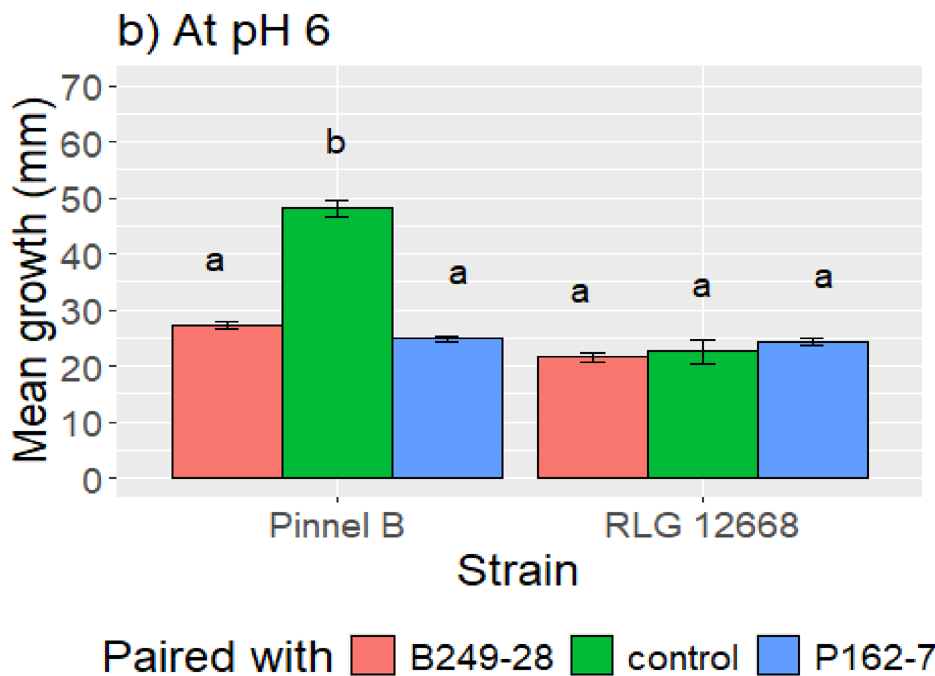


Figure 24. Mean growth of *Hy. fasciculare* in single and paired colony cultures with 2 strains of *A. ostoyae* (B249-28 and P162-7) at pH 5 and pH 6 on day 11. Bars above and below each mean indicate ±1 SE. Same letters above the bars indicate no significant difference between the mean growth of strains while different letters indicate significant differences at p = 0.05 (Tukey's test).

