INVESTIGATING THE BIOCONTROL POTENTIAL AND REPRODUCTIVE BIOLOGY OF *PHLEBIOPSIS GIGANTEA*

by

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Under the Direction of

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ABSTRACT

This study examined the ability of *Phlebiopsis gigantea* to act as a biocontrol agent for Heterobasidion Root Disease in the southeastern U.S., and also explored the reproductive biology of *P. gigantea*. The biocontrol efficacy of a *P. gigantea* isolate native to Alabama was tested in a field trial, and provided significant control of Heterobasidion Root Disease, reducing colonization of the pathogen by about two-thirds, even when treatment was delayed by 3 days. The impact of *P. gigantea* stump treatments on the prevalence of airborne *P. gigantea* spores in the environment was tested in a field trial, and it was determined that the stump treatments did not result in an increase in *P. gigantea* present in the environment. Investigation of homothallic behavior in *P. gigantea*, a species thought to be a heterothallic, revealed evidence of bonafide sexual fruiting in *P. gigantea* colonies that were generated from single basidiospores.

INDEX WORDS: *Phlebiopsis gigantea, Heterobasidion irregulare*, Heterobasidion Root Disease, pseudo-homothallic

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Heterobasidion Root Disease is an important problem in commercial conifer forests throughout the Northern Hemisphere. The disease causes root and butt rot in conifers, resulting in reduced growth and eventual death. Losses due to infection can vary greatly from stand to stand, but overall economic losses in Europe are estimated to be roughly one billion U.S. dollars per year (Woodward *et al*.1998). The impact of Heterobasidion Root Disease in the United States has not been researched as thoroughly, but a survey from South Carolina in 2006 estimated financial losses in that state alone totaled nearly 2 million dollars (Forest Health Protection 2007). Similarly, the disease is considered a major problem in both Alabama and Georgia. In 2006, it was responsible for more mortality in Georgia pines than any other disease (Forest Health Protection 2007).

Increasingly, intensive management of conifer stands, along with global climate trends, point towards an increase in the prevalence of Heterobasidion Root Disease in coming years. Intensive stand management involves planting denser stands and thinning them more aggressively, both of which serve to increase the risk of this disease entering a stand (Rishbeth 1951). Global climate change is expected to result in milder winters and an increase in drought conditions. Mild winters favor the spread of Heterobasidion Root Disease, while drought conditions are thought to make conifers more susceptible to it (La Porta *et al*. 2008). The European forest industry is well equipped to handle the increased risk of Heterobasidion Root Disease because they possess a control method for the disease that is already well developed and is being utilized extensively to prevent its spread. The control method revolves around the use of a wood-rotting fungus, *Phlebiopsis gigantea*, as a biocontrol agent (Holdenrieder and Greig 1998). In contrast, foresters in the U.S. are not regularly using control methods for the

disease and do not have access to the same biocontrol agent (Michelle Cram, USDA Forest Service, personal communication). With the potential increased risk of Heterobasidion Root Disease in mind, it is vital that a practical and effective control method be available for use in the US.

Heterobasidion Root Disease is caused by a family of closely related fungal pathogens found within the genus *Heterobasidion*. The disease has traditionally been attributed to a single species, *Heterobasidion annosum*, but recent research concludes that *H. annosum* is actually a species complex consisting of five slightly differing species (Otrosina and Garbelotto 2010). *Heterobasidion irregulare* is the species prevalent in the southeastern U.S.

Heterobasidion species cause disease by infecting and decaying the roots of trees. *Heterobasidion* species are not typically effective at entering healthy adult trees, but in managed stands they are able to bypass this obstacle by establishing themselves in the stumps left behind by thinnings (Rishbeth 1950; Rishbeth 1951). *Heterobasidion* spores land on the stump surfaces and then the fungus grows down into the root systems left by the thinned trees (Rishbeth 1951). The pathogen can then infect the roots of healthy surrounding trees by moving through root junctions into their roots (Rishbeth 1951). In this manner, *Heterobasidion* species can quickly infect sizable portions of a stand despite initially infecting relatively few stumps. Georgia stands infected in this way were observed to suffer a 10-20 % loss of the residual stand following a thinning (Driver and Dell 1961; Ross 1973). Frequent thinning has become standard practice for foresters in the southeastern US and due to its economic benefits it is only likely to increase in the future (James Johnson, Georgia Forestry Commission, personal communication), thus the need to manage Heterobasidion Root Disease is also likely to persist.

Once it became known that *Heterobasidion* species were entering stands via stumps, researchers began hunting for something that would prevent infection if applied to the surfaces of stumps following a thinning. Chemicals such as borax and creosote were quickly found to prevent infection, acting as stump sealants by creating a layer of chemicals on the stump

surface toxic to *Heterobasidion* (Driver 1963; Rishbeth 1959a). Effectiveness depended on covering the entire stump surface with the treatment and applying it immediately after thinning. Borax in particular showed nearly 100% control of *Heterobasidion* infections under these conditions (Driver 1963; Rishbeth 1959b). Despite their potential effectiveness, these chemical treatments are not the preferred method of control for modern foresters due to a number of issues. Chief among these is the fact that incomplete stump surface coverage, delayed application, or subsequent damage to the stump can all render the treatment ineffective (Rishbeth 1959a). All of those scenarios can lead to infection due to some portion of untreated stumps being exposed either before or after treatment. Another concern is that these treatments do little to stop the further spread of *Heterobasidion* in an already infected stand (Rishbeth 1959a). In fact, chemical treatment tends to slow the decay of stumps and roots, which in turn increases the ability of endemic *Heterobasidion* to spread through a stand. Finally, there are ecological issues associated with widespread application of chemicals in forest settings, as runoff and spills are inevitable (Pratt *et al*. 1998). Spillage associated with the spraying of Timbor, a commercially produced borax derivative, has been shown to cause severe damage to ground-vegetation species surrounding stumps (Westlund and Nohrstedt 2000).

While a truly satisfactory chemical treatment for preventing *Heterobasidion* infection has not been found, researchers have discovered and developed an effective biological control in the form of *Phlebiopsis gigantea*. Like *Heterobasidion species, P. gigantea* is a wood-rotting fungus native to the coniferous forests of the northern hemisphere, but it differs in that it is purely saprobic and will not attack living trees. Even before the need for a stump treatment became obvious, *P. gigantea* had been identified as a strong competitor with *Heterobasidion* for space in root systems (Rishbeth 1950). Later, when the need for a stump treatment became apparent, *P. gigantea* was quickly identified as an effective biocontrol agent. One early experiment showed that if a stump was inoculated with equal numbers of *Heterobasidion* and *P. gigantea* spores, within three months there would be virtually no trace (2%) of *Heterobasidion*

left in the stump while the *P. gigantea* would have colonized it almost completely (Meredith 1960). Further experimentation confirmed this result, showing that *P. gigantea* could achieve complete control of *Heterobasidion* when applied in high concentrations as a stump treatment (Rishbeth 1963). It is not yet clear how *P. gigantea* limits the growth of *Heterobasidion*. One theory is that *P. gigantea* achieves control by out-competing *Heterobasidion* for nutrients and space in the stump (Holdenrieder and Greig 1998). Alternatively, inoculation with *P. gigantea* may induce a resistance reaction in stumps, making them more difficult for *Heterobasidion* to colonize (Sun *et al.* 2011)

In Europe, *P. gigantea* has become the preferred method of controlling Heterobasidion Root Disease in the countries where it is available, such as Finland, Norway, Sweden, and England (Bendz-Hellgren *et al*. 1998; Redfern and Ward 1998). It is sold in the Scandinavian countries commercially, under the name Rotstop, which is produced by the Finnish company Verdera Oy. Rotstop consists of a concentrated powder containing dried spores from a Finnish isolate of *P. gigantea* and has been shown to provide over 99% control of *Heterobasidion* (Korhonen *et al*. 1994). Other European countries, such as Italy and Lithuania, are actively developing native *P. gigantea* isolates of their own for use as biocontrol agents (Annesi *et al*. 2005; Fiodorov 1998). As a biocontrol agent, *P. gigantea* has a number of advantages over chemical stump treatments. Its ability to spread after inoculation suggests that it would be more effective than chemical agents in situations where there was only partial coverage of the stump surface or a delay in application. It is also expected to have a beneficial effect in stands where Heterobasidion Root Disease is already present, as *P. gigantea* will accelerate the decay of stumps and roots rather than slowing it like the chemical treatments do. And finally, *P. gigantea* displays none of the negative ecological impacts that chemical agents do (Westlund and Nohrstedt 2000). However, one potential concern associated with use of *P. gigantea* in this fashion is that the widespread use of a single isolate could alter the genetic make up of the naturally occurring population in a given location (Holdenrieder and Greig 1998).

P. gigantea isolates native to the U.S. have been shown to be effective as biocontrol agents for Heterobasidion Root Disease (Ross and Hodges 1981), but no *P. gigantea* based products are currently certified for use in U.S. forests. This is due in large part to the lack of Environmental Protection Agency (EPA) approval, which is required for the use of biocontrol agents in the U.S. (Covert and Higgins 2007). One of the EPA's concerns mirrors the one described in the previous paragraph regarding the influence of widespread use of a single isolate of *P. gigantea* on the natural population's genetics and prevalence. In this study we attempt to address this concern by conducting field trials that compare *P. gigantea* populations at sites which have been sprayed with *P. gigantea* to the populations at sites which have not.

The EPA is also requiring that a native *P. gigantea* isolate be used in the U.S., instead of a European one. With that in mind, our lab has collected a number of *P. gigantea* isolates native to the southeastern U.S. and performed growth and competition assays in order to determine which isolates compete with *H. irregulare* most effectively in laboratory studies. We identified three native isolates that control *H. irregulare* just as effectively as the European isolates in the lab (Higgins and Covert, unpublished data). In the present study, the control efficacy of one of these, *P. gigantea* isolate 11061-1, was tested in a competitive field trial. More specifically, *H. irregulare* colonization in stumps treated with both *P. gigantea* and *H. irregulare* was compared to that of stumps treated with *H. irregulare* alone.

Even if it is approved by the EPA, use of *P. gigantea* as a stump treatment for Heterobasidion Root Disease in the U.S. may hinge upon its ability to work when application is delayed (James Johnson, Georgia Forestry Commission, personal communication). Sprayers built into logging equipment have made immediate stump treatment after cutting standard practice in Europe (Jim Pratt, UK Forestry Commission Research Agency, personal communication), but U.S. loggers are not likely to invest in such attachments until it is clear that this additional expense is warranted by significant customer demand for stump treatments. As a consequence, *P. gigantea* stumps treatments in the U.S. are most likely to be applied manually

after the large harvesting equipment has left the property. For this reason it is crucial that we determine if a *P. gigantea* product can be applied 3-7 days after thinning and still effectively control Heterobasidion Root Disease. With that goal in mind, we included delayed treatments of *P. gigantea* isolate 11061-1 in our competitive field trial. These treatments were applied to stumps that had already been treated with *H. irregulare* either 3 or 7 days prior. This trial gives us insight regarding both the control efficacy of this native *P. gigantea* isolate in southeastern U.S., and an idea of how effective delayed applications will be.

