The effect of *Sirex* spp. woodwasps and their fungal associates on Alabama forest health

by

Andrea B. C. Wahl

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Approved by

Dr. Lori G. Eckhardt, Professor of Forestry and Wildlife Sciences
Dr. Bernard Slippers, Professor of Genetics
Dr. Kathy Lawrence, Professor of Plant Pathology
Dr. Andrew Liebhold, Research Entomologist

Abstract

Forestry is an industry in the Southeastern United States that provides products and jobs to people throughout the region. These forests, while are mostly well managed for various outcomes, are susceptible to a variety of pests and pathogens that have the potential to do great ecological and economic damage.

One pest species that has been studied extensively in the Southern Hemisphere is *Sirex noctilio*. This is a species of woodwasp, native to Eurasia, which has become invasive in many parts of the world. This insect, along with its mutualistic fungal pathogen, *Amylostereum areolatum*, is a cause of concern because they have the potential to attack healthy pine stands, ending in the mortality of vast amounts of trees.

In the southeast, no *S. noctilio* have been detected. A survey was undertaken to gain a better understanding as to which species of woodwasps are found in Alabama forests. Traps were deployed in three localities throughout the state, and were visited year-round for at least one year. Woodwasp and other insect species of concern were morphologically identified and cataloged from all sites.

Woodwasp species were dissected and sampled for nematode infestation and for associated fungal species. These samples (wasp, nematode, and fungi) were all molecularly analyzed, and then phylogenetic relationships were determined. A novel relationship between *S. nigricornis* and *Deladenus siricidicola* was observed.

Isolates of *Amylostereum* from Alabama and other localities around the world were subjected to chemicals emitted by the pine substrates in which they grow. Certain defense chemicals were shown to significantly reduce growth rates of *Amylostereum* spp. hyphae compared to a dH_2O control treatment.

Amylostereum spp. tended to be poor competitors to *Leptographium* spp. fungi when plated in direct contact with each other. One isolate of *A. chailletii*, 15B from Alabama, outcompeted both *L. terebrantis* and *L. procerum.* A VCG study also was performed, where isolates of the same species of *Amylostereum* were plated together to determine if isolates were clonal. Two distinct vegetative compatibility groupings of *A. chailletii* were determined from *S. nigricornis* captured in Alabama.

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Introduction and Literature Review

1.1. Siricid Distribution

1.1.1. Siricids Worldwide

While there are 100 species within the family Siricidae worldwide, their native ranges are limited to the northern hemisphere. (Schiff et al., 2006). The accidental movements of species into forests in the southern hemisphere have had devastating effects (Hurley et al., 2007). When *Sirex noctilio* F. was introduced into Australia, New Zealand, and Tasmania in the early 1900s, thousands of hectares of planted pine were lost (Bain, 2005; Talbot, 1977). In a three year span in the States of South Australia and Victoria, 4.8 million trees were killed during a *S. noctilio* outbreak in Australia (Rawlings, 1955). Slippers et al. (2003) showed that a single introduction of *S. noctilio* was made into the Southern hemisphere and then later spread to the mentioned areas. Pine species planted for industrial forestry purposes are primarily affected including *Pinus radiata* D.Don (Burnip et al., 2010). *Sirex noctilio* is endemic in Europe and North Africa, where it is a secondary pest (Spradberry and Kirk, 1978).

1.1.2. Siricids in North America

In North America, 23 species of siricid woodwasps have been recorded, including introduced species (Schiff et al., 2012). Emergence of adults, mating, and oviposition occurs in

the late fall to early winter months of October to November in the southeast (Haavik et al., 2014). Emergence occurs slightly earlier in the year, July to September, in the north (Zylastra et al., 2010). Native species of siricids are found throughout the southeastern United States, but are only attracted to dead and dying stands of trees. Three native species are found in the eastern United States: *Sirex nigricornis* F., *S. cyaneus* F., and *S. nitidus* Harris (Schiff et al., 2012). These native insects are secondary pests, only entering trees that are giving off semiochemicals (kairomones) that indicate the tree is damaged or stressed. Native populations of siricids tend to cause little if any real economic or ecological threats to forests in which they are found. Unfortunately, the invasive *S. noctilio* has been documented as a primary pest in areas outside of its native range, resulting in these wasps regularly attacking, and many times eventually killing previously healthy trees (Crook et al., 2012).

In 2002, a *S. noctilio* adult female was found in a warehouse in Bloomington, Indiana (Haugen and Hoebeke et al., 2005). In 2004, *S. noctilio* was captured in Oswego County, New York, and has since spread to surrounding states and Canadian provinces (Haugen and Hoebeke et al., 2005; Ayres et al., 2009). It was thought that the siricids were brought in via shipping material, as sightings of siricid larvae in imported packing material have been reported regularly (Ciesla, 2003). It is likely that the invasion of *S. noctilio* will continue to spread throughout the eastern United States because there is an abundance of host material (Carnegie et al., 2006).

Sirex nigricornis, a native species of woodwasp is found throughout the eastern United States. Its range extends from Quebec, south to Florida, and west to Texas north up to Saskatchewan (Schiff et al., 2006). Previous studies suggest that this species is more abundant in the southeast (Keeler, 2012). However, these observations could be artifacts of a bias in

collecting methods, where most sampling in the southeast has been conducted on thinned or cut land, while studies in the northeast have been conducted in unaltered forests (Haavik et al., 2014). Another species previously identified as native to the eastern United States, *S. edwardsii* Brullé has recently been reclassified as a color form synonym of *S. nigricornis* (Goulet, 2012).

1.1.3. Siricids in Alabama

Previous surveys for native siricid species have been conducted in the southeastern Unites States, but no formal trapping studies have been conducted in the State of Alabama. Other Siricid species reported from the southeastern United States (Louisiana) include *S. nigricornis* (Fig. 1.1), *Eriotremex formosanus* Matsumura, *Tremex Columba* L., and *Urocerus cressoni* Norton (Johnson et al. 2013). It can be hypothesized that these same species, along with *S. nigricornis*, would be present in Alabama.

Since *S. noctilio* is known to colonize the same host tree species as utilized by native siricids in the United States, it can be hypothesized that a certain amount of competition may occur for resources between the species. There also is evidence of indirect competition between populations of native and invasive siricids in the northeast United States. The native *S. nigricornis* has been documented to carry a strain of *Amylostereum areolatum* (Chaillet ex Fr.) Boidin fungus commonly associated with *S. noctilio* (Hajek et al., 2013). Different Sirex species also are known to be parasitized by the same nematode species.

Figure. 1.1. Pinned female *S. nigricornis* trapped in Mary Olive Thomas Demonstration Forest in Auburn, Alabama.

It is possible that *S. noctilio* may enter the southeastern United States via Alabama since the port of Mobile is a major port in the region, and many products are imported with either wood packing material, or are wood products themselves. In 2013 alone, 25.3 million tons of material were moved through this port (Alabama State Port Authority, 2015). With this amount of movement of goods, it is likely that a wood pest could be imported without detection.

1.2. Ecology of Siricids

1.2.1. Lifecycle

Female wasps emerging from trees are photopositive, exiting to the surface, where they begin flight searching for a male to mate with (Madden, 1974). Mating tends to occur in the upper canopy of trees (Böröczky et al., 2009). Females initiate mating by walking up to a male, to which males respond by tapping the female's body with their antennae and front legs, perhaps sensing a pheromone produced by females (Morgan and Stewart, 1966). Female wasps are known to attract males with a sex pheromone, but a long range pheromone has not been identified (Böröczky et al., 2009). Simpson (1976) suggests that siricids find mates when both

male and female wasps are both attracted to host attractants (kairomones) emitted by host trees.

Females begin oviposition after a flight period, irrespective of whether they have mated. Reflecting the haplodiploid sex determination system in the Hymenoptera, unfertilized eggs laid by females develop exclusively into males (Myers et al., 2014). Because of this and perhaps other factors, ratios of male to female wasps emerging may be as high as ten to one (Morgan, 1968).