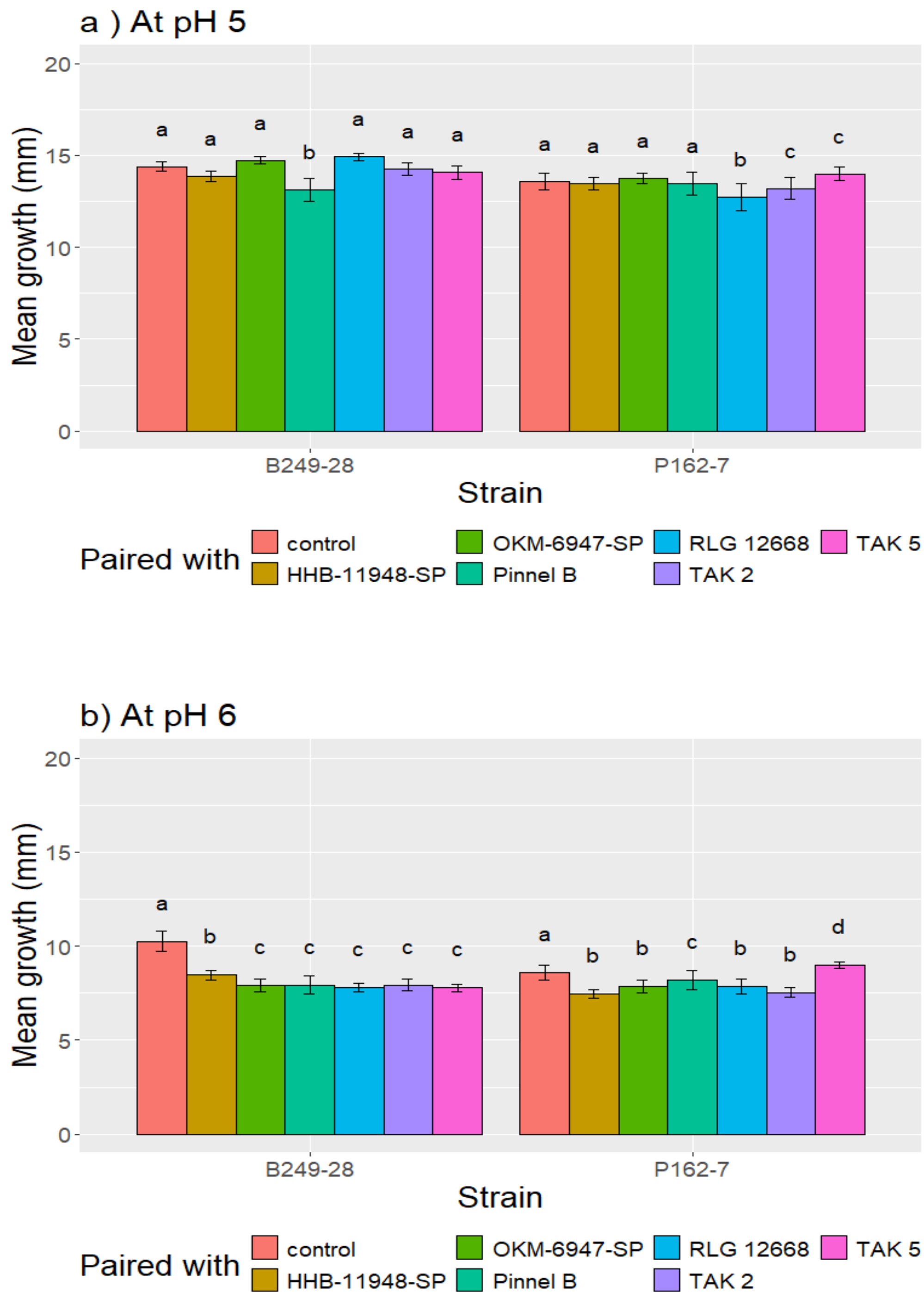


Figure 25. Mean growth of *A. ostoyae* strains B249-28 and P162-7 in single and paired colony cultures with *Hypholoma* spp. – *Hy. fasciculare* (Pinnel B, RLG-12668-SP), *Hy. capnoides* (TAK 2 and TAK5), and *Hy. sublateritium* (HHB-11948-SP, OKM-6947-SP) at pH 5 and 6. Bars above and below each mean indicate ±1 SE. Same letters above the bars indicate no significant difference between the mean growth of strains while different letters indicate significant differences at p = 0.05 (Tukey's test).

3.4.3.1.1 Interactions of 2 strains of each *Hypholoma* spp., *Hy. fasciculare*, *Hy. capnoides* and *Hy. sublateritium* against *A. ostoyae* (B249-28 and P162-7) on substrate pH 5 and 6

The biocontrol efficacy of *Hypholoma* spp. strains was assessed by evaluating their ability to inhibit the growth of two *A. ostoyae* strains when paired together at two different pH levels (pH 5 and pH 6) on day 10. The mean growth of *A. ostoyae* strains was determined in both control (single colony cultures) and when paired with the biocontrol species and strains. The percentage inhibition of *A. ostoyae* strains in the presence of competitors was calculated by following the methodology of Rahman et al. (2009).

There was a significant decrease in the growth of *A. ostoyae* in the presence of *Hy. fasciculare* strains at pH 6, indicating effective inhibition (Table 11 &12). However, *Hy. fasciculare* showed limited effectiveness in inhibiting *A. ostoyae* growth at pH 5 on day 10 (Table 11 &12), suggesting that *Hy. fasciculare* may be more effective against *A. ostoyae* at higher pH (6) than lower pH (5), possibly due to higher growth of *A. ostoyae* on more acidic substrates (Singh, 1983; Tsykun, 2012). *Hypholoma capnoides* strains TAK 2 and TAK 5 effectively inhibited the growth of both *A. ostoyae* strains at pH 5 and 6. However, the inhibition of B249-28 strain was more at pH 6 compared to pH 5 for both biocontrol strains. In the case of P162-7 strain, TAK 2 inhibited its growth at both pH 5 and pH 6, while TAK 5 was unable to inhibit the growth of this strain. This suggested variations in the response of strains of the same biocontrol species on different pH levels. The study also highlights that the growth and virulence *A. ostoyae* strains vary in the presence of different biocontrols on different pH levels. Despite B249-28 being considered more virulent, the growth of B249-28 was successfully inhibited by *Hy. capnoides* strains, whereas the growth of P162-7 could not be inhibited by TAK 5 at both pH 5 and 6 (Table 13 &14). *Hypholoma sublateritium* strains