While *P. gigantea* has been studied thoroughly as a biocontrol agent, other aspects of its biology are relatively unexplored. In particular, complete understanding of its reproductive behavior is lacking. *P. gigantea* has been described as heterothallic (Korhonen and Kauppila 1988), but this description is at odds with the behavior it exhibits in culture. By definition, heterothallic cultures can not undergo sexual reproduction in the absence of a compatible mating partner. According to this definition, *P. gigantea* cultures that are derived from single sexual basidiospores, presumed to be haploid, should not be able to form fruiting bodies. But in our studies of *P. gigantea*, and those of other researchers, this hasn't been the case (Korhonen and Kauppila 1988).

In our study of *P. gigantea*'s reproductive behavior we examined the potential for cultures derived from single basidiospores to form sexual fruiting bodies. We looked for evidence of sexual reproduction in these cultures by examining cultures for the presence of basidiospores, cystidia, and basidia, which are characteristic of fruiting *P. gigantea* cultures (Erikkson *et al*. 1981). We also investigated a potential explanation for *P. gigantea*'s apparent homothallic fruiting by examining the nuclear condition of its basidiospores.

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CHAPTER 2

DEVELOPMENT OF *PHLEBIOPSIS GIGANTEA* AS A BIOCONTROL AGENT FOR HETEROBASIDION ROOT DISEASE IN THE SOUTHEASTERN UNITED STATES

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Abstract

Heterobasidion Root Disease is a problem in commercial conifer forests throughout the northern hemisphere and is of increasing concern in the southeastern United States. In Europe, the wood decay fungus *Phlebiopsis gigantea* is registered as a biocontrol agent for Heterobasidion Root Disease, but in the U.S. *P. gigantea* has not been approved by the Environmental Protection Agency (EPA) for this use. As a step towards eventual EPA registration, the biocontrol efficacy of a *P. gigantea* isolate collected in Alabama was tested in a field trial in which stumps of loblolly pine were treated with the pathogen, *Heterobasidion irregulare*, and then treated with *P. gigantea*. To test the efficacy of delayed treatment, *P. gigantea* was applied to stumps at three different intervals: immediately after *H. irregulare* application, three days later, and seven days later. After 3 to 4 months incubation, the *P. gigantea* treatments on day 0 and day 3 reduced *H. irregulare* colonization by about two-thirds, while the day 7 treatment reduced pathogen colonization by about one-third. To assess the impact of *P. gigantea* stump treatments on the prevalence of airborne *P. gigantea* spores in the environment, loblolly pine stumps at 5 sites were treated with *P. gigantea* or water, followed by monthly monitoring of the surrounding environment for airborne spores. There was no difference between sites that were treated with *P. gigantea* and those that were treated with water. These results suggest that *P. gigantea* has the potential to be an effective biocontrol agent in the southeastern U.S., and its use is unlikely to significantly increase the amount of *P. gigantea* present in the environment.

Introduction

Heterobasidion Root Disease is the source of significant damage to commercial conifer stands in Europe and the U.S. The disease is caused several closely related fungal pathogens found within the genus *Heterobasidion*. It results in root and butt rot in conifers, leading to reduced growth and eventual death. Losses due to *Heterobasidion* infection in Europe are estimated to be roughly one billion U.S. dollars per year (Woodward *et al*. 1998). The disease is also considered a major problem in the southeastern U.S. (Forest Health Protection 2007), and it is likely to become more prevalent in coming years as increasingly common stand management practices, such as thinning densely-planted stands, serve to increase the risk of the disease (Rishbeth 1951). *Heterobasidion* species typically infect by way of aerial spores, which land on the stump surfaces left by thinned trees (Rishbeth 1951). The pathogen then spreads through the stumps into their root systems, where it can transfer to nearby healthy trees by moving through root junctions (Rishbeth 1951).

A number of treatments aimed at preventing Heterobasidion Root Disease have been investigated, with a focus on stopping the initial infection of the stump surface. Chemical treatments, such as Borax and creosote, have been shown to completely prevent infection if they are applied to stumps immediately after thinning, and stumps are completely coated (Driver 1963; Rishbeth 1959a). But these chemical methods aren't preferred, as they can be unreliable in cases where stumps are only partially treated or damaged after treatment (Rishbeth 1959a), and there are ecological issues associated with the widespread use of chemical treatments in a forest setting (Pratt *et al*. 1998; Westlund and Nohrstedt 2000). Biological control, using *Phlebiopsis gigantea*, has emerged as a viable alternative to chemical control methods. *P. gigantea* is a wood-rotting fungus native to northern coniferous forests, but unlike *Heterobasidion* species, it is saprobic, meaning that it will not attack living trees. *P. gigantea* was initially identified as a strong competitor with *Heterobasidion* species for colonization of roots (Rishbeth 1950), and later it was shown to effectively prevent *Heterobasidion* growth when

equal numbers of spores from each species were applied to stumps (Meredith 1960). The mechanism by which *P. gigantea* limits *Heterobasidion* growth isn't known, but it has long been suspected that *P. gigantea* out-competes *Heterobasidion* for nutrients and substrate in the stump (Holdenrieder and Greig 1998), although recent research suggests that inoculation with *P. gigantea* could be inducing a resistance reaction in stumps, making them more difficult for *Heterobasidion* to colonize (Sun *et al*. 2011).

As a biocontrol agent, *P. gigantea* has some advantages over chemical stump treatments. Its ability to spread after inoculation suggests that it would be more effective than chemical treatments in stumps that have been only partially treated, stumps that are damaged after treatment, and stumps that receive delayed treatment. *P. gigantea* also displays none of the negative ecological impacts that chemical treatments do (Westlund and Nohrstedt 2000). Due to these advantages, *P. gigantea* has become the preferred method of controlling Heterobasidion Root Disease in the countries where it is available. This includes England, Poland, and the Scandinavian countries, where it is sold commercially under the name Rotstop (Bendz-Hellgren *et al*. 1998; Pratt *et al*. 2000). Rotstop, produced by the Finnish company Verdera Oy, consists of a concentrated powder containing dried spores from a Finnish isolate of *P. gigantea*, and has been shown to provide over 99% control of Heterobasidion Root Disease (Korhonen et al 1994).

Despite widespread adoption in Europe, no *P. gigantea* based products are currently certified for use in the U.S. This is due in large part to the lack of Environmental Protection Agency (EPA) approval, which is required for the use of biocontrol agents in the U.S. (Covert and Higgins 2007). EPA approval depends on a number of factors, one being that the *P. gigantea* isolate used must be native to the U.S., not Europe. Another issue that the EPA has raised is the potential ecological effect that commercial use of *P. gigantea* could have on the natural *P. gigantea* population. The use of *P. gigantea* as a stump treatment for Heterobasidion Root Disease in the U.S. faces another barrier in its ability to be successfully applied by U.S.

loggers. Sprayers built into logging equipment have made immediate stump treatment after cutting standard practice in Europe (Jim Pratt, UK Forestry Commission Research Agency, personal communication). But in the U.S., logging techniques dictate that stump treatments will likely be performed manually after the feller-bunchers and skidders have completed a thinning. For this reason it is important that a *P. gigantea* product developed for use in the U.S. can be applied 3-7 days after thinning and still effectively control Heterobasidion Root Disease.

In this study we investigated the ability of a native U.S. *P. gigantea* isolate to control Heterobasidion Root Disease, even when application of the *P. gigantea* was 3-7 days after the stump was created. We also examined the impact of *P. gigantea* release on the prevalence of *P. gigantea* in the surrounding environment.

Methods

Preparation of Fungi

P. gigantea isolates 11061-1, originally collected in Macon County, AL, and 31044b-10, originally collected in Talbot County, GA, were sent to Verdera Oy (Finland), where their spores were prepared as a commercial-grade product (Pratt *et al*. 2000) containing 2×10^6-10^7 colony forming units/gram. On the day of stump treatment, 1 g of this *P. gigantea* product was mixed with 1 L of water just before use. Isolate 11061-1 was used in the biocontrol efficacy trial, while isolate 31044b-10 was used in the impact of *P. gigantea* release on *P. gigantea* prevalence trial.

Three *H. irregulare* isolates were used in the biocontrol efficacy trial: FB (collected in Fort Benning, GA), and NC1 and NC2 (collected in North Carolina) (M. Cram, USDA Forest Service, personal communication). They were grown on 2% Bacto malt extract agar at room temperature for two to three weeks. On the day of stump treatment, approximately equal numbers of viable spores from each isolate were mixed in water just before use so that the final *H. irregulare* spore concentration was predicted to be 6×10^5 spores/L. The typical number of spores produced by each *H. irregulare* isolate grown under these conditions was determined previously by collecting spores from a single Petri dish in 5 ml of water and counting them with a

haemocytometer. Spores from each isolate were collected from at least three Petri dishes on at least five different dates, and the average number of spores/mL collected was determined. Viability of the spores was estimated by plating 200 spores on medium semi-selective for hymenomycetes (1.5% Bacto malt extract, 1.5 % agar, 2 ml/L lactic acid, 0.06 g/L benlate, and 0.1 g/L streptomycin sulfate) (Worrall 1991), storing the plates at room temperature (23º C) for seven days, and then counting the number of colonies formed. This process was repeated four times for each *H. irregulare* isolate, with an average percent spore viability calculated for each.

Biocontrol Efficacy Trial

An experiment to test the biocontrol efficacy of *P. gigantea* 11061-1 was replicated at six independent sites populated with 20-25 year old loblolly pine, within the University of Georgia's B.F. Grant Memorial Forest in Putnam County, GA. The first four replicates were established in the winter of 2009-2010, in managed pine stands undergoing operational thinning. At these sites, the loggers were instructed to leave stumps over 30 cm high. Only stumps without split wood or damaged bark were selected for further use in the trial. The fifth and sixth replicates were established in the winter of 2010-2011 at sites thinned by chainsaw operators, who were also instructed to leave stumps over 30 cm high. One to two days after the stumps were first created, they were cut to 30 cm to create uniform, level surfaces. On the same day, the first fungal treatments were applied. Each of the six sites was divided into 25 blocks, with each block containing four stumps. The four stumps in each block were then randomly assigned to one of four treatments: "*P. gigantea* on Day 0", "*P. gigantea* on Day 3", "*P. gigantea* on Day 7", or "No *P. gigantea* Control". Interspersed among these blocks were 20 stumps, selected to serve as "sentinel stumps" for monitoring stump colonization by *H. irregulare*.