When females land on a tree prior to oviposition, they first make an exploratory drill into the trunk of a tree. Following this first drill, females randomly drill into the host tree, depositing a mixture of eggs, venom, and *Amylostereum* spores. The female continues to drill until no more egg mixture remains in her abdomen, so her last drill is normally an empty hole in the tree (Madden, 1974). *Sirex noctilio* females typically have around 400 eggs in their ovaries (Chrystal, 1928). This number differs slightly by species.

Figure 1.2. Emergence hole from mature *S. noctilio* in pine tree from Indiana County, Pennsylvania.

The typical lifecycle of siricid wasps takes one year to complete, but may take up to three (Ryan and Hurley, 2014). Siricids overwinter as mid- to late-instar larvae (Myers et al., 2014). Once in the larval stage, siricids typically go through twelve larval instars before pupation, but

this period may be cut short if a food supply is depleted or the substrate conditions are not favorable (Taylor, 1981). Larvae are completely developed before leaving the egg, and chew their way out of their protective covering with developed mandibles (Chrystal, 1928). Larvae feed by chewing galleries deep into the wood of trees, where they eventually pupate (Madden and Coutts, 1979). When exiting the tree, the adult wasp tunnels out of the heartwood leaving a trail of tightly packed frass (Fig. 1.2) (Chrystal, 1928). Adult females have an organ in their abdomens that acts as a reservoir for a phytotoxic venom noctilisin that aids the *Amylostereum* fungus in degrading the tree for larvae to feed upon (Bordeaux et al., 2014).

It appears that the size of the adult female plays an important role in its egg laying behavior. Unmated females have been recorded as being less active in oviposition behavior as mated females of an equivalent size. Also, smaller wasps that were mated lived shorter lives and were ovipositted less than larger, mated females (Madden, 1974). Early pupation, for various reasons, also causes a decrease in adult body size (Yousuf et al., 2014). The fungal growth rate affects the size of the emerging adult, with larger adults emerging from sites with greater fungal growth (Madden and Coutts, 1979).

Siricids are generally documented to be attracted to stressed trees (Madden, 1968a). Examples of abiotic causes of tree stress are fire, flood and drought, as well as other environmental conditions (Madden, 1988).

1.2.2. Attraction to volatized chemicals

Monoterpenes are chemicals produced by trees. Production of these compounds tend to be induced when trees are under some sort of stressful situation, whether it be drought, insect infestation, fungal infection, or other problems. Various types of tree pests are known to be

attracted to these chemicals, such as α-pinene and β-pinene and these compounds are known attractants of *Sirex* species (Bashford, 2008). Johnson et al., (2013) reported that *S. nigricornis* in the southeast United States are responsive to these kairomones released by *Pinus taeda* L. An interesting aspect of this interaction is that these chemicals that attract the pests also are hypothesized to be toxic to the fungi associated with these pests (Eckhardt et al., 2009). Other secondary metabolites produced by *Pinus* species such as camphene, myrcene, limonene and β-Phellandrene also are known to be toxic to the associated fungus of wood infesting insects (Eckhardt et al., 2009).

1.2.3. Competition between tree pests

It may be hypothesized that siricids compete with other wood boring and bark pests for resources of declining trees. This would explain why at least some siricid species are attracted to pheromones released by such competitors (Miller and Asaro, 2005). Miller et al. (2005) determined that siricids are most likely to compete with *Ips* engraver beetles (*Ips avulsus* Eichhoff, *I. grandicollis* Eichhoff, *I. calligraphus* Germar) for declining tree material. In Australia, Youseuf et al. (2014) correlated the presence of *I. grandicollis* with smaller *Sirex* brood, higher larval mortality, reduced *Amylostereum* growth, and ineffective parasitism by *D. siricidicola*. This disruption of Siricid development is most likely due to the *Ips* associated fungus, *Ophiostoma ips* (Rumb.), outcompeting *A. areolatum* and causing the host substrate to dry quickly resulting in xylem that is not suitable for siricid development.

In North America, approximately 260 insect species are known to colonize the boles of *Pinus* species (de Groot and Turgeon, 1998), creating a great deal of competition for the niche of siricids. Some beetles that may most directly compete with siricids would be *I. grandicollis,*

Pissodes nemorensis Germar, and *Gnathotrichus materiarius* Fitch (Ryan et al. 2012a).

Even if the siricids oviposit in the same region of the bole, direct encounters with beetles are unlikely, because Curculionidae typically feed in the phloem, away from wasp larvae (Ryan et al., 2012a). As mentioned above, the more likely competition will be indirect. Competition has been documented between the *Sirex- Amylostereum* association and other fungi commonly found in pines of the *Ophiostoma* H.&. P. Sydow and *Trichoderma* Persoon genera. These two mentioned genera have been shown to inhibit *Sirex* oviposition at localized sites on a tree (Spradberry and Kirk, 1978). *Amylostereum areolatum* has been shown to be a weaker competitor than *Ophiostoma* species (Hurley et al., 2012). Interestingly, the presence of *Armillariella* fungus does not deter siricid oviposition or development (Hanson, 1939).

1.3. Mutualistic Associations with *Sirex*

Amylostereum is a genus of mutualistic fungi associated with siricids and is a Basidiomycete, a common grouping of fungi infecting trees. Three known species in this genus that are associated with woodwasps are *A. chailletii* Pers. Boidin, *A. areolatum*, and *A. laevigatum* Fr. Boidin (Gaut, 1970). These species are morphologically similar, especially *A. areolatum* and *A. chailletii*, which are commonly identified using polymerase chain reaction (Slippers et al., 2003). Once thought to be species specific, Wooding et al. (2013) discussed that siricid species are able to capture and thereby switch the species of *Amylostereum* they carry. This may be dependent on the geographical region the siricids occur in, since certain strains of *Amylostereum* are more dominant in certain regions of the world (Slippers et al., 2015).

Characteristics of *Amylostereum* spp. include smooth amyloid basidiospores, hyaline

encrusted cystidia, and resupinate fruiting bodies (Boidin, 1958). Fungi in this genus have a tetrapolar nuclear state when they reproduce, and are heterothallic (Boidin and Lanquetin, 1984). In nature, many cultures isolated have low genetic diversity as a result of the wasps spreading clones of a few select isolates (Vasiliauskas et al., 1998). A single clone of *A. areolatum* may be the widest spread clone of a fungus, having been documented on three continents (Slippers et al., 2015).

Sirex have an obligatory relationship with this fungus, as it provides food for larval wasps, a critical necessity in the lifecycle of this pest (Slippers et al., 2006). The wasps are, in turn, also critical for the proliferation of the fungus, providing a means of dispersal. The asexual *Amylostereum* oidia spores are spread in organs called mycangia that are found internally at the base of the ovipositor (Vasiliauskas et al., 1998; Edmonds et al., 2011). These glands have been well described, and were first identified for their structure in the late 1920s (Crystal, 1928). Studies by van der Nest et al. (2012) have shown that *A. areolatum* by itself is not a pathogen capable of causing tree mortality as is the case with other basidiomycetes.

This fungus is effective, like other pathogens, at restricting water flow in the vascular system of the infected tree by causing tissues to degrade into a white rot (van der Nest et al., 2012). White rots are typically effective at decomposition of both lignin and cellulose (Campbell, 1932). Tize (1970) suggests that the *Amylostereum* metabolism of wood substrate is anaerobic. While tree tissue is drying out and beginning to decompose, larvae feed on this decaying material (Edmonds et al., 2011). Kukor and Martin (1983a) suggested that *Amylostereum* species produce cellulase that enables cellulose to be broken down and more easily consumed by the siricid larvae. *Streptomyces* bacteria were shown to play a role in

breaking down cellulose when associated with *Amylostereum* (Adams et al., 2011).