HHB-11948-SP and OKM-6947-SP were able to inhibit the growth of *A. ostoyae* (B249-28 and P162-7) when paired on pH 6 (Table 15&16). However, both the strains were not much effective in inhibiting the growth on pH 5 especially OKM-6947-SP which could not inhibit the growth of both the strains of *A. ostoyae* at pH 5. This study suggests that *Hy. sublateritium* may potentially inhibit the growth of *A. ostoyae* at higher pH such as pH 6 but not at lower pH such as pH 5.

Table 11. Interactions of *Hy. fasciculare* strains Pinnel B and RLG-12668 when paired with B249-28 strain of *A. ostoyae* at pH 5 and pH 6.

pH	Strain of biocontrol *Hy. fasciculare*	Mean growth of B249-27 in Control (mm)	Mean growth of B249-27 when paired (mm)	Percentage inhibition
5	**Pinnel B**	14.4	13.11	9.21
5	**RLG- 12668**	14.4	14.9	NA
6	**Pinnel B**	10.26	7.93	22.7
6	**RLG-12668**	10.26	7.8	23.97

Table 12. Interactions of *Hy. fasciculare* strains Pinnel B and RLG-12668 when paired with P162-7 strain of *A. ostoyae* at pH 5 and pH 6.

pH	Strain of biocontrol *Hy. fasciculare*	Mean growth of P162-7 in Control (mm)	Mean growth of P162-7 when paired (mm)	Percentage inhibition
5	**Pinnel B**	13.6	13.46	NA
5	**RLG- 12668**	13.6	12.7	6.39
6	**Pinnel B**	8.6	8.2	4.65
6	**RLG-12668**	8.6	7.8	9.3

Table 13. Interactions of *Hy. capnoides* strains TAK 2 and TAK 5 when paired with B249-28 strain of *A. ostoyae* at pH 5 and pH 6.

pH	**Strain of biocontrol *Hy. capnoides***	**Mean growth of B249-28 in Control (mm)**	**Mean growth of B249-28 when paired (mm)**	**Percentage inhibition**
5	**TAK 2**	14.4	14.21	1.38
5	**TAK 5**	14.4	14	2.77
6	**TAK 2**	10.26	7.93	22.7
6	**TAK 5**	10.26	7.8	23.97

Table 14. Interactions of *Hy. capnoides* strains TAK 2 and TAK 5 when paired with P162-7 strain of *A. ostoyae* at pH 5 and pH 6.

pH	**Strain of biocontrol *Hy. capnoides***	**Mean growth of P162-7 in Control (mm)**	**Mean growth of P162-7 when paired (mm)**	**Percentage inhibition**
5	**TAK 2**	13.6	13.2	2.9
5	**TAK 5**	13.6	14	NA
6	**TAK 2**	8.6	7.5	12.7
6	**TAK 5**	8.6	9	NA

Table 15. Interactions of *Hy. sublateritium* strains HHB-11948-SP and OKM-6947-SP when paired with B249-28 strain of *A. ostoyae* on pH 5 and pH 6.

pH	**Strain of biocontrol *Hy. sublateritium***	**Mean growth of B249-28 in Control (mm)**	**Mean growth of B249-28 when paired (mm)**	**Percentage inhibition**
5	**OKM-6947-SP**	14.4	14.7	NA
5	**HHB-11948-SP**	14.4	13.86	3.75
6	**OKM-6947-SP**	10.26	7.9	23
6	**HHB-11948-SP**	10.26	8.4	18.12