On the first day of treatment, all 120 stumps at a given site were treated with *H. irregulare* applied at a rate of 60 spores/cm^2, simulating two weeks' worth of natural *H. irregulare* accumulation in Georgia (Ross 1972). This was followed on the same day with an application of *P. gigantea* to all "*P. gigantea* on Day 0" stumps. Three days later, all "*P. gigantea*

on Day 3" stumps were treated with *P. gigantea*, and seven days later all "*P. gigantea* on Day 7" stumps were treated with *P. gigantea*. Stumps assigned to the "No *P. gigantea* Control" or "Sentinel Stump" treatments were not treated with *P. gigantea*, only with *H. irregulare. P. gigantea* was applied at 200-2000 spores/cm^2, the commercial rate suggested by Verdera Oy (Pratt *et al*. 2000). Both treatments were applied in the form of spore suspensions, using hand-held spray bottles, which were calibrated to deliver 0.1 ml of spore suspension per 1 cm^2 of stump surface. To measure spore viability, diluted spore suspensions were plated on medium semi-selective for hymenomycetes (above) in volumes predicted to contain 100 colony forming units of *P. gigantea*, or 110 colony forming units of *H. irregulare*. Five samples of each fungus were plated on each treatment day, and the percent spore viability was calculated by counting the number of colonies formed on each plate after three to four days incubation at room temperature (23º C).

Internal stump temperature in the forest was monitored using Tinytag (Gemini Monitors) electronic monitors that were placed in stumps 1 cm below the surface at sites 1, 2, 5, and 6. The Tinytag monitors recorded internal stump temperature every 15 minutes. Ambient climate data were collected from a climate monitoring station approximately two miles from the field sites, at the Central Georgia Research and Education Center, operated by the University of Georgia.

Two to three months after a site was treated with *H. irregulare*, its sentinel stumps were collected to determine the depth of maximal *H. irregulare* colonization. This was performed by sectioning the stumps from top to bottom into discs 1-2 cm thick with a chainsaw. The chainsaw carried an attachment (Figure 2.1), provided by Jim Pratt (UK Forestry Research, retired), which allowed for the cutting of discs of a defined thickness. Each disc was labeled according to its depth in the stump and returned to the lab. Disks were rinsed with water, wrapped in newspaper, and incubated at room temperature (23º C) for seven days. The disc surfaces were scored for the presence of *H. irregulare* by laying a transparent grid (1 cm x 1 cm) on them and

using an Olympus SZH10 dissecting microscope to examine every third square for the distinctive *spiniger* conidiophores produced by *H. irregulare*. *H. irregulare* colonization is reported as a percentage of squares scored positive.

Three to four months after *H. irregulare* treatment, each site was fully harvested. This full harvest involved collecting one disc from each stump at the depth where *H. irregulare* was predicted to be most prevalent by each site's sentinel stump results. These discs were then treated and scored in the same manner as the sentinel stump discs. Following full harvest, the resulting stumps at each site were treated with *P. gigantea* in an effort to prevent further development of Heterobasidion Root Disease in the residual stands.

Measuring the Impact of *P. gigantea* Release on *P. gigantea* Prevalence

To determine the impact of *P. gigantea* use on the prevalence of airborne *P. gigantea* spores in the environment, plots in two thinned loblolly pine stands in central Georgia were treated with aqueous suspensions of *P. gigantea* spores. In July 2007, three experimental sites were installed in Shady Dale, GA, at locations which had been thinned one to five weeks earlier. At each of the three experimental sites, 30 stumps were treated with a *P. gigantea* spore suspension. The spore suspension was sprayed onto stumps manually, with a colored dye (Turf Mark supplied by Verdera Oy) included to ensure that the surface of each stump was covered completely. Approximately half a mile away from each experimental site, another 30 stumps were treated with water containing only Turf Mark dye. Three additional thinned sites, approximately 0.25 to 1.25 miles from the treated sites, were also selected and left completely untreated. Monitoring of *P. gigantea* prevalence at all of these sites began in September 2007 and ended in July 2009.

In February 2008, two more experimental sites were installed in UGA's B.F. Grant Memorial Forest in Putnam County, GA. These sites were treated as described above, except that 100 stumps at each site were treated with *P. gigantea* or water, and the treatments were applied the same day as the trees were cut. One additional thinned site, approximately 0.2 miles

from the nearest treated site, was also selected and left completely untreated. Monitoring of *P. gigantea* prevalence at these sites began in February 2008 and ended in January 2010.

P. gigantea prevalence was monitored using petri dishes containing agar semi-selective for hymenomycetes (above). Once a month, during the morning, four to eight dishes were placed on the ground at each treated site. An additional four to eight dishes were also placed at a location halfway between the *P. gigantea*-treated and water-treated stumps at each site. This was labeled the "middle" site, and was included to monitor potential effects of wind-blown spores moving from the *P. gigantea* treated sites to the other sites. The petri dishes were left exposed to the environment for one to two hours before being collected, wrapped in parafilm, and returned to the lab. They were incubated at room temperature (23º C) for five to seven days. After incubation, the dishes were examined visually and the number of *P. gigantea* colonies recorded. Colonies were identified by their morphology, which is characterized by a dense white mycelium that lacks aerial hyphae and is spotted with small mycelial aggregations. These identifications were confirmed in some cases by microscopic examination of spores using an Olympus CH-2 light microscope.

Statistical Methods

R statistical software was used to analyze the data from the biocontrol efficacy trial. A linear mixed-effects model was used to look at the effects of site, block, and treatment across all sites. A series of analyses of variance were used to examine the effects of treatment within each site.

Data from the study measuring the impact of *P. gigantea* release were analyzed using SAS statistical software to run an analysis of variance and then evaluate a series of general linear models. In this analysis, spore counts were transformed using the natural log in order to avoid bias due to the counts being heavily and positively skewed.

Results

Biocontrol Efficacy Trial

To determine if *P. gigantea* isolate 11061-1 is capable of controlling *H. irregulare* infections in the field, even if *P. gigantea* application is delayed until several days after thinning, loblolly pine stumps were inoculated with *H. irregulare* and treated with *P. gigantea* 11061-1 zero to seven days later. Before the field experiment began, the amount of viable conidia typically produced by three different *H. irregulare* isolates during growth in culture was determined so that the appropriate number of spores could be applied to stumps in the field without a need to count them on the day of application. On average, isolate FB produced 2.3 x 10^5 conidia/mL, isolate NC1 produced 3.2 x 10^5 conidia/mL, and isolate NC2 produced 1.9 x 10^5 conidia/mL after 17 days of growth. Three to four months after the stumps were treated, discs were cut from each stump and the amount of *H. irregulare* colonization in each disc was recorded as the percentage of stump surface area that produced *H. irregulare* conidiophores after 7 days incubation. *H. irregulare* colonization in the No-*P. gigantea* controls varied significantly from site to site, ranging from an average of 91% at site 1 to 11% at site 3 (Figure 2.2). When results from all sites were considered, the Day 0 and Day 3 treatments each reduced the *H. irregulare* colonization by about two-thirds relative to the control, versus a drop of about one-third for the Day 7 treatment (Figure 2.3).

The amount of *H. irregulare* colonization for all discs across all sites was analyzed using a linear mixed-effects model, with treatment included as a fixed effect, while block and site were considered random effects (Table 2.1). Both site and treatment were found to have significant effects ($p < 0.0001$), while block was not a significant source of variance ($p = 0.9994$) (Table 2.1). A comparison of standard deviations within this model revealed that our treatments were responsible for roughly 46% of the total variation seen in the results, with site-to-site differences accounting for the rest. The model was also tested with disc surface area included as a covariate, but it was not a significant source of variance ($p = 0.5125$). A series of pair-wise

comparisons between the different treatments revealed that all three *P. gigantea* treatments (Days 0, 3, and 7) had significant effects on *H. irregulare* colonization relative to the control and to one another ($p < 0.0001$ for all comparisons except for Day 0 vs. Day 3, for which $p = 0.005$). The Bonferroni correction was included, setting the bar for significance at $p = 0.0083$. In addition, a linear model was used to compare treatment effects within each site. The Day 0 and Day 3 treatments both significantly reduced *H. irregulare* colonization relative to the control at all sites ($p < 0.05$ in all cases), except for site 3 (Day 0, $p = 0.2061$; Day 3, $p = 0.5995$). Day 7 treatments were inconsistent, having a significant effect at only half of the sites (sites 2, 5, and 6; $p \leq 0.0380$).

The viability of the *P. gigantea* and *H. irregulare* spores applied to the stumps was monitored by plating five samples of the spore suspensions used in the field and counting the resulting colonies. *P. gigantea* viability varied from site to site and by treatment, and was generally at the low end of the expected range, especially at sites 1-4 (Table 2.2). Despite this variation, there was not a strong correlation between *P. gigantea* viability and treatment effect (R^2 values: Day 0 = 0.270, Day 3 = 0.004, Day 7 = 0.096). *H. irregulare* viability was more consistent at each site and always higher than expected (Table 2.2). There was a moderate, although not clearly significant, correlation between *H. irregulare* viability and *H. irregulare* colonization in control stumps ($R^2 = 0.452$, $p = 0.073$).

Stump temperature readings were gathered in the treatment plots, while a nearby climate station provided ambient temperature data. Stump temperatures and ambient temperatures varied, as expected, with sites established in December (sites 1-3, and 5) generally experiencing lower temperatures throughout the experiment than those established in January (sites 4 and 6) (Figure 2.4). Possible effects of site-to-site temperature variation on site-to-site variation of treatment effect were examined by correlating various temperature measures (average maximum, average minimum, absolute maximum, absolute minimum, and overall average) with average percent change in *H. irregulare* colonization relative to the control across

all *P. gigantea* treatments for each site. None of these correlations were statistically significant, but the relationship between treatment effect and absolute minimum temperatures was positive for both stump data ($R^2 = 0.501$, $p = 0.11$), and ambient data ($R^2 = 0.393$, $p = 0.18$). Effects of site-to-site temperature variation on *H. irregulare* growth were examined by running the same set of correlations, comparing the various temperature measures to the average *H. irregulare* colonization of the controls at each site. These correlations were very weak ($p = 0.22 – 0.96$), with one exception: absolute maximum ambient temperature, which had a significant negative correlation with *H. irregulare* growth ($R^2 = 0.709$, $p = 0.036$).