Amylostereum species are presumed to compete with other fungi for a substrate to grow within the tree. *Ophiostoma* species of fungi, commonly known as blue stain, are vectored by bark beetles. These fungal species kill resin canal cells and ray parenchyma (Edmonds et al., 2011), and its growth may be inhibited by the *Amylostereum* fungi colonizing and drying out the wood it grows on (Coutts and Dolezal, 1965).

1.4. Control Agents

1.4.1. Parasitic nematodes

Since siricids live inside wood and the adult stage of life is ephemeral, a nonconventional pest management plan had to be developed to control this insect (Spradberry and Kirk, 1978). *Deladenus siricidicola* is a species of nematode that has been reared and intentionally used as a biological control agent of *Sirex noctilio* in the southern hemisphere (Hurley et al., 2007). These nematodes are effective in controlling *S. noctilio* populations because one stage of their life cycle infects the eggs inside a female's body before oviposition (Fig. 1.3), rendering those eggs sterile (Morris et al., 2013). Infected male siricids are a dead end host for *Deladenus* species, as they cannot act as a vector to a new host tree (Bedding, 2009). Different strains of *D. siricidicola* affect various species of siricids. Some strains have proven to be more virulent than others, and are currently being tested for use as a biological agent that may be applied to infected stands to control outbreak *S. noctilio* populations (Slippers et al., 2012). Trees infested with *S. noctilio* larvae are inoculated with *D. siricidicola* adult females, that then feed on the fungus and lay their eggs in the siricid larvae (Yousuf et al., 2014).

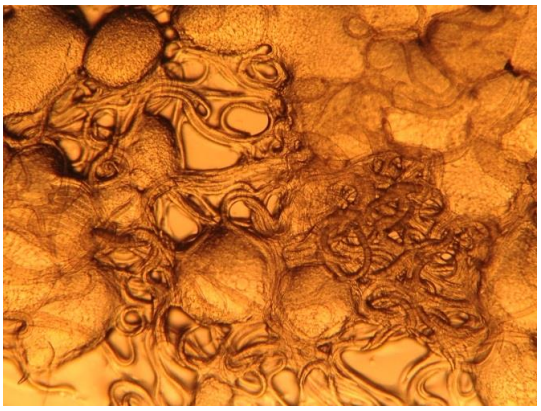

Figure 1.3. *D.siricidicola* nematodes infesting eggs of mature *S. noctilio* captured in Pretoria, South Africa.

Two stages of this nematode have been found, one parasitic phase as previously described, and another less damaging phase that feeds on the symbiotic *A. areolatum* fungus associated with the woodwasps (van der Nest et al., 2012). This free living, mycetophagous stage reproduces outside of the wasp's body in the tracheids of the host tree (Morris et al., 2013), while the entomophagous phase reproduces inside the body of wasp larvae (Slippers et al., 2012). Both the mycetophagous and entomophagous stages of these nematodes mate via amphimixis (Morris et al., 2013). The dicyclic lifecycle exhibited by these species is beneficial because the nematodes are able to live off of the fungus that lives within the host pine tree, and then the nematodes are able to switch over to living inside the siricid wasp in order to disperse to a new host substrate (Morris et al., 2013).

Other species of *Deladenus* nematodes have been reported to colonize siricids. *Deladenus proximus* and *D. wilsonii* are associated with *S. nigricornis* and its associated fungi, *A. chailletti* (Morris et al., 2014; Dodds and de Groot et al., 2012). *Deladenus proximus* was first identified by Bedding and Akhurst (1978) parasitizing *S. nigricornis* in South Carolina. In his 1974 paper, Bedding described seven species of *Deladenus* nematodes that parasitize insects. Morris et al. (2013) suggests that only three species of *Deladenus* occur in the United States: *D. proximus* Bedding, *D. siricidicola*, and *D. canii* Bedding.

Four species of nematodes, including the previously mentioned *D. siricidicola*, are known to parasitize woodwasps in North America. *Deladenus proximus* Bedding has been found to parasitize *S. nigricornis*., but are not always capable of sterilization (Morris et al. 2013). The third and fourth species, *D. canii and D. wilsoni* Bedding are mostly found to parasitize *S. cyaneus* F., whose range spans from Canada south to North Carolina, and across to New Mexico (Morris et al. 2013; Schiff et al. 2006).

1.4.2. Parasitic Wasps

Four species of parasitic wasps that are utilized as biological controls of *S. noctilio* are *Ibalia leucospoides* (Hochenw), *Rhyssa persuasoria* L., *Megarhyssa nortoni nortoni* (Cresson), and *Schlettererius cinctipes* (Cresson) (Taylor, 1976) . The first of these is known to attack eggs and first instar larvae, while the latter two parasitize later instars of larvae and pupae (Madden, 1968b). Because of its ability to tunnel through the bole of the tree to locate the siricid larvae and has a similar emergence period, *I. leucospoides* is the most efficient parasitoid (Ryan et al., 2012b). Unfortunately, to date, introductions of these species have not been able to provide sufficient control of introduced populations of *S. noctilio* (Yousuf et al., 2014).

1.4.3. Silvicultural Practices

Silvicultural practices aimed at reducing stress within a stand are known to lessen the consequences of a *S. noctilio* population establishment (Hurley et al., 2015). Thinning forests to reduce already infested host tree material and improve the quality of healthy trees in a stand would likely reduce the chance of *S. noctilio* from becoming established, as siricids are known to

colonize stressed trees (Dodds and de Groot, 2012). This thinning should take place in a season other than when siricids are emerging (Neumann et al., 1987). Fortunately for the southeastern United States, these silvicultural practices are already in place to manage for southern pine beetle (*Dendroctonus frontalis* Zimmerman), further reducing the chances of a serious outbreak of *S. noctilio* (Chase et al., 2014).

1.5. Forestry in the South

The commercial forest industry represents a major component of the economy in the Southeastern United States (Eckhardt et al., 2007). A large portion of trees planted for the commercial forest industry in the southeast are loblolly pines, (*Pinus taeda* L.) which is a native species. This species is dominant on 11.7 million hectares in this region (Baker and Langdon, 1990). This species also comprises almost half of the harvested pine in its current planted range, from New England to Florida, and west to Texas (Shultz, 1997). Common species found in the southeast in addition to *P. taeda* are *P. palustris* Mill. (Longleaf Pine), *P. echinata* Mill. (Shortleaf Pine), and *P. elliottii* Engelem. (Slash Pine), which are all susceptible host species for *S. noctilio* (Haugen and Hoebeke, 2005).

This vast industry creates jobs, provides wood and paper products, and also provides an ecosystem for native species of mammals, birds, reptiles, and other wildlife during the span of a rotation. One estimate showed an annual economic loss of $3.1 billion to invasive forest and wood boring insects in the United States (Pimentel et al., 2005). One of the principal causes of the extensive damages sometimes caused by exotic pests is that native tree species have not coevolved with the pests and are therefore lacking physiological defense mechanisms to protect

them from specific non-native pest attacks (Gandhi and Herms, 2009). The two main pathways that these pests and disease are able to enter new territory are through infested wood packing material and on live plants (Lovett et al., 2016).

1.6. Monitoring, detection, and isolation

1.6.1. Prevention of Movement

Recently, the United States Animal and Plant Health Inspection Service (APHIS) has issued a standard for incoming wood packing material known as the International Standards of Phytosanitary Measures Guidelines for Regulating Wood Packaging Material in International Trade (ISPM-15). These standards have been in place since 2002 (Haack et al., 2014). Although these policies are a move in the direction to lessen the chance of infested material arriving in American ports, many introductions are still happening because of the huge amount of resources it would take to make sure all imported material is free of contamination. Leung et al. (2014) predicts the cost of ISPM-15 will be $5 billion for implementation through the year 2050, and could reduce pest introductions by up to 52%.