Table 16. Interactions of *Hy. sublateritium* strains HHB-11948-SP and OKM-6947-SP when paired with P162-7 strain of *A. ostoyae* on pH 5 and pH 6.

pH	**Strain of biocontrol *Hy. sublateritium***	**Mean growth of P162-7 in Control (mm)**	**Mean growth of P162-7 when paired (mm)**	**Percentage inhibition**
5	**OKM-6947-SP**	13.6	13.71	NA
5	**HHB-11948-SP**	13.6	13.4	1.47
6	**OKM-6947-SP**	8.6	7.8	9.3
6	**HHB-11948-SP**	8.6	7.4	13.95

3.4.3.2 Mean growth of *He. irregulare* paired with *P. gigantea* on pH 5, 6, 7, and 8 on Day 6

The mean growth of *P. gigantea* was significantly higher than that of *He. irregulare* ($F (1) = 670.54$, $p < 0.0001$). Differences in radial growth between the two species were also significant when paired ($F (2) = 21.25$, $p < 0.0001$). Also, both species showed a significant variation in growth at different pH levels ($F (3) = 217.35$, $p < 0.001$). The growth of both species at pH 6 was higher than at other pH across all days ($F(3) = 13.58$, $p<0.0001$).

Table 17. One-way repeated measures ANOVA Table: Mean growth of *He. irregulare* paired with *P. gigantea* on pH 5, 6, 7, and 8 on Day 6, n=5.

Effect	df	F	P
Strain	1	670.54	<0.001
pH	3	217.35	<0.001
Pairing	2	21.25	<0.001
Strain x pH	3	13.58	<0.001
pH x Pairing	6	10.12	<0.001

Figure 26 indicates that the growth of *He. irregulare* was higher in single cultures (control) than in paired cultures at pH 7. The difference was not significant at pH 5, 6, and 8 on day 6, suggesting that *He. irregulare* growth was most inhibited at pH 7 (Fig. 26 & Table 18). However, growth was not significantly inhibited at other pH levels. Mean growth of *P. gigantea,* when paired with *He. irregulare,* was significantly higher at pH 6 and pH 7 (Fig. 27). This suggests that the high growth rate of *P. gigantea* at pH 7 on day 6 could be the reason for the decline in *He. irregulare* growth at this pH. The mean growth of *He. irregulare,* when paired with *P. gigantea,* decreased significantly at pH 7, while the decrease

at pH 5 and 6 was not significant. The growth of *He. irregulare* was similar on pH 8 in both single and paired cultures.

Contrary to our hypothesis, at higher pH, the presence of *P. gigantea* could not inhibit the growth of *He. irregulare*. Despite *P. gigantea* showing higher growth, *He. irregulare* growth was not inhibited. The growth of *He. irregulare* was highest at the end of the experiment at pH 6, followed by pH 7. Growth was not inhibited at pH 6 and 5, indicating that *P. gigantea* may not efficiently inhibit *He. irregulare* at these pH levels. The inhibition of *He. irregulare* growth when in close proximity of *P. gigantea* might be due to the chemical interactions such as release of the antifungal toxins by *P. gigantea* (Kalvo et al., 2018). Since these interactions are influenced by different pH (Boddy, 2000), this study revealed that in the event of climate change and an increase in pH, *P. gigantea* may not effectively control the growth and spread of *He. irregulare*. This needs further investigation in field conditions.

The growth of *P. gigantea* when paired with *He. irregulare* was measured and showed higher growth in the presence of *He. irregulare* on pH 6, 7, and 8. However, the increase was not statistically significant at pH 8. At pH 5, the growth of *P. gigantea* decreased when paired with *He. irregulare*, indicating antagonism between the two species at pH 6, 7, and 8. *P. gigantea* showed faster growth rate in presence of the pathogen compared to solo growth. However, on lower pH 5, *P. gigantea* growth did not increase in the presence of *He. irregulare,* while *He. irregulare* growth decreased. This implies that at lower pH, both species growth experience decreased growth at a distance. Nevertheless, *P. gigantea* still managed to inhibit the growth of *He. irregulare* from a distance by 24.09% and further overgrowth may be observed over time at this pH.