Measuring the Impact of *P. gigantea* release on *P. gigantea* Prevalence

The effect of *P. gigantea* release on the prevalence of its airborne spores in the environment was measured by treating stumps in a given area with *P. gigantea* and subsequently monitoring those areas, as well as nearby untreated areas, for the presence of airborne spores. This monitoring was accomplished by leaving exposed Petri plates in those areas to capture spores and then counting the number of *P. gigantea* colonies following a short incubation. Average *P. gigantea* spore captures for each site, treatment, and month were calculated (Figure 2.5). Location was clearly a major factor in the results, as the sites at B.F. Grant (sites 6-8) captured nearly 60% more spores per plate than the sites at Shady Dale (sites 1-5,9). *P. gigantea* treatment did not appear to increase the prevalence of *P. gigantea* in the environment; only at site 2 did the plates placed in the *P. gigantea*-treated area capture more spores than the plates at the water-treated and untreated middle areas (Figure 2.5). At sites 1 and 3, spore captures were highest in the middle area, and at sites 6 and 7 spore captures were highest in the water-treated area (Figure 2.5). There was no clear pattern in the significant month-to-month variation in spore captures, although capture rates were especially high in July (Figure 2.5b). An initial one-way analysis of variance comparing treatments across all sites, and not controlling for any other variables, suggested that treatment did have a significant effect on spore capture rates ($p = 0.0025$). But when a general linear model was used, factoring in both

location and date as covariates, treatment was no longer a significant source of variance in the spore captures ($p = 0.4850$).

Discussion

All three *P. gigantea* treatments, day 0, day 3, and day 7, resulted in a statistically significant reduction in the *H. irregulare* colonization of treated stumps relative to *H. irregulare* colonization of stumps without *P. gigantea* treatment. Control efficacies of day 0 and day 3 treatments were comparable, reducing *H. irregulare* colonization by 64% and 67%. The day 7 treatments were clearly less effective, only reducing *H. irregulare* colonization by 32%. While these results are statistically significant, judging their practical significance is more difficult, as there is no established standard for control of Heterobasidion Root Disease in stump trials. Overall these control efficacies are somewhat lower than those reported in previous studies of a similar nature. One hundred percent control efficacy was reported in one comparable study conducted in the UK, in which Corsican pine stumps were treated with Rotstop, and Rotstop S, isolates *of P. gigantea,* as well as *H. annosum* (Tubby *et al*. 2008). Control efficacy in excess of 90% was found in a similar Finnish study (Korhonen *et al*. 1994), but in that study *Heterobasidion* was not directly applied to the stumps; instead natural inoculation was relied upon. This method more accurately mimics the colonization of stumps in nature than our trial, which involved delivering a large dose of *H. irregulare* to all stumps on the first day of treatment. Presumably the gradual dose of *Heterobasidion* presented by natural inoculation would present less of a challenge to *P. gigantea*, and this difference could be partially responsible for our relatively low control efficacies. In a similar study of Rotstop, conducted in Sweden, control efficacy ranged from 58% to 71% (Ronnberg *et al*. 2006). The lower ranges reported by this study suggest that the performance of the day 0 and day 3 treatments in our study could be strong enough to be of practical significance. The low efficacy shown by the day 7 treatments would likely rule it out as a useful treatment in a practical setting, but exploring why the day 7 treatment was less effective could be of value. It is possible that changes in stump condition,

such as reduced surface moisture or sealing, could limit the ability of *P. gigantea* to colonize a 7-day old stump and compete successfully with *H. irregulare*. However, it is also possible that the specific design of our study, particularly the application of a large dose of *H. irregulare* on day 0, resulted in the *H. irregulare* having too much time to establish itself before the day 7 treatments were applied. A more accurate measure of the practical effectiveness might be gathered by using the natural inoculation method of applying *H. irregulare*.

Another possible cause of the relatively low control efficacy in our experiment is the unexpectedly high ratio of *H. irregulare* to *P. gigantea* that we applied to the stumps. Spore viability data collected from suspensions of both species used during the experiment revealed that we applied slightly more *H. irregulare* than intended at all sites, while simultaneously applying less *P. gigantea* than expected on a number of occasions. This resulted in an actual average *H. irregulare* to *P. gigantea* ratio of 0.35, higher than the 0.3-0.03 range we expected. There is evidence that this ratio being skewed in favor of *H. irregulare* can have a large impact on *P. gigantea* control efficacy; Sun *et al*. (2009) reported that a *H. irregulare* to *P. gigantea* ratio of 0.4 (as opposed to the 0.1 ratio they expected), was associated with much lower control efficacies than expected (i.e. 19.5-68.5% observed vs. >90% expected). In our experiment there was a negative, although not statistically significant, association between this ratio and the *P. gigantea* control efficacy, which may explain in part the variance in control efficacy between sites and treatments (data not shown). Variation in *H. irregulare* viability may also help explain some of the variation seen in *H. irregulare* colonization from site-to-site. While there was not a statistically significant correlation between *H. irregulare* viability and colonization, there was a positive trend, highlighted by the fact that the two sites with the highest rate of *H. irregulare* colonization also had the highest levels of *H. irregulare* viability (Table 2.2; Figure 2.2).

Temperature is another possible source of the observed site-to-site variation, because *H. irregulare* is thought to be sensitive to high temperatures, with its growth reduced at 30º C, and arrested at sustained temperatures over 40º C (Gooding *et al*. 1966). This sensitivity to heat

is also evident in the sharp decline of aerial *H. irregulare* spores during the summer months in Georgia (Ross 1973). Vulnerability to heat could explain why *H. irregulare* presence in the controls was highest at sites 1 and 4, because stumps at those sites experienced the lowest temperatures on average and were never exposed to temperatures above 25º C, whereas stumps at the other sites were all exposed to temperatures of 35º C and greater. The effect of temperature on the biocontrol efficacy of *P. gigantea* remains unclear, as it is difficult to distinguish between an effect on *H. irregulare* growth and *P. gigantea* effectiveness in stumps that have been treated with both. With that said, there was a positive, although not quite statistically significant, correlation between higher temperatures and high control efficacy. The inclusion of stumps treated only with *P. gigantea*, and scoring of *P. gigantea* colonization, would be helpful in determining how much of this correlation was due to increased *P. gigantea* growth.

Our spore capture results suggest that targeted use of *P. gigantea* to treat stumps has no impact on the prevalence of airborne spores in the surrounding environment. Spore captures were highest in the *P. gigantea*-treated area at only one of the five experimental sites where *P. gigantean* and water treatments were applied. Of the other four sites treated this way, spore captures were highest at the water treated areas in two instances and at the middle areas in the other two. On average, the water-treated areas had the highest rate of *P. gigantea* spore captures, but the differences between treatments was found to be insignificant when month and location were taken into account. Indeed, spore captures varied significantly from site-to-site and month-to-month, but these variations can be explained by natural phenomena. Because the *P. gigantea* stump treatments had no apparent effect on the prevalence of airborne *P. gigantea* spores in the environment, it is to be expected that differences between sites in their natural *P. gigantea* populations would have a large effect on the spore capture results. The high variation in month-to-month spore captures, with no clear seasonal pattern, is consistent with previous studies of *P. gigantea* sporulation in Georgia (Ross 1973; Boyce 1963).

Overall, the results presented here suggest that *P. gigantea* treatment of stumps is an effective and environmentally sound method for control of Heterobasidion Root Disease in the southeastern U.S. The results from the day 0 and day 3 *P. gigantea* treatments are promising, suggesting that *P. gigantea* isolate 11061-1 is a good candidate for biocontrol use, and that delayed treatment is a practical possibility. Further testing using conditions closer to those found in operational thinnings would be ideal, and could possibly show that a delay of *P. gigantea* treatment beyond 3 days is still viable. Ideally, future studies will include the use of stumps flush with the ground, as opposed to elevated as they were in our study, and the use of *H. irregulare* inoculation methods more representative of the rate of inoculation that occurs in nature. While our spore capture results indicate that the use of *P. gigantea* as a biocontrol agent is unlikely to significantly increase the prevalence of airborne spores in the environment, monitoring over a longer time period in an operational setting would be optimal for making the most accurate predictions about long term effects of *P. gigantea* use.

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Tables and Figures

Table 2.1: Linear Mixed-Effects Model

	df	L. Ratio	p-value
Site[1]	1	419.5	<.0001
Block	1	5.7E-07	0.9994
Treatment	3	175.1	<.0001
Disc Area	1	0.429	0.5125

[1]Site, block, treatment, and disc area are included as covariates explaining variation in *H. irregulare* colonization across all sites.

Table 2.2: Viability of Fungal Spores Used to Treat Sites 1-6 in the Biocontrol Efficacy Trial

		Number of Colonies / Plate					
	Day	**Site 1**	**Site 2**	**Site 3**	**Site 4**	**Site 5**	**Site 6**
P. gigantea[1]							
	0	8	62	ND[2]	8	79	159
	3	ND	29	48	24	62	59
	7	ND	8	23	75	61	49
H. irregulare[1]							
	0	166	105	120	159	111	130

[1]Measured in colonies per plate. Averages from five plates created from diluted spore suspensions on sites. Expectation for *P. gigantea*: 20-200 colonies/plate. Expectation for *H. irregulare*: 100 colonies/plate. [2]Not determined.

Figure 2.1 – Chainsaw with specialized attachment. Used to cut discs of an exact thickness.

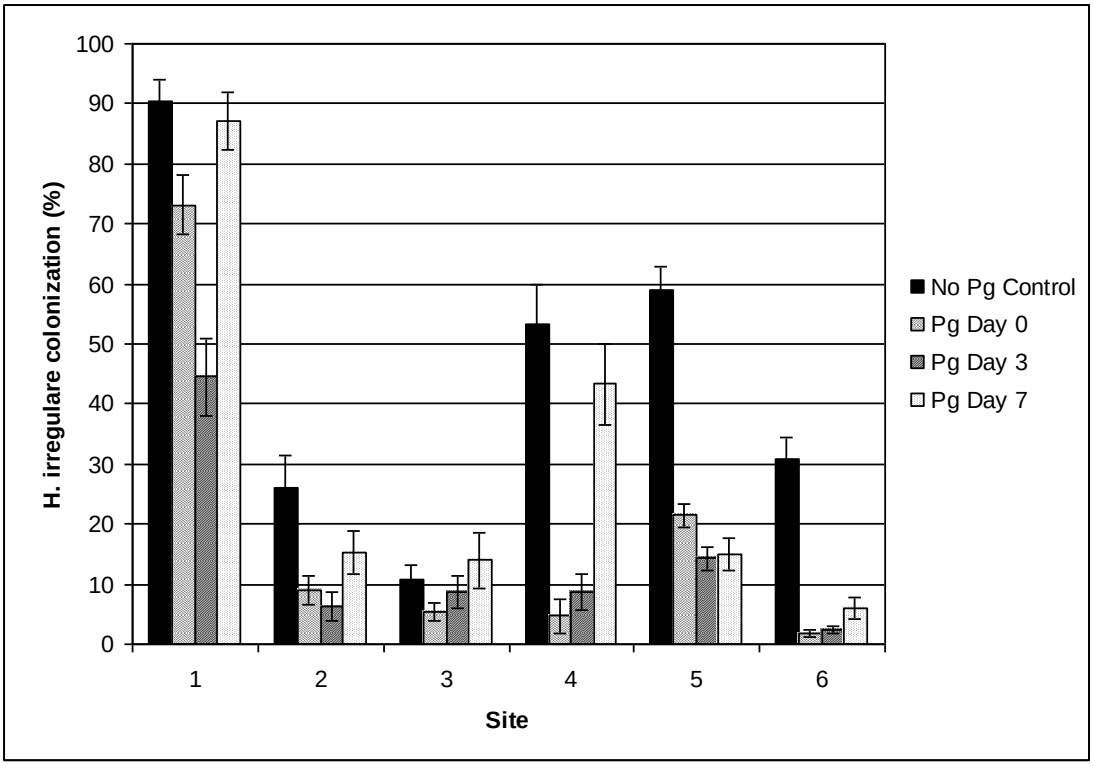

Figure 2.2 - Average *H. irregulare* colonization in treated stumps at each site. Error bars display the standard error.