1.6.2. Trapping techniques

Standard insect trapping procedure utilizes black intercept panel traps, baited with lures; flying insects attracted to lures strike panels and fall into a wet collection cup containing a mixture of propylene glycol and water (3:1 v/v) (Lindgren, 1983; Haavik et al., 2014). This method has proved to be most effective for sampling siricid populations in a forest setting (Hurley et al., 2015). Lindgren multiple funnel traps also may be used to collect dead specimens (Johnson et al., 2013). In their 2014 paper, Haavik et al. determined that multiple funnel or panel

traps are equally effective in trapping both *S. nigricornis* and *S. noctilio*.

Female siricids have been shown to be attracted to a kairomone mixture of 70:30 α-pinene: β-pinene (Simpson and McQuilkin, 1976). Previous findings by Johnson et al. (2013) suggest that verbonone, a pheromone given off by scolytids, may increase the effectiveness of these chemical traps. Studies have been conducted to test whether or not a male pheromone lure would be effective in trapping females, but so far kairomone lures are still more effective (Hurley et al., 2015). Mesh bags filled with freshly cut pine billets also have been shown to be an effective lure for siricids (Barnes et al., 2014; Chase et al., 2014).

When using traps baited with volatile kairomones, one may have issues in catching a specific species desired. Dodds and de Groot (2012) state that catching *S. noctilio* with this type of trap is less likely to be successful in North America than in the southern hemisphere. This is hypothesized to be because the density of *S. noctilio* populations would be lower, and native siricids would also be drawn to the lure. Creating an artificially stressed tree environment also is standard practice for collecting siricids. The herbicide dicambia may be injected, mechanically girdling of the bark, or felling a whole tree is effective in creating an environment suitable for siricid oviposition (Barnes et al., 2014).

1.6.3. Alternative methods

Methods such as analysis of satellite imagery also have been utilized to monitor the spread of *S. noctilio* populations by mapping the direction and spread of dying trees (Ismail and Mutanga, 2011). This sort of method is not feasible to use when infestations are of low number, or are being caused by more than one species of pest.

Chapter 2

Flight phenology of *Sirex nigricornis* (Hymenoptera: Siricidae) and other woodwasps in Alabama

2.1. Abstract

Woodwasps (Hymenoptera: Siricidae) are found throughout the world and play important roles in deciduous and coniferous forests ecosystems. Currently only native Siricidae are known to be present in the southeastern United States, but the Eurasian Siricid species, *Sirex noctilio*, is already established in the northeastern United States and anticipated to spread throughout the United States in the next few decades. In this study, a survey for *Sirex* species was conducted throughout Alabama. Traps were baited with a mixture of kairomones determined to specifically attract siricids. Traps were collected and all specimens were identified to species level. Bark and ambrosia beetles known to be species of concern for forest health also were identified and cataloged, as to determine how native *Sirex* species overlap temporally with beetle populations. Siricid species identifications were later confirmed with molecular techniques.

2.2. Introduction

Woodwasps (Hymenoptera: Siricidae) are an important component of forested ecosystems in the Northern Hemisphere by aiding in the decomposition of dead and dying trees. In North America, 23 species of siricid woodwasps have been recorded, including four introduced species

(Schiff et al., 2006). *Sirex noctilio* is one of these invasive species, native to Eurasia and northern Africa, first reported in North America in 2005 from Oswego County, New York, United States (Hoebeke et al., 2005). Subsequently, *S. noctilio* currently is found in the northern States of New York, Pennsylvania, Vermont, Connecticut, Michigan, and in the Canadian province of Ontario. *Sirex noctilio* has the potential to cause significant damage to pine forests of the southeastern United States; based on the economic impact it has historically had in commercial pine plantations in the Southern Hemisphere where it has previously invaded (Olatinwo et al., 2013; Hoebeke et al., 2005).

Sirex noctilio causes damage and the death of pines following oviposition within the xylem, where eggs develop into larvae. During oviposition a symbiotic fungus, *Amylostereum areolatum*, and phytotoxic venom also are injected into the tree, along with eggs, resulting in wood decay providing nutritious material that *S. noctilio* larvae feed upon (Edmonds et al., 2011; Slippers et al., 2006). Fungal spores of *A. areolatum* are carried in the mycangium of adult *S. noctilio* located internally at the base of the ovipositor (Edmonds et al., 2011). Growth of *A. areolatum* restricts water flow in the vascular system of infected pine trees by degrading tissues into a white rot (van der Nest et al., 2012).

Forestry is of significant economic importance in the southeastern United States contributing economic, environmental, social, and aesthetic benefits to these States. The USDA [United States Department of Agriculture] estimates that 244-million square feet of timber could be killed by *S. noctilio*, with $1.9 billion dollars projected to be lost to local economies (USDA 2008). A study by Olatinwo et al. (2013) predicted that *S. noctilio* could cause up to $48- 606 million losses in the State of Georgia over a thirty-year time period, and Yemshanov et al. (2009)

estimated a potential loss of $760 million throughout the whole United States over the same time period.

The potential economic and ecological concerns about the future invasions by non-native *Sirex* spp. into the southeastern United States should motivate an increase in the monitoring and surveillance for siricids in this area. The State of Alabama is specifically at risk of invasion because of the potential for infested wood packing material to enter through the port of Mobile (Yemshanov et al., 2009). The objectives of this study were to 1) monitor and survey native and potentially non-native woodwasp populations in various latitudes across Alabama, 2) determine the length of siricid flight seasons, and 3) survey bark and ambrosia beetle catch in traps to determine if emergence spikes overlap temporally.

2.3. Materials and Methods

2.3.1. Site Design

Surveys were conducted in three regions across the State of Alabama ranging from Talladega in the north and Andalusia in the south. Sampling was conducted at a total of 33 sites continuously from August 2014 to February 2016. Surveys were conducted in Tuskegee (August 2014- March 2016) and Talladega (Oakmulgee Ranger District) National Forests (February 2015- March 2016), and at Auburn University's Solon Dixon Center in Andalusia, Alabama (February 2015- March 2016). These regions were chosen in order to provide coverage throughout the State of Alabama (Fig. 2.1). All sites chosen were located in mixed species forests, dominated by loblolly pine (*Pinus taeda*) mixed with other pines and a few hardwood

species (Fig. 2.2).

Figure 2.1. Map of the State of Alabama, USA, indicating the sites where sampling was conducted from 2014-2016.

Figure 2.2. Intensely managed field site in Tuskegee National Forest that had recently been burned. Black panel trap can be seen in left background.

Black cross vane panel insect traps (Forestry Distributing Inc., Boulder, CO, U.S.A.) were placed at each site and monitored every two weeks. Traps were hung at a 45° angle from a six foot tall metal pipe driven in the ground, which was curved at the top to elicit hanging traps with ease (Fig. 2.3). Collection cups were attached at the trap bottom to catch insects that were lured to the traps. These collection cups were filled with 250 mL of a 30% propylene glycol

solution to preserve specimens. Traps were baited using 8 mL glass vials filled with a mixture of 70% α-Pinene and 30% β-Pinene, previously determined by Simpson and McQilkin (1976) to be optimal for attracting *S. noctilio*. This collection method, for surveying woodwasp populations, was undertaken in accordance with Barnes et al. (2014). Specimens were collected from traps every other week continuously through the entire duration of the trapping survey.

Figure. 2.3. Black cross vein panel trap set in Tuskegee National Forest, with white collection cup on bottom.

2.3.2. Identification

Specimens of siricids and other insects captured were identified to the species level and curated at the Forest Health Dynamics Laboratory at Auburn University. Specimens were later taken to the Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa for molecular analysis for identification confirmation. Species identifications were determined using Schiff et al. (2006).

By catch beetles that are deemed as species of concern were morphologically identified and cataloged throughout the duration of the trapping survey (Table 2.1). Species of concern include *Hylobious pales, Pachylobious picivorous, Ips* spp., *Hylastes* spp., and several common species of ambrosia beetles. Species were determined using taxonomic keys from Wood (1982).