At pH 6, growth of *P. gigantea* increased in the presence of *He. irregulare,* leading to a decrease in the growth of *He. irregulare* and successful inhibition by 15.38%. At pH 7, the

growth of *He. irregulare* was maximally inhibited by 55.76%. This implies a considerable increase in growth of *P. gigantea* at this pH, reducing the growth of *He. irregulare* to half of its solo growth. At pH 8, the growth of *He. irregulare* was only inhibited by 15.09%, with lowest inhibition observed at this pH.

In the event of soil alkalization in future, *P. gigantea* might be unable to efficiently inhibit the growth of *He. irregulare*. However, further investigation in a natural environment and using several strains of *He. irregulare* is needed to test the efficiency of *P. gigantea* at higher pH levels.

Table 18. Interactions of *P. gigantea* when paired with *He. irregulare* on pH 5, 6, 7 and 8

pH	**Growth of *He. irregulare* in control (mm)**	**Growth of *He. irregulare* when paired with *P. gigantea* (mm)**	**Percentage inhibition**
5	8.3	6.31	24.09 (N.S.)
6	18.2	15.4	15.38 (N.S.)
7	15.6	6.9	55.76 *
8	1.06	0.9	15.09 (N.S.)

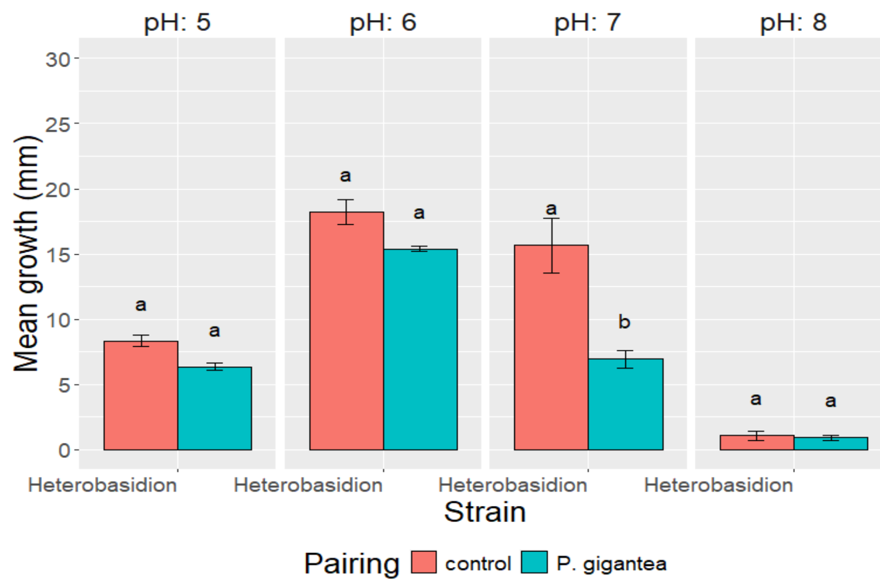


Figure 26. Mean growth of *He. irregulare* in Control (single culture) and when paired with *P. gigantea* at pH 5, 6, 7 and 8 on day 6. Bars above and below each mean indicate ±1 SE. Same letters above the bars indicate no significant difference at p = 0.05 (Tukey's test).