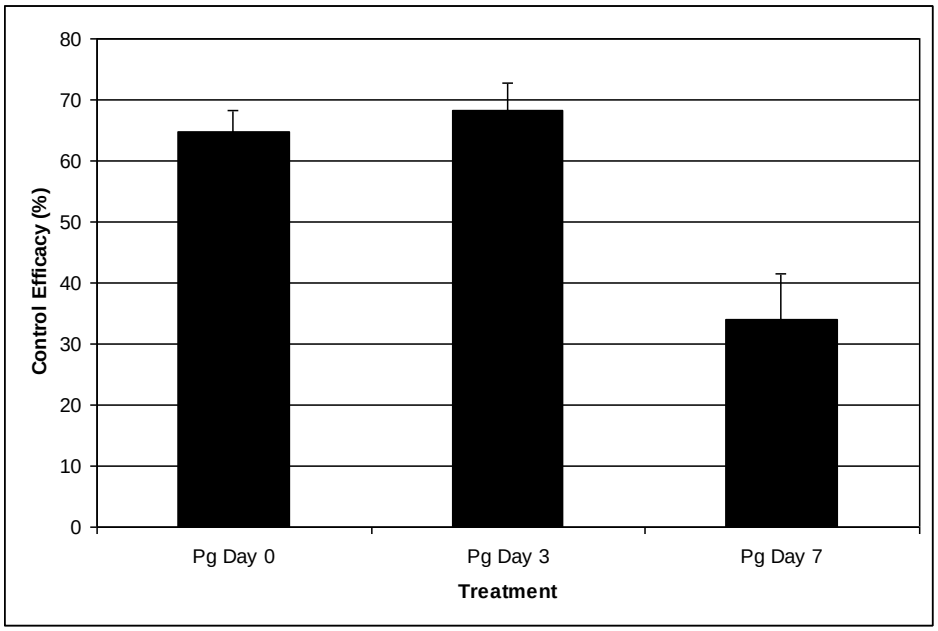

Figure 2.3 – Average control efficacies of *P. gigantea* treated stumps across all six sites. Control efficacy is expressed as the percent difference between the average amount of *H. irregulare* found in the No *P. gigantea* controls at a given site, and the amount of *H. irregulare* found in each of the *P. gigantea* treated stumps at that site. Error bars display the standard error.

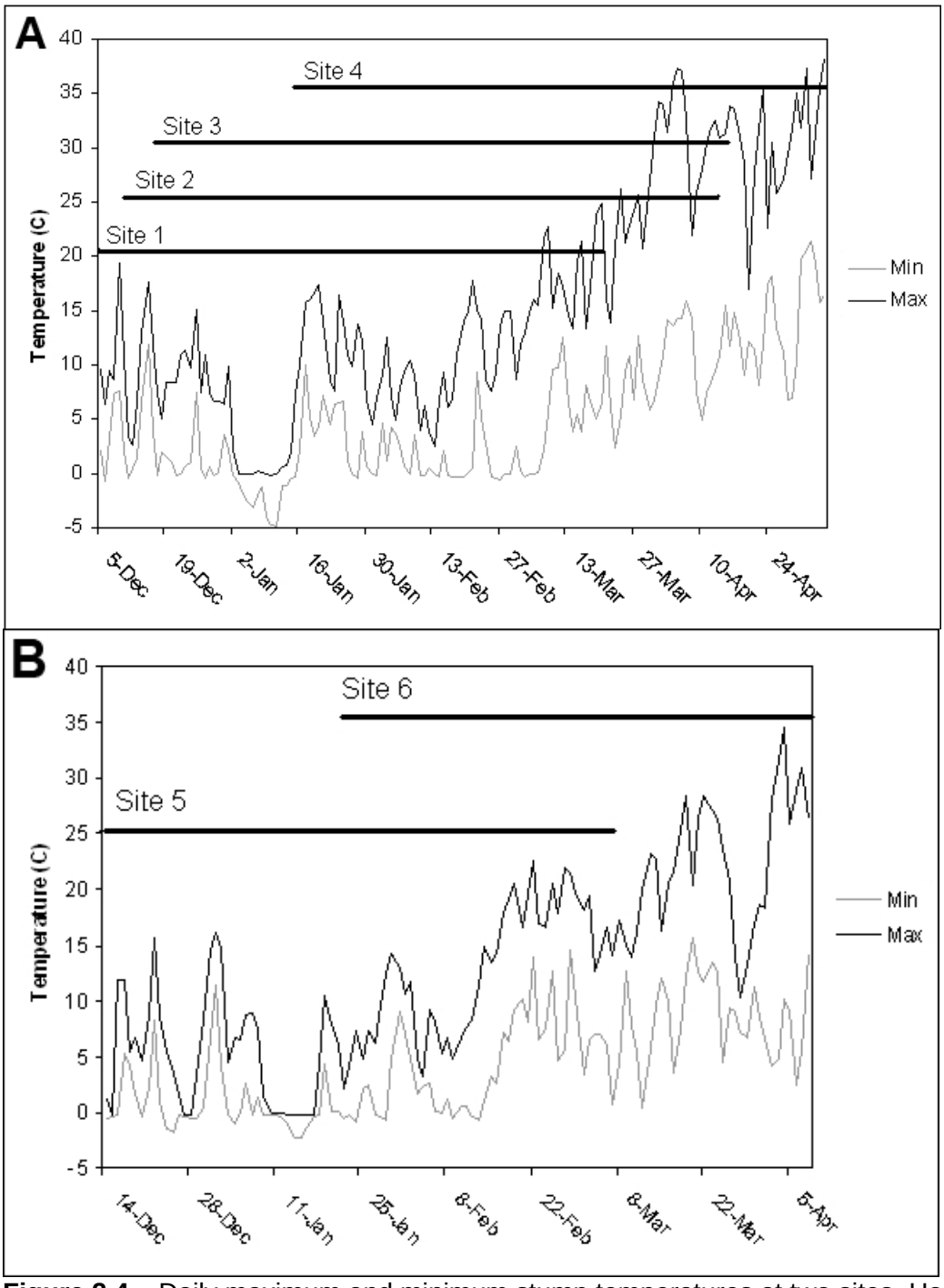

Figure 2.4 – Daily maximum and minimum stump temperatures at two sites. Horizontal bars labeled with site numbers display the duration of the experiment at each site, from installation to final harvest. Panel A: Data collected from a stump probe at Site 1, Dec 2009 – Apr 2010. Panel B: Data collected from a stump probe at Site 5, Dec 2010 – Apr 2011.

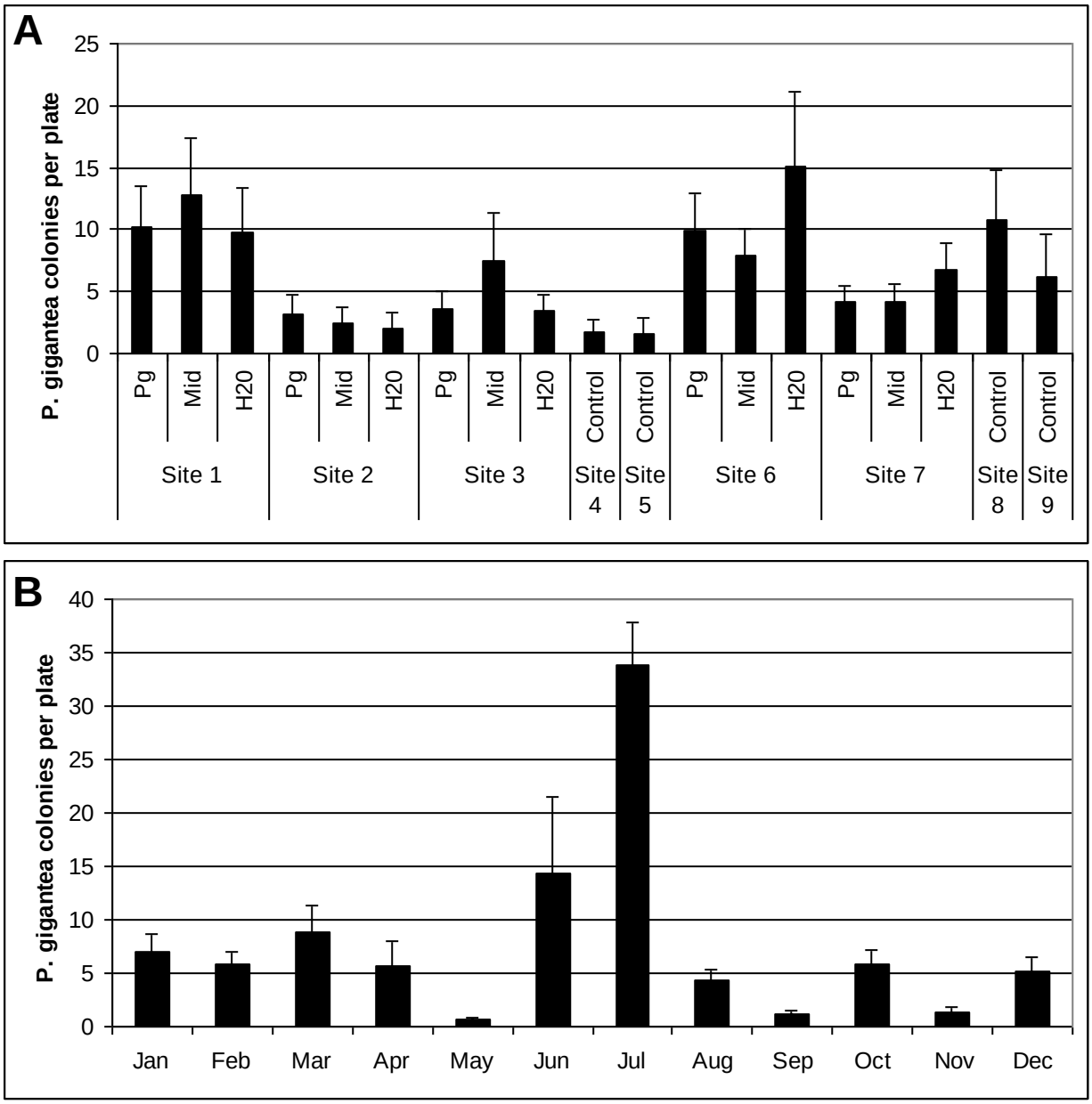

Figure 2.5 - Spore Capture Results. Panel A: Average number of *P. gigantea* colonies per plate for each treatment at each site. Includes all observations at each site. Pg = Sites treated with *P. gigantea*, Mid = Sites midway between *P. gigantea* treated sites and water treated sites, H2O = Sites treated with water. Panel B: Average number of *P. gigantea* colonies per plate across all sites for each month. Error bars display standard error.