Table 2.1. Species of concern captured as by-catch and cataloged throughout the duration of the study.

Designation in Graph	**Species**
Hylastes	*Hylastes salebrosus, Hylastes porculus, Hylastes tenuis*
Hylobiini	*Pachylobius picivorous, Hylobious pales, Pissodes nemorensis*
Ips	*Ips avulsus, Ips grandicollis, Ips calligraphus*
Ambrosia	*Xyleborus pubescens, Xyleborus ferrugineus, Orthotomicus caelatus, Xyleborinus saxeseni, Xylosandrus crassiusculus, Xylosandrus compactus, Xylosandrus germanus, Xyleborus mutilates, Monarthrum fasciatum, Monarthrum mali, Pityoborus comatus, Trypodendron scabricollis, Dryoxylon onoharaensum*

2.3.3. Statistical Analyses

The mean number of female siricid per trap were analyzed using a repeated measures analysis of variance (ANOVA), in accordance with Hurley et al. (2015). The catch size of each species per week between field sites were analyzed by conducting a factorial ANOVA. The dependent variable was quantity while the independent variable was time. All statistical analyses were run using the SAS program (SAS 9.4, 2013).

2.4. Results

A total of 131 woodwasps were collected over the duration of this study, all specimens were females (Fig. 2.7). Six of these wasps were identified as *Tremex columba* L.; three were captured in Tuskegee National Forest (Fig. 2.6), two were captured in Talladega National Forest (Fig 2.8), and the last was captured at the Solon Dixon Center (Fig. 2.5). One *Urocerus cressoni* Norton was collected from Tuskegee National Forest (Fig. 2.6). All remaining wasps (n=124) were identified as *S. nigricornis*.

Captures of *S. nigricornis* in Tuskegee National Forest varied from October 2, 2014 to December 11, 2014 and October 15, 2015 to December 10, 2015, but not significantly (p= 0.5291). In Talladega National Forest (Fig. 2.8), the captures ranged from October 17, 2015 until November 28, 2015. In this locale, *S. nigricornis* capture was significantly higher than *T. columba* capture (p= 0.0346). At the Solon Dixon Center, the only captured female was collected on November 19, 2015 (Fig. 2.9).

The earliest capture of a woodwasp was June 13, 2015 in Talladega National Forest (Fig. 3). This specimen was *T. columba*. The latest recorded emergence in the survey was *S. nigricornis* on December 11, 2014 in Tuskegee National Forest (Fig. 2.7). The only *U. cressoni* captured in the study was collected from Tuskegee National Forest on October 2, 2014 (Fig. 2.7).

Mean capture of *Hylastes* spp. and *Ips* spp. were significantly higher than other bark and ambrosia beetle captures at all three sights. Peaks of these populations occurred earlier in the year than peak *S. nigricornis* flight season (October- December).

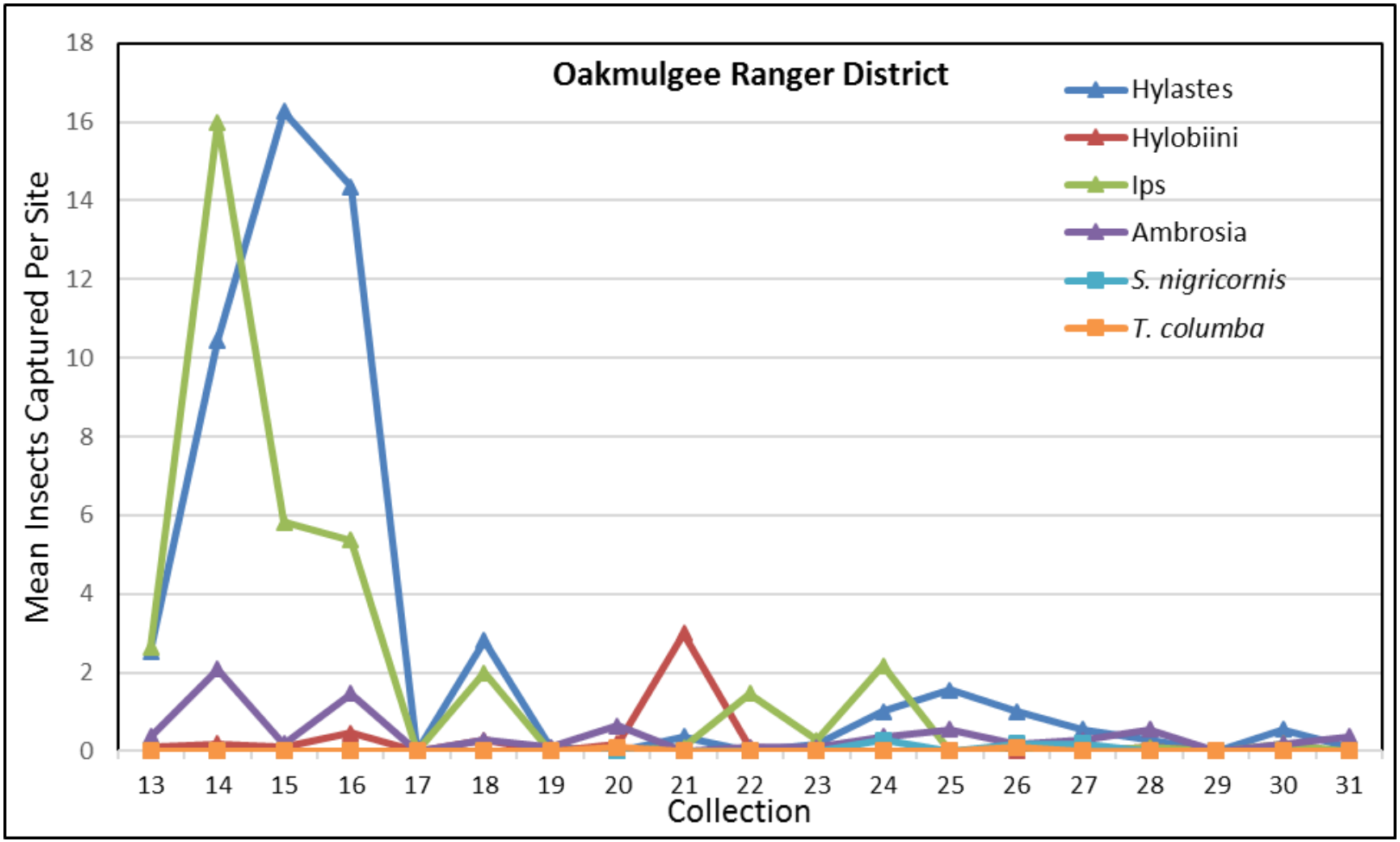

Figure 2.4. Mean insects captured at the Oakmulgee Ranger District of Talladega National Forest. *Ips* spp. and *Hylastes* spp. peak early in the summer, before Hylobiini or *S. nigricornis* in the fall. Collections from 3/7/15 to 1/20/16.