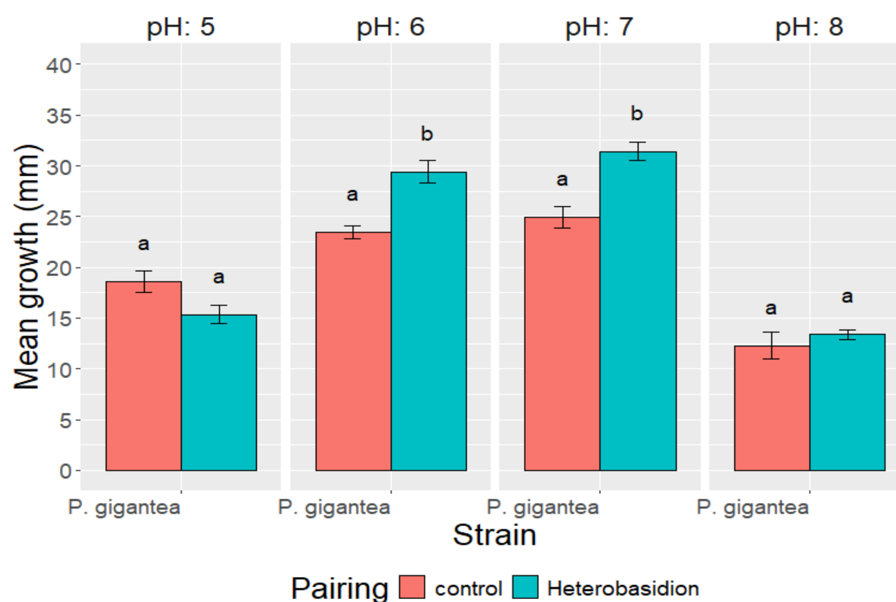


Figure 27. Mean growth of *P. gigantea* in Control (Single culture) and when paired with *He. irregulare* at pH 5, 6, 7 and 8 on day 6. Bars above and below each mean indicate ±1 SE. Same letters above the bars indicate no significant at p = 0.05 (Tukey's test).

3.5 Conclusions

This study revealed the impact of pH on the growth and interactions of *Hypholoma* spp. and *P. gigantea* with *A. ostoyae* and *He. irregulare*. The mean growth of *Hypholoma* spp. varied between species and among strains of the same species across different substrate pH levels. Both *A*. *ostoyae* strains showed a preference for acidic substrates, with optimal growth observed at pH 5. P162-7, in particular, exhibited only marginal growth at higher pH, indicating that B249-28 strain might be comparatively more virulent in alkaline soil conifer plantations. All *Hypholoma* species and strains showed growth at higher pH, such as 8, but their optimal growth occurred at pH 5, revealing that both *A. ostoyae* and *Hypholoma* spp. prefer lower substrate pH.

Paired culture studies at pH 5 and 6 revealed that *Hypholoma* spp. may effectively inhibit *A. ostoyae* growth at pH 6 but might be less effective at lower substrate pH, such as, pH 5. In the case of *He. irregulare* and *P. gigantea*, *P. gigantea* showed growth across all substrate pH levels tested, with optimal growth at pH 6. *Heteroabsidion irregulare* showed optimal growth at pH 6 but limited growth at higher pH, such as 8. Paired studies revealed that *P. gigantea* successfully inhibited the growth of *He*. *irregulare* most effectively at pH 7. These findings need to be further investigated in field conditions to validate our lab results, considering the potential pH-lowering abilities of *Hy. fasciculare* and *Heterobasidion* spp. as observed in this study.

Chapter 4 Conclusions

Soil temperature and pH can impact *Armillaria* root rot and Annosus root rot disease-causing pathogens, *Armillaria* spp. and *Heterobasidion* spp., in conifer plantations by modifying conditions for spore production and disease proliferation (Redfern, 1978; Rishbeth, 1978; Singh 1983; Wargo and Harrington, 1991; Taubert, 2008; Hietala et al., 2016; Boddy, 2016; Myers et al., 2018a). *Armillaria ostoyae* and *Heterobasidion irregulare* are the most widespread and highly pathogenic species infecting and killing red pine plantations in Simcoe County, Ontario forests (McLaughlin et al., 2010).

Various lab and field studies have shown the efficacy of biocontrol *Hypholoma* spp. such as *Hypholoma fasciculare,* in reducing the growth of the pathogen *A. ostoyae* (Chapman et al., 2004; Keca, 2009; Stevens, 2019). Another biocontrol, *Phlebiopsis gigantea* VRA 1992, is currently registered and used for controlling the growth of *He. irregulare* in the forests of Canada (Government of Canada, 2014). Since there is limited information on the effect of these abiotic factors on the growth of the pathogen, their potential biocontrols, and their interspecific interactions, this study was carried out to shed light on the issue.