CHAPTER 3

REPRODUCTIVE BIOLOGY OF *PHLEBIOPSIS GIGANTEA*

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Abstract

Aspects of the reproductive biology of *Phlebiopsis gigantea* remain unclear. European isolates of this basidiomycete have been described as heterothallic, but they also have been reported to readily produce basidiospores from homokaryotic cultures. To determine if North American isolates of the species can undergo sexual reproduction after being initiated from single basidiospores, we examined the reproductive structures made by 85 single basidiospore cultures. Sixty-nine of these cultures displayed evidence of sexual reproduction in the form of fruiting body and basidiospore production. Microscopic examination indicated that these fruiting bodies contained cystidia and basidia, thus verifying the occurrence of homothallic sexual reproduction. In an effort to understand the mechanism underlying this apparent homothallism, the nuclear condition of the basidiospores was assessed via staining. The majority of them (61.6%) contained two nuclei. Overall these results indicate that *P. gigantea* from North America does not consistently behave in a heterothallic manner, and that it may be pseudo-homothallic.

Introduction

Phlebiopsis gigantea is well studied in its role as a biocontrol agent for Heterobasidion Root Disease. Its general morphology has been described in large monographs (Stalpers 1978; Erikkson *et al*. 1981), but direct investigation of its reproductive biology is limited, and few to no micrographs of its sexual structures have been published.

Korhonen and Kauppila (1988) described European isolates of *P. gigantea* as heterothallic, a paradigm that has been accepted ever since. However, they also reported that homokaryotic cultures of *P. gigantea* fruited readily, which is not consistent with designation of the species as heterokaryotic.

We present morphological evidence of sexual reproduction by *P. gigantea* isolates from North America: i.e. the formation of fruiting bodies containing cystidia, and the development of basidia bearing basidiospores. We also determined that North American *P. gigantea* isolates frequently fruit in cultures arising from single basidiospores, and we investigated a potential explanation for *P. gigantea*'s apparent homothallic fruiting by examining the nuclear condition of its basidiospores.

Methods

Fungal Isolates

Aerial spore capture was used to collect *P. gigantea* isolates 11061-1 and 11058-1-1 in Macon County, Alabama, and isolate 31044b-10 in Talbot Country, Georgia. All three were confirmed as *P. gigantea* isolates by sequencing their ribosomal RNA internal transcribed spacer region (Higgins and Covert, unpublished data). *P. gigantea* strain CR-5-4 was started from a single basidiospore, which was collected from the lid of a *P. gigantea* 11061-1 culture. All isolates were cultured on 2% Bacto malt extract agar, wrapped in parafilm, and incubated upside down at room temperature (23ºC).

Visualizing Reproductive Structures

The reproductive structures made by *P. gigantea* cultures were investigated using both scanning electron microscopy (SEM) and light microscopy. Cultures were prepared for SEM examination via growth on nylon membranes. One cm x one cm pieces of nylon membrane (ICN Biotrans) were placed around the edges of agar plates prior to inoculation with an agar plug. Four weeks after inoculation, nylon squares containing suspected fruiting bodies were collected. Upon removal from the agar, the nylon squares were stored overnight at 4ºC in a 1:1 mixture of 5% glutaraldehyde and 0.1M potassium phosphate buffer. The samples were then washed three times for 15 minutes, in 0.05M potassium phosphate buffer. Samples were fixed for two hours at 4ºC with a 1:1 mixture of osmium tetroxide and 0.1 potassium phosphate buffer. Next the samples were rinsed three times for 15 minutes with double-distilled water. Samples were then dehydrated, first through a series of ethanol exchanges (25% ethanol, 50%, 75%, 95%, 100%, and 100% again), each of which lasted 15 minutes, and then with a critical point dryer. Fixed samples were mounted on stubs, coated with gold using a sputter coater, and visualized using a JSM 5800 scanning electron microscope.

Basidiospores were collected from the lids of fruiting *P. gigantea* cultures, and arthroconidia were collected on agar plugs. Both types of samples were visualized with a Zeiss1450EP variable pressure SEM. These samples were prepared as outlined previously, with the following exceptions: 2% glutaraldehyde was used in place of 5%, initial fixation and secondary fixation only lasted 30-60 minutes instead of overnight and two hours, respectively, and all washes, rinses, and dehydration steps were done for 10 minutes instead of 15.

Fresh, unfixed fruiting bodies were collected on agar plugs from the edges of Petri dishes. These plugs were angled on the stage of an Olympus BH-2 microscope so it was possible to get a side view of the fruiting area.

A Zeiss Axioplan light microscope, equipped with a Zeiss AxioCam MRc camera, was used to examine arthroconidia collected from the surface of agar cultures, and basidiospores

collected from the lids of agar cultures. To visualize nuclei, the basidiospores were placed in a fixative (3.7% formaldehyde, 0.2% TritonX-100, 50mM phosphate buffer, pH 7) for 30 minutes, rinsed with water twice, stained with Hoechst solution (1 mg/ml Hoechst 33258 [Sigma] in water) for five minutes, and then rinsed with water twice before being placed on slides. The nuclei were viewed under ultraviolet (UV) light. At least 300 basidiospores from each culture were surveyed, and the number of visible nuclei in each was recorded.

Production of Cultures Derived From Single Basidiospores

Basidiospores on the lids of *P. gigantea* agar cultures were collected in water and scanned using an Olympus CH-2 light microscope to ensure that no asexual arthroconidia were present among them. These spore suspensions were diluted to various concentrations (0.02, 0.05, 0.1, 0.5, and 1.0 spores/µl), and then 1ml from each dilution was spread on an agar plate. Single colonies were isolated from these plates and moved to new plates, resulting in cultures that were thought to be the products of single basidiospores. Basidiospores were then collected from the lids of three of these progeny and the entire process was repeated, resulting in 27 second generation cultures thought to be the product of single basidiospores.

Because multiple basidiospores may stick together during plating we also isolated single basidiospores with a MARK I Micromanipulator (Lawrence Precision Machine), attached to an Olympus CH-2 microscope. This approach allowed us to confirm visually that each culture originated from a single basidiospore. Basidiospores were collected in water from the lids of 4 week old *P. gigantea* 11061-1 and *P. gigantea* 31044b-10 cultures. They were then spread on glass slides that were coated with a thin film of agar. The micromanipulator was used to move individual spores into pre-marked areas on the glass slides, which were devoid of other spores. Once seven spores were isolated this way the slide was removed from the microscope and the pre-marked areas were cut from the slide and transferred to fresh agar plates.

Results

All three *P. gigantea* field isolates produced abundant arthrocondia (Figure 3.1) after only five to seven days of growth. Agar cultures that were three to four weeks old, however, sometimes produced dense white hyphal mats on the outer perimeter of the medium. If these cultures were incubated upside down, a white haze appeared on the lid of the petri dish opposite the white hyphal mats. Microscopic examination of this white material collected in water revealed spores, whose morphology was distinct from that of the arthroconidia, and consistent with a previous report of *P. gigantea* basidiospore morphology (Erikkson *et al*.1981). Further examination of spores collected this way from cultures of *P. gigantea* isolates 11061-1 and 31044b-10 was conducted using light microscopy, and the spores consistently fit the previously described basidiospore morphology (Figure 3.2a,c). Scanning electron microscopy revealed the presence of spores with that morphology in a sample of fruiting body taken from a *P. gigantea* 11061-1 culture (Figure 3.3). SEM was also used to visualize spores collected from the lid of *P. gigantea* CR-5-4 (Figure 3.4). While some of these spores matched the expected basidiospore morphology, others varied in appearance, and were echinulated or covered with mucilage.

Cultures derived from these single basidiospore frequently fruited, i.e. they produced dense hyphal mats on the culture periphery after three to four weeks of incubation and they shed spores with the characteristic morphology of *P. gigantea* basidiospores on the lids of their Petri dishes. Fruiting was seen in 32 of the 40 progeny collected from field isolates 11061-1 and 11058-1-1 via dilution plating. The same method was used to create a second generation of 27 progeny from the 32 first generation progeny. All 27 of the second generation progeny fruited. Single basidiospore progeny were also generated from 11061-1 and 31044b-10 using a micromanipulator to guarantee that single basidiospores were being isolated. Eighteen progeny were generated in this way and at the time of this writing those cultures are six weeks old. Ten of them are fruiting.

The high frequency of apparent homothallism seen in cultures derived from single basidiospores was inconsistent with the previous description of *P. gigantea* as a heterothallic species. In order to gather more evidence regarding sexual reproduction that was occurring in these cultures, fruiting areas were studied microscopically for the presence of other sexual structures. Cystidia were readily apparent in fruiting bodies from all three field isolates (Figures 3.5 & 3.6). Fresh, unfixed fruiting bodies were examined for the presence of basidia bearing basidiopores. Intact basidia were found on samples taken from a *P. gigantea* 31044b-10 fruiting body (Figure 3.7).

To get a clearer picture of the mechanism behind *P. gigantea*'s apparent homothallic behavior, nuclear staining was used to reveal the nuclear condition of the basidiospores. The majority of the basidiospores (62%) had two nuclei, but there were also some basidiospores that contained zero (14.5%), one (22.8%), or three or more (1.1%) visible nuclei (Table 3.1, Figure 3.2b, d).

Discussion

With the visual confirmation of the presence of basidiospores, cystidia, and basidia combined, it is evident that what was suspected to be sexual fruiting in our *P. gigantea* cultures was indeed sexual reproduction. When considered along with the previously reported incidence of sexual reproduction in presumed homokaryotic cultures (Korhonen and Kaupilla 1988), it is apparent that *P. gigantea* does not fit the purely heterothallic paradigm that has been attributed to it. This discrepancy has also been noted in other heterothallic fungi when pure homokaryon cultures were isolated in laboratory conditions (Stahl and Esser 1976), raising the possibility that this behavior is a byproduct of those conditions and rarely seen in nature as part of the natural life cycle. This is consistent with previous reports that all naturally occurring *P. gigantea* fruiting bodies are heterokaryotic (Grillo *et al*. 2005; Korhonen and Kaupilla 1988).