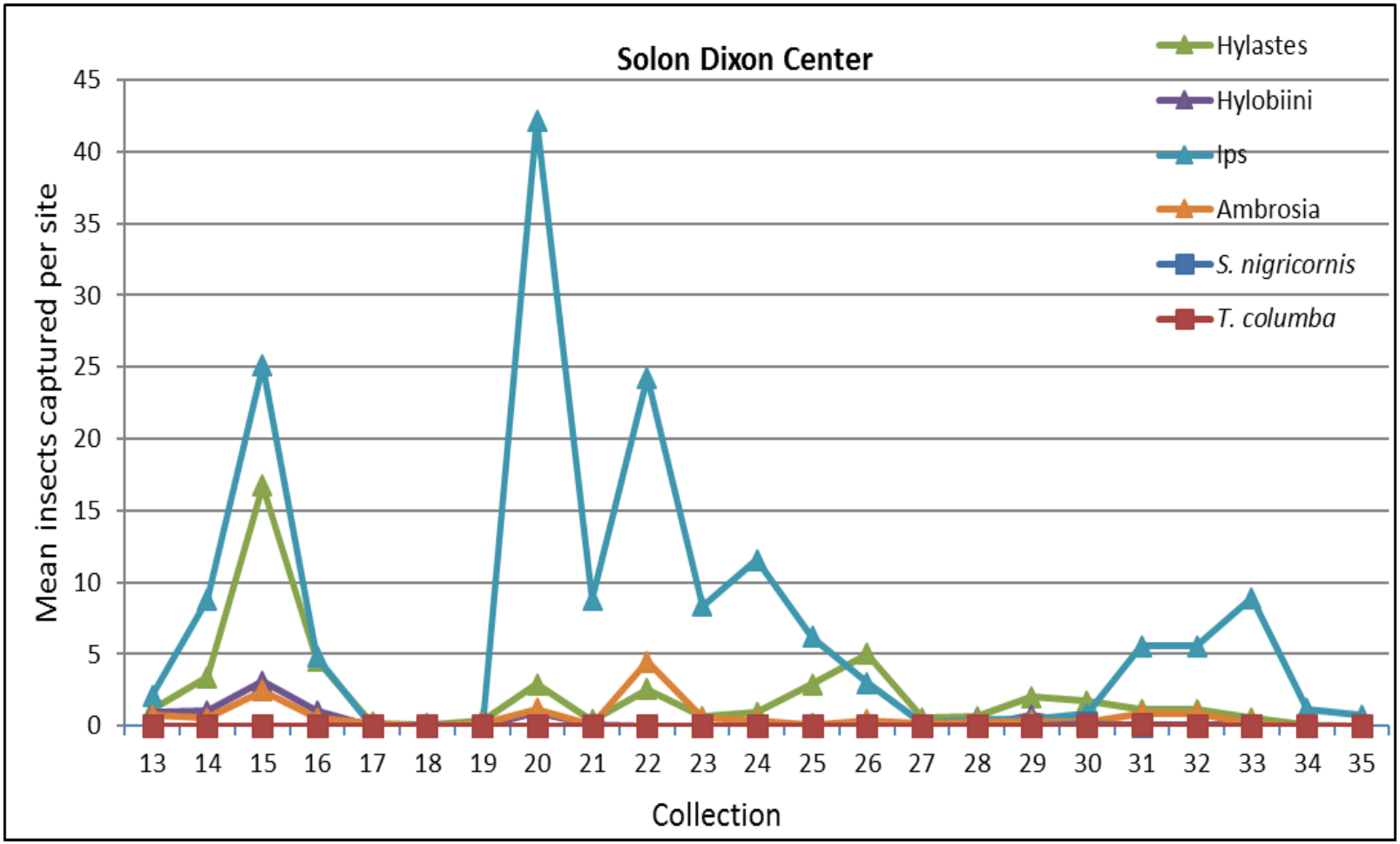

Figure 2.5. Mean capture of species of concern per site in Solon Dixon. *Ips* spp. and *Hylastes* spp. mean catch per site is significantly higher, and occurred earlier in the summer than more northern sites in Tuskegee and Oakmulgee. Collections from 3/11/15 to 1/28/16.

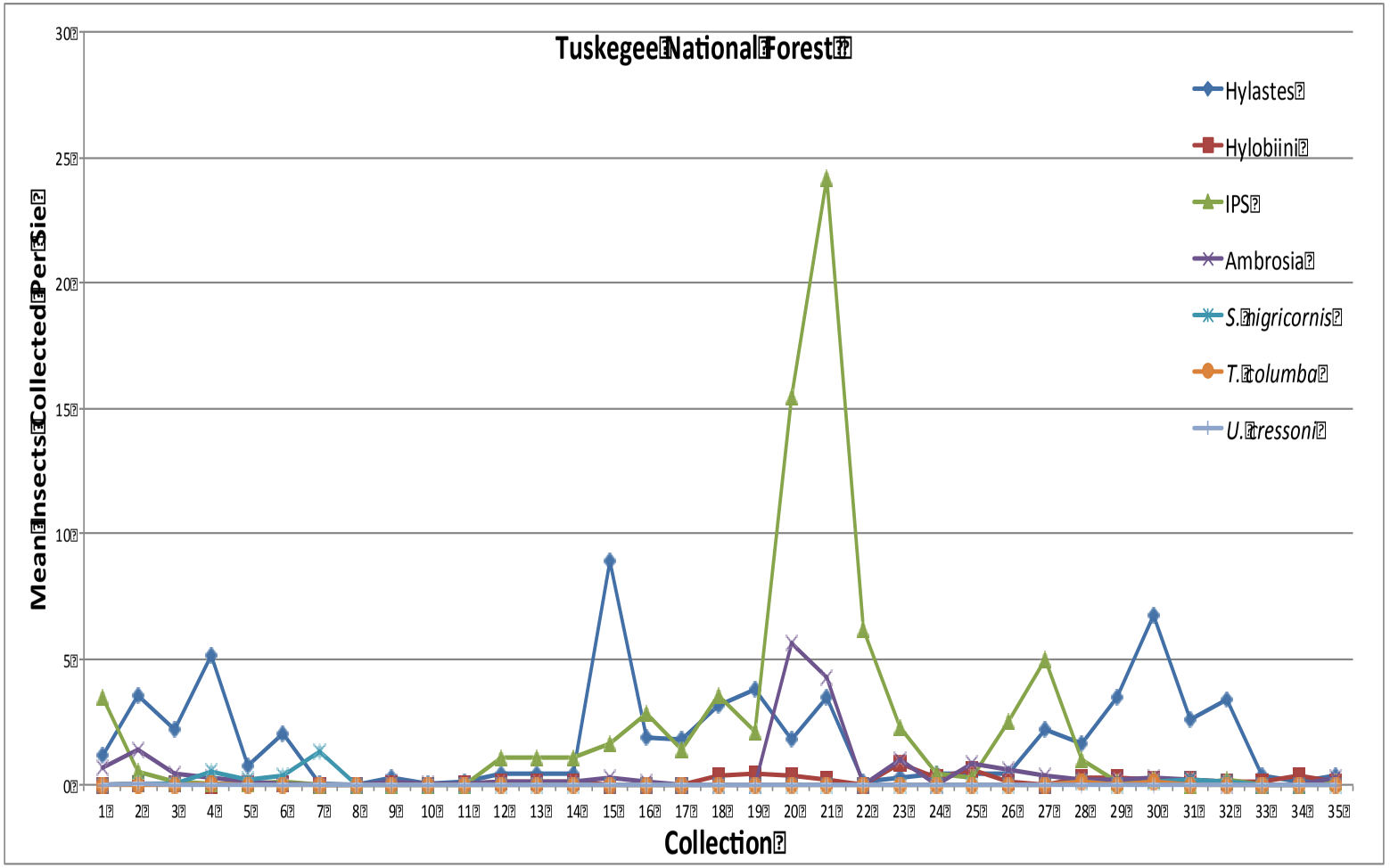

Figure 2.6. Mean capture of insects of concern per site in Tuskegee National Forest. Mean *S. nigricornis* capture significantly lower than *Ips* spp. and *Hylastes* spp. Peaks in these two genera occur during mid-summer, before *S. nigricornis* emergence.