To determine the effect of temperature and pH on the growth of the pathogen *A. ostoyae* and biocontrol strains of *Hypholoma fasciculare*, *Hy. capnoides* and *Hy. sublateritium*, and pathogen *He. irregulare* and biocontrol *P. gigantea* VRA 1992, these species were exposed to various temperature and pH conditions approximately similar to soil pH and average air temperature in southern Ontario, Canada. Our first objective was to provide the information on the effect of temperature on these species when growing in single colonies and in paired pathogen + biocontrol cultures. Our study showed that growth and spread of the pathogens and biocontrols are greatly influenced by changes in temperature. This study supports the assumption that potential rise in temperature due to climate change may increase the risk of spread of *Armillaria* root rot in Ontario since *A. ostoyae* survived

temperatures as high as 30°C. The biocontrol *Hypholoma* spp., however, could not survive such high temperature, though it showed higher growth at all other temperatures compared to *A. ostoyae* strains. The growth of this biocontrol was also significantly affected when in the presence of the pathogen at different temperatures. At the lowest and highest temperatures studied, *Hypholoma* spp. growth increased in presence of *A. ostoyae* strains except for *Hy. fasciculare*, which decreased. This study suggests that some *Hypholoma* spp. may be effective as a biocontrol against *A. ostoyae* at higher temperatures but not as high as 30°C. Changes in the growth of *A. ostoyae* need to be further investigated to determine the effectiveness of these biocontrol species. The pathogen *He. irregulare* exhibited low growth at higher temperatures and did not grow at 30°C, whereas its biocontrol, *P. gigantea*, continued to grow across all temperatures examined, even at 30°C. This study does not support the prediction that Annosus root rot would increase with an increase in temperature. This study also suggests that *P. gigantea* is a good candidate to control the growth of *He. irregulare* even at higher temperatures in Simcoe County forests since *P. gigantea* was able to inhibit the growth of *He. irregulare* at all temperatures. Furthermore, the results also encourage scheduling thinning and silviculture operations in late winter or early spring if possible since pathogen growth is extremely low during this time and biocontrols, if applied at this time, may overgrow and reduce the overall inoculum of the pathogen throughout the year and would be more economically viable.

My second objective was to provide information on the effect of variable pH on the species under study in single and paired colony cultures. This study showed that variation in pH significantly affected the growth of all the species under study. The pathogen *A. ostoyae* and biocontrol *Hypholoma* spp. displayed acidophilic behaviours and had minimal growth at higher pH such as 8. When paired together, all species of *Hypholoma* spp. under study were able to inhibit the growth of the B249-28 strain of *A. ostoyae* but were not able to

successfully inhibit the P162-7 strain. This was interesting since B249-28 is considered to be more virulent than P162-7, but in paired cultures, the P162-7 strain was more resistant to growth reductions in the presence of *Hy. capnoides* (TAK 5) and showed increased growth. This needs to be further investigated. The pathogen *He. irregulare* and biocontrol *P. gigantea* showed preference for neutral pH. However, *P. gigantea* was able to inhibit *He. irregulare* growth at all pH levels when paired together. This study showed that the presence of respective biocontrols reduced the growth of pathogens even at optimal pH levels for the pathogens. In the case of potential increase in temperate forest soil pH associated with climate change, the growth of *A. ostoyae* and *He. irregulare* can be successfully inhibited in the presence of *Hypholoma* spp. and *P. gigantea* respectively.

Our study suggests that the temperature and pH are important modifiers of the fate of pathogens both in the presence and absence of antagonists. Changes in these abiotic factors may alter the growth and competition outcomes of fungal species during pathogen-biocontrol interactions. This study provides insight of what to expect in case of change in environmental conditions. Predicted global warming will most probably increase the *Armillaria* root rot infection, but it may be kept in check if *Hypholoma fasciculare* (especially Pinnel B) and/or *Hy. sublateritium* application measures are applied early and during low- temperature seasons. Testing the site pH to ensure optimal growth conditions for biocontrol agents is recommended to ensure optimal pH for the growth of the biocontrol for successful disease management. Most importantly, different strains of biocontrol should be tested in local soils before using against pathogens to mitigate the risk of unintended consequences, such as an increase in disease aggressiveness in the presence of a biocontrol agent.

Future studies may involve:

- Replicating these temperature studies in natural soils in different seasons to verify the responses of the pathogens in the presence of their respective biocontrols
- Replicating pH studies using soil pH samples from different horizons since soil pH also varies with depth.
- Determining the interactive effects of these abiotic factors along with other climate change factors such as soil moisture and biotic agents, which will provide better insight into the future of the forest.
- Investigating the mechanisms responsible for pathogen inhibition by the biocontrols

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