Even if we accept that *P. gigantea* behaves like a heterothallic fungus in the wild, the issue of it fruiting while in homokaryon form remains unresolved. Our observation of

basidiospores containing two nuclei supports the hypothesis that *P. gigantea* usually behaves like a pseudo-homothallic fungus. Pseudo-homothallic fungi package two nuclei in each basidiospore, thus allowing colonies that arise from single basidiospores to undergo sexual reproduction (Ullrich and Raper 1975). A more thorough survey of basidiospore nuclear condition could strongly support this hypothesis if it is found that the basidiospores consistently contain two nuclei.

Direct genetic analysis, comparing parental isolates to progeny generated from single basidiospores collected from those isolates, would be a powerful step forward in characterizing *P. gigantea*'s reproductive behavior. One possible approach would be to compare the single nucleotide polymorphisms in conserved regions of the parents to those of the sexual progeny. This would provide valuable insight as to the nuclear condition of the basidiospores and would indicate if *P. gigantea* is pseudo-homothallic or homothallic.

Acknowledgements

This work was supported by a grant from the U.S. Forest Service's Special Technology Development Program. We thank Rebecca Roulo for assistance in managing *P. gigantea* cultures and creating single basidiospore isolates, Dr. John P. Shields for assistance with the Zeiss1450EP variable pressure SEM, Beth Richardson and Mary Ard for assistance with the JSM 5800 scanning electron microscope, and Susan Hastings for assistance in staining and photographing *P. gigantea* basidiospores.

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Tables and Figures

Table 3.1: Nuclear Condition of Basidiospores

# Nuclei / Basidiospore	*P. gigantea* 11061-1[1]	*P. gigantea* 31044b-10[2]
0	85	52
1	151	59
2	392	188
3+	7	3

[1]Average of three replicates, each including 608 to 687 spores
[2]Single replicate, including 302 spores

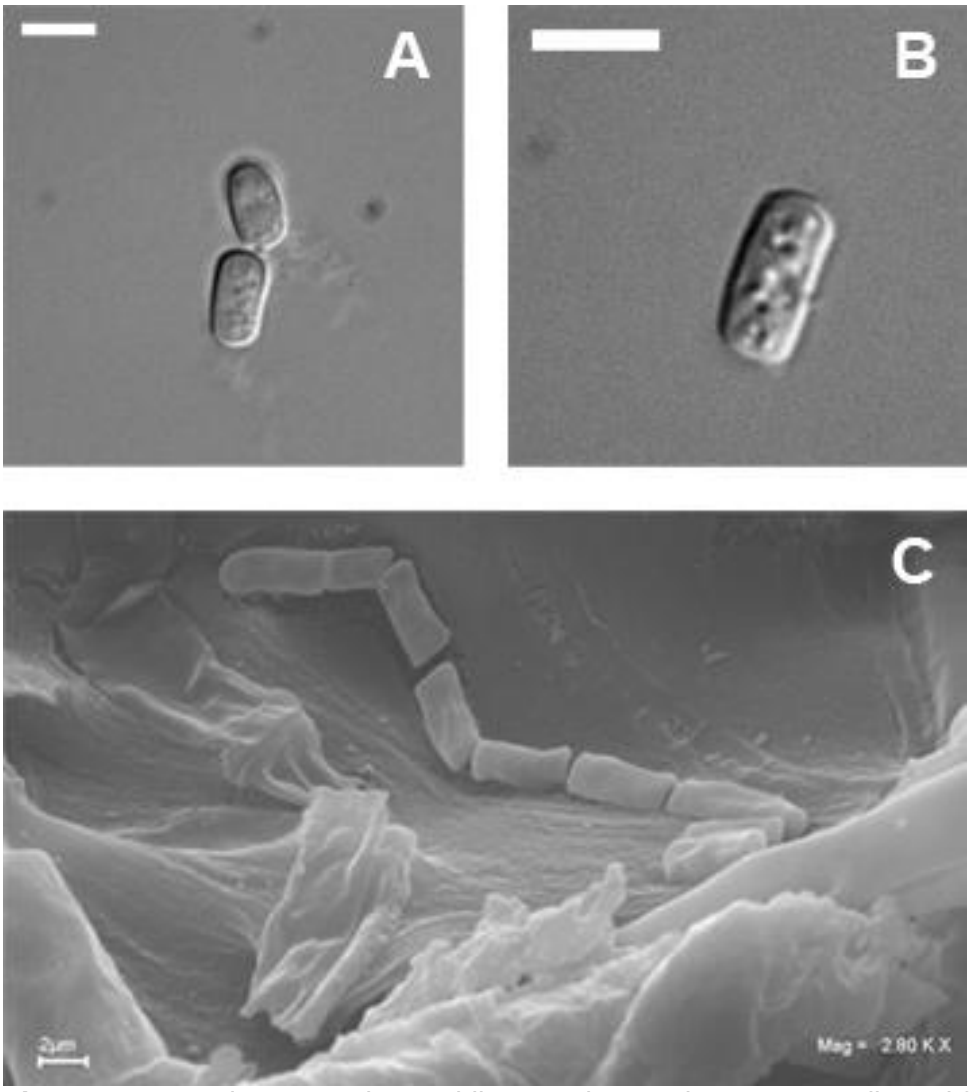

Figure 3.1 – *P. gigantea* arthroconidia. Panels A and B: Spores collected from a 7 week old *P. gigantea* 31044b-10 culture. Scale bars = 5 µm. Panel C: A *P. gigantea* 11058-1-1 hypha fragmented into arthroconidia. Scale bar = 2 µm.

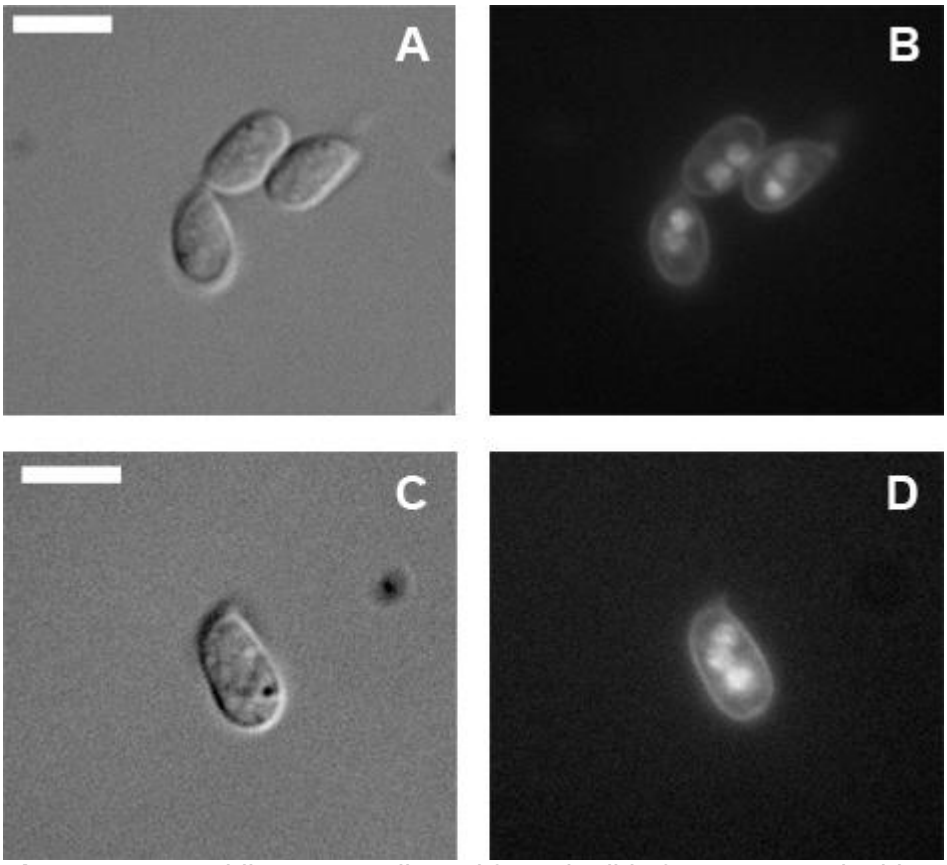

Figure 3.2 – Basidiospores collected from the lid of a seven week old *P. gigantea* 31044b-10. Visualized by light microscopy. Panels A and C: Viewed without UV. Panels B and D: Viewed with UV light. Scale bars display 5 µm.

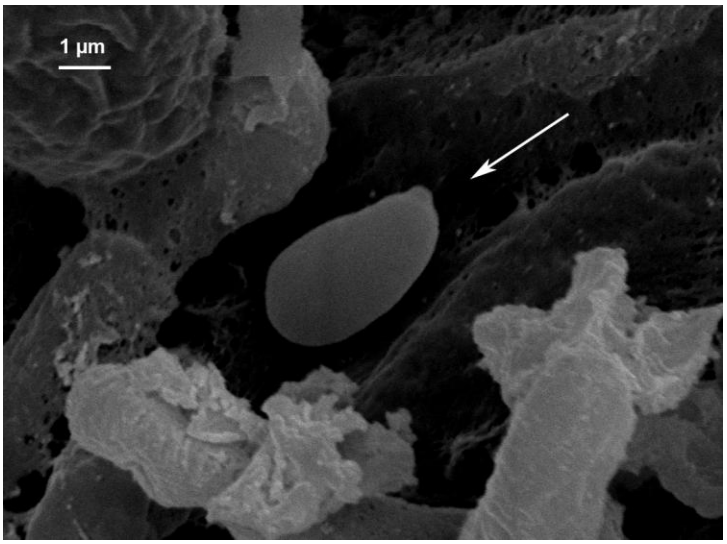

Figure 3.3 – Basidiospore from four week old *P. gigantea* 11061-1 fruiting body, collected on nylon membrane. Visualized by scanning electron microscopy.