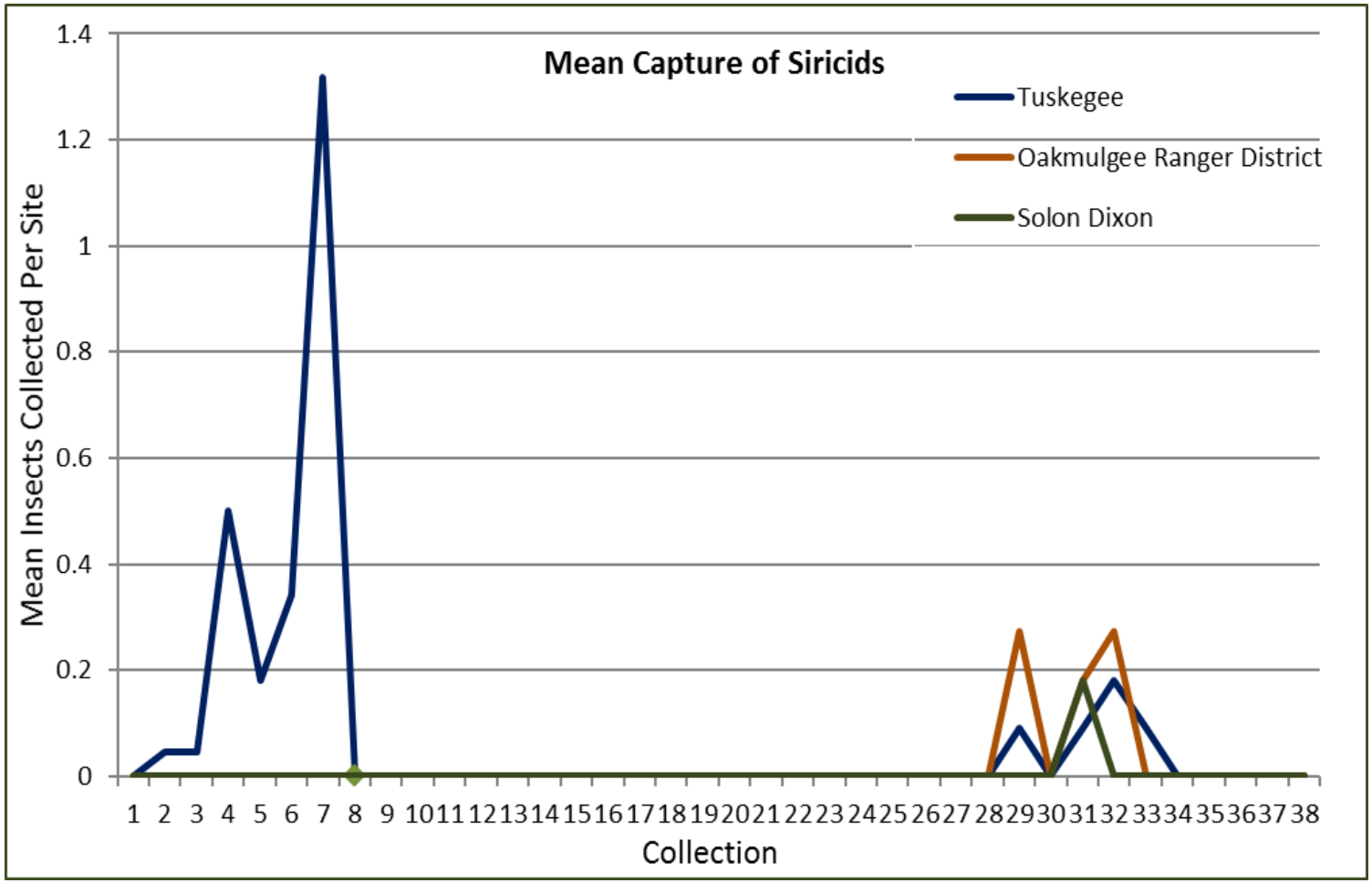

Figure 2.7. Mean capture of siricids per site in from September 2014 to March 2016. *S. nigricornis* peaks are between 10/30/14 and 12/11/14, then 10/15/15 to 12/10/15.

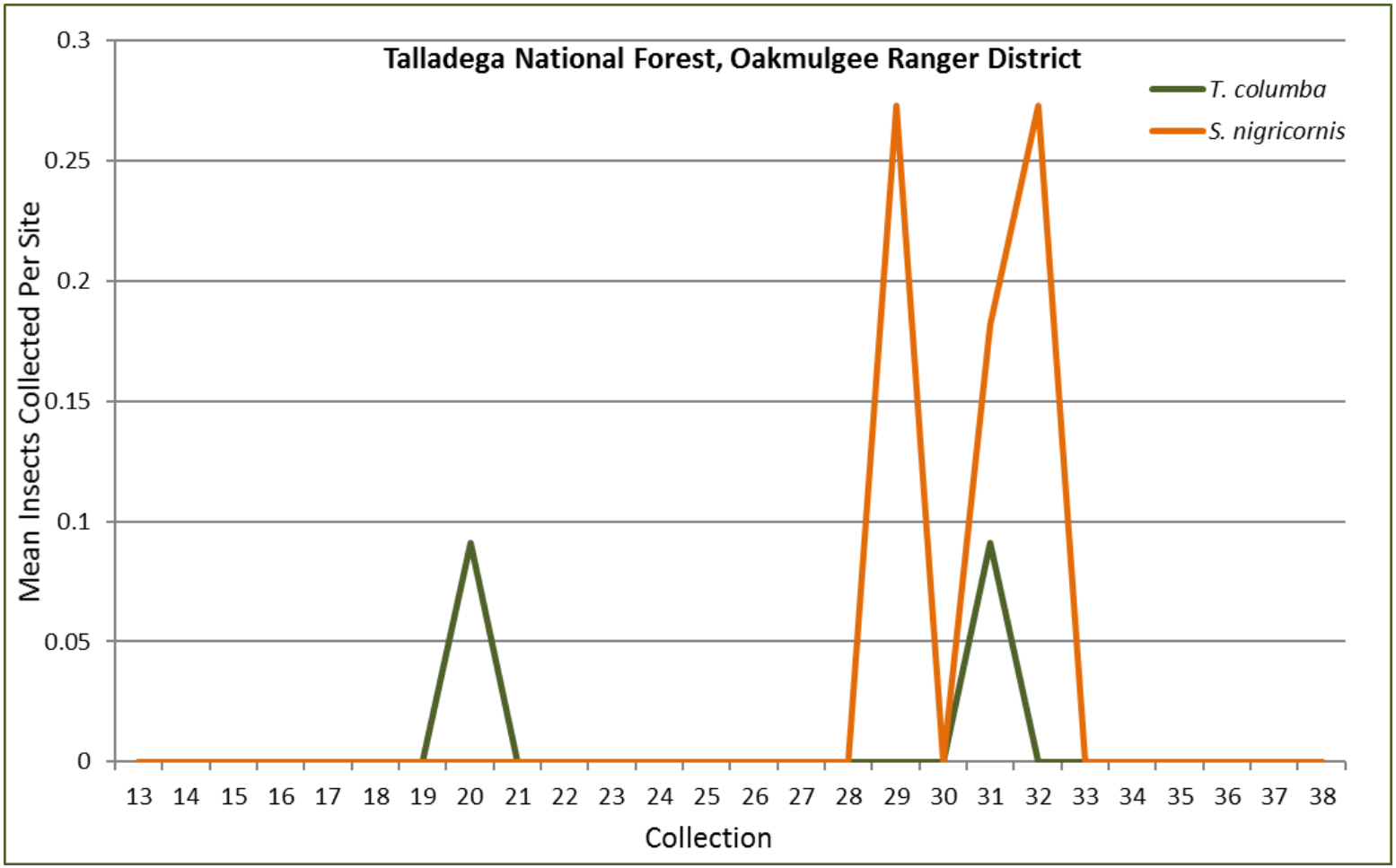

Figure 2.8. Mean capture of siricids per site in Oakmulgee Ranger District of Talladega National Forest. *S. nigricornis* peaks are 10/17/15 and 11/28/15.

Beetle populations were higher than woodwasps by number in all localities. In all cases, beetle populations peaked earlier in the year than *S. nigricornis*.

2.5. Discussion

No *S. noctilio* were captured in any traps through the duration of the trapping survey with only three woodwasp species captured in Tuskegee National Forest in 2014. Of these species, only S. *nigricornis* and *U. cressoni* utilize pines as hosts (Schiff et al. 2006).