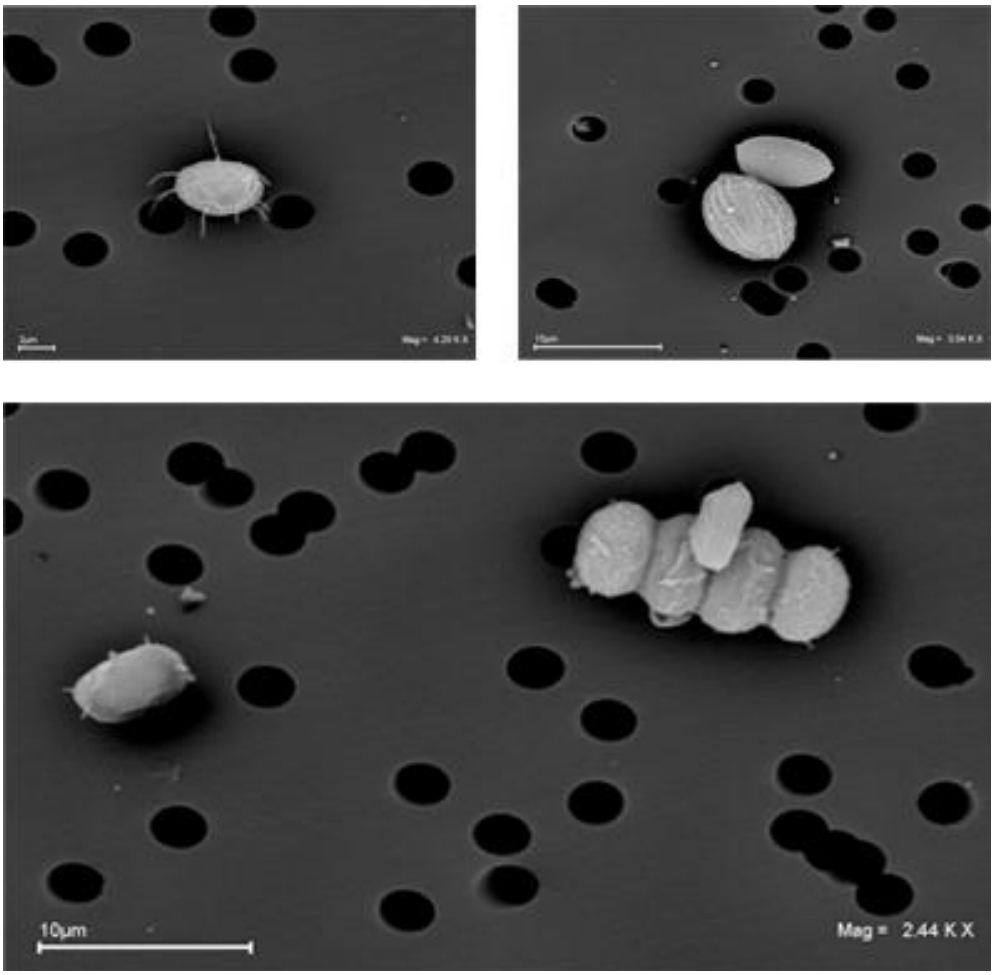

Figure 3.4 – Basidiospores collected from the lid of a six week old *P. gigantea* CR-5-4 culture. Visualized by scanning electron microscopy.

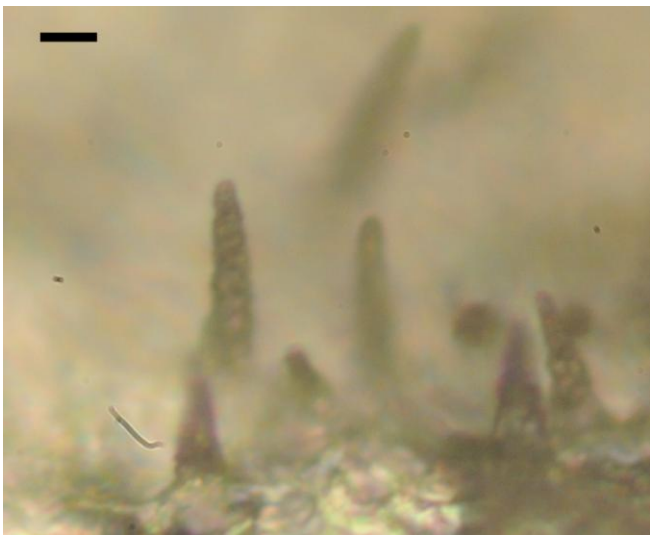

Figure 3.5 – Cystidia from a three week old *P. gigantea* 31044b-10 fruiting body, collected on an agar plug. Visualized by light microscopy. Scale bar displays 10 µm.

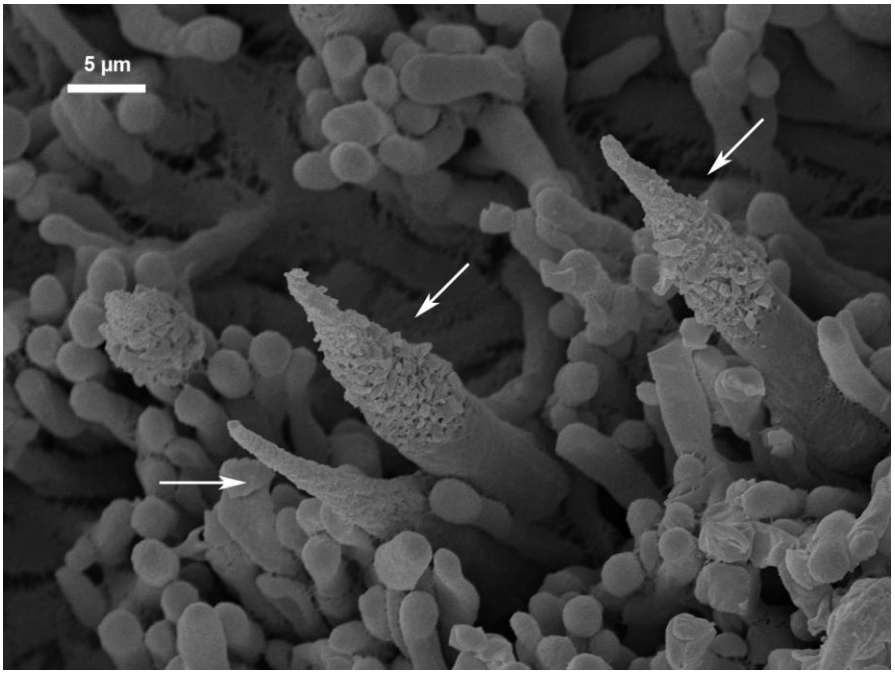

Figure 3.6 – Cystidia from a four week old *P. gigantea* 11061-1 fruiting body, collected on nylon membrane. Visualized by scanning electron microscopy.

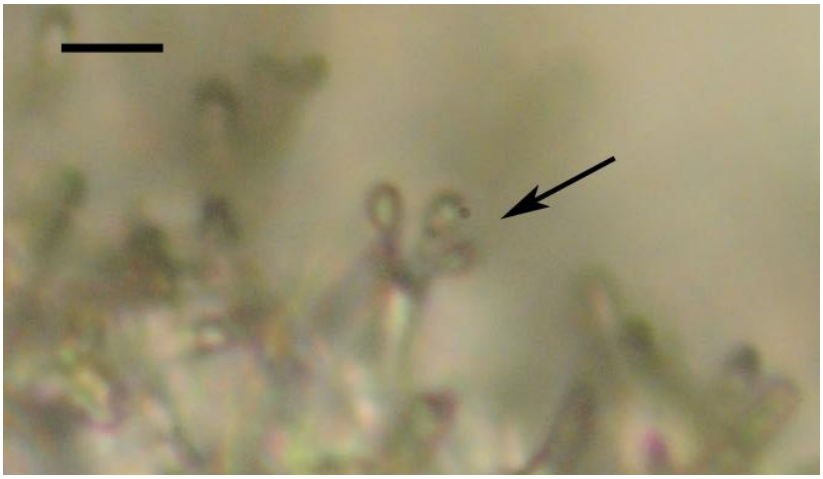

Figure 3.7 – Basidium bearing three basidiospores. From a 3 week old *P. gigantea* 31044b-10 fruiting body, collected on an agar plug. Visualized by light microscopy. Scale bar displays 10 µm.

CHAPTER 4

CONCLUSION

These investigations of *Phlebiopsis gigantea*'s biocontrol efficacy, and the effect of its release on its prevalence in the surrounding environment, combine to suggest that *P. gigantea* has the potential to be an effective and environmentally friendly method of controlling Heterobasidion Root Disease in the southeastern U.S. The evidence of *P. gigantea* maintaining its biocontrol efficacy after a three day delay in application is also a promising result, one that could be key in the development of *P. gigantea* biocontrol products developed for use in the U.S. (James Johnson, Georgia Forestry Commission, personal communication).

Despite these positive findings, some significant barriers still need to be overcome before use of *P. gigantea* as a biocontrol agent for Heterobasidion Root Disease in the U.S. can hope to match its widespread adoption in Europe (Pratt *et al*. 2000). It is especially important that the findings of this study be tested in an operational setting, removing some of the artificial conditions that could have skewed our results. Elevated stumps are one such condition; they were necessary for sampling purposes in this study, but being elevated instead of flush with the ground could have altered their moisture and temperature levels, thus influencing the development of *P. gigantea* and *Heterobasidion irregulare* in the stumps. Another big difference between our study and an operational thinning is that we applied two weeks worth of *H. irregulare* to stumps immediately after they were cut, as opposed to the gradual deposition of spores that would occur naturally. Our method of inoculation would seem to present more of a challenge to *P. gigantea's* control abilities than the *P. gigantea* would face in an operational setting, and as a result we may be underestimating its control efficacy, particularly that of the delayed treatments.

It is also vital that foresters in the southeastern U.S. are made aware of the need to treat for Heterobasidion Root Disease. Managed pine forests are a major commercial crop in the region, and many of the forests are in areas that are considered high risk for *H. irregulare* infection (Pratt *et al.* 2011). But despite this risk, willingness to invest in treatments for Heterobasidion Root Disease remains low, probably because the damage caused by the disease is mistakenly attributed to bark beetles (Pratt *et al*. 2011). Increasing forester awareness of the damages caused by Heterobasidion Root Disease, and the future threat it poses, is crucial in the development and survival of a *P. gigantea* biocontrol product.

Findings from our investigation of *P. gigantea*'s reproductive behavior shine a light on the inconsistencies between the heterothallic paradigm attributed to it and the actual reproductive behavior it displays in culture. Fruiting and production of basidiospores were regular occurrences in our cultures that were generated from single sexual basidiospores and presumed to be homokaryotic. That leaves us to question what is going on at the nuclear level that allows for fruiting in these cultures. We took an initial step in exploring that question by examining the nuclear condition of basidiospores, finding that the majority of basidiospores surveyed contained two nuclei. This result opens up the possibility that *P. gigantea* is pseudo-homothallic, packaging two compatible nuclei in each basidiospore so that it constantly maintains the heterokaryotic state.

To gain a deeper understanding of this system it seems likely that a genotypic approach would be best. By comparing DNA sequence information from parental isolates and their single basidiospore progeny it would be possible to determine if the progeny were homokaryons or heterokaryons. Molecular markers, such as single nucleotide polymorphisms, or previously identified microsatellites (Liu *et al*. 2009), could be targeted and amplified for this purpose. If DNA sequences collected from single basidiospore progeny were variable it would suggest that the progeny were heterokaryons, supporting the idea that *P. gigantea* is pseudo-homothallic.

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