Sirex nigricornis and *T. columba* had similar flight periods, with the exception of the early emergence trapping of *T. columba* in Talladega National Forest on June 13, 2015. There is little likelihood of competition occurring between these species even though their flight periods overlap; *T. columba* only attacks deciduous hardwood trees (Schiff et al., 2006). The flight season observed in *S. nigricornis* was in accordance with Hartshorn et al. (2016) in Arkansas. The observed emergence period of *S. nigricornis* adult females was slightly longer lasting (into December) than previously reported by Haavik et al. (2013), which conducted their survey in a controlled environment in Louisiana.

Woodwasps capture in 2015 in Tuskegee National Forest was lower compared with 2014, but this difference was not significant. Greater numbers could be due to prescribed burns that were conducted in the spring of 2015. Many of the sites and surrounding wooded areas within the study were burned, so traps had to be removed and reconstructed during this time.

To further address this difference, weather data from neighboring Lee County were obtained,

because data from Macon County were unavailable. Monthly average temperature and rainfall data were obtained from the National Oceanic and Atmospheric Administration (NOAA, www.ncei.noaa.gov; accessed 21 April 2016). There was a deep freeze event in December 2014 that effectively ended the flight season, and caused the liquid solution in all of the traps to freeze solid. December 2014 also proved to be much wetter than any of the other months during the trapping survey, with over 45 cm of precipitation accumulating.

Bark and ambrosia populations peaked earlier in the year than *S. nigricornis*, suggesting there would not be direct competition for substrate for rearing larvae. These spring and earlier summer peaks could potentially overlap with *S. noctilio* emergence, if they were to be found in the area. If *S. noctilio* were to be found in the area, a high population density could potentially affect bark and ambrosia beetle populations, as there would be more stressed and dying tree material for beetles to be attracted to. It could be hypothesized that the establishment of *S. noctilio* in an area would exacerbate a beetle outbreak, causing even more tree death in an area.

2.6. Conclusion

Only females were captured, which also was the case in trapping done in Mississippi by Chase et al. (2014) and is generally the case for all siricid trapping studies (Hurley et al., 2015; Johnson et al., 2013; Coyle et al., 2012). Martínez et al. (2014) suggests that male flight is primality restricted to the tops of the trees in the canopy, where mating occurs. Male siricids do not have the exact temporal emergence period that females do, depending on outside temperature conditions. *Sirex nigricornis* males emerged a predicted two days after females according to the degree day models of Haavik et al. (2013).

To conclude, this study provides a greater understanding of native woodwasp populations, primarily of *S. nigricornis*, in three forests in the State of Alabama. The survey shows that emergence periods of female *S. nigricornis* adults were fairly uniform for the duration of the project, even though the total number of specimens caught differed significantly. Cataloged catch of bark and ambrosia beetle populations in the area suggests that these insects peak in population at an earlier time of the year than *S. nigricornis* populations. Emergence habits and population size estimates of woodwasps are important details to know in this region, where planted pine forests make up a great deal of the economy. A more intensive and exhaustive collection survey is warranted in the future, as the limited time scale on this project cannot determine the regular population sizes of endemic woodwasps.

Chapter 3

Deladenus species associated with native Siricid Woodwasps in Alabama

3.1. Abstract

Sirex nigricornis woodwasp populations in the southeastern United States have been studied to gain a better understanding of how this species interacts in the pine forest ecosystem. In this study, specimens collected from three forests in Alabama were dissected in order to sample for the mutualistic fungi, *Amylostereum* spp. and parasitic nematode, *Deladenus* spp. Molecular and phylogenetic analyses were performed in order to determine relationships between the woodwasps collected, and fungal and nematode species determined to coexist inside the wasps. *Sirex nigricornis* was found to carry both *A. chailletii* and *A. areolatum,* and was found to be parasitized by both *D. proximus* and *D. siricidicola*. A *Tremex columba* specimen was found to carry *Cerrena unicolor,* and also was parasitized by *D. siricidicola*.

3.2. Introduction

The invasive woodwasp *Sirex noctilio* L. (Hymenoptera: Siricidae) was first discovered in the State of New York in 2004 (Hoebeke et al., 2005). *Sirex noctilio* is known to kill pine trees by using its ovipositor to drill into the xylem of the pine tree where it subsequently deposits a

mixture of eggs, fungal spores, and phytotoxic venom resulting in cell death due to reduced moisture present within the impacted tree (Madden, 1968c). This woodwasp is native to Europe and Northern Africa (Spradbery and Kirk, 1978), and has become a pest of significant economic importance in commercial forestry plantations within the Southern Hemisphere (Slippers and Wingfield, 2012). Such nonnative planted pine plantations are usually established by planting a single pine species over vast areas (Carnegie et al., 2006). The economic impact caused by this pest in the Southern Hemisphere has not yet occurred within the mixed pine and hardwood forests of the northeastern United States. It is still unclear as to whether *S. noctilio* could have a significant impact on commercial pine forestry in the southeastern United States, which are comprised predominantly of loblolly pine (*Pinus taeda* L.). Since the initial introduction of *S. noctilio*, researchers throughout the United States have been conducting studies to determine as to how this pest could potentially impact native ecosystems, and other woodwasps found within overlapping geographic areas as to where *S. noctilio* is found. The complex of the siricid woodwasp in relation to their mutualistic fungi and nematode species has been studied extensively (Bedding and Akhurst, 1974; Madden, 1968c; Hurley et al., 2008). Historically, there was thought to be a strong species specific relationship between *Sirex* spp. and *Amylostereum* spp. This fungal symbiont typically associated with native North American siricids is that of *A. chailletii* Pers. Boidin (Smith and Schiff, 2002; Hajek et al., 2013). Recently, however, several fungal species including *A. areolatum* were found to occur with the North American native *S. nigricornis* F. (Olatinwo et al., 2013). Fruiting basidiocarps are very rarely seen in nature (van der Nest et al., 2012), so it is unlikely that spores could have been transmitted in this manner.

A sterilizing strain of nematode, *Deladenus siricidicola* Bedding (Tylenchida:

Neotylenchidae), is commonly used in the southern hemisphere as a biological control agent on *S. noctilio* (Bedding and Iede, 2005). This parasitic strain of nematode is thought to be species specific thus unlikely to infest *Sirex* spp. native to North America. Currently in North America, there are seven native *Sirex* spp. and one invasive species in addition to *S. noctilio*, *S. juvencus juvencus* L. (Schiff et al., 2006). This presence of *D. siricidicola* in native siricids was however, found to occur on eggs dissected from female *S. nigricornis* in this study. This association is most likely due to vertical transmission within the tree during the larval stage, when adult *S. nigricornis* wasps carry *A. areolatum* spores internally. It is currently unclear as to whether *D. siricidicola* associated with *S. nigricornis* is the sterilizing strain commercially used in the Southern Hemisphere, or a non-sterilizing strain of *D. siricidicola* found to occur in *S. noctilio* in Canada (Yu et al., 2009). Studies report that there is a strict relationship between the species of nematode and the fungus for which they are associated. Bedding and Akhurst (1978) found that *D. siricidicola* was strictly associated with *A. areolatum*, and *D. canii* Bedding was strictly associated with *A. chailletii*. These linkages support the idea that siricids are associated to the species of nematode that are endemic to the same areas in which they are found. In addition other Siricidae have a strict relationship with associated nematodes and fungus, examples include *Tremex columba* L., the pigeon horntail, a woodwasp native to the United States that generally inhabits hardwoods such as oak, elm, and beech (Schiff et al., 2006). *Tremex columba* is typically associated with *Cerrena unicolor* (Bull.) Merrill, a similar saprophytic white rot (Stillwell, 1965; Pažoutová et al., 2010). This fungus is native to the United States, and is commonly found to colonize dead or dying hardwoods (Enebak and Blanchette, 1989).

3. 3. Materials and Methods

3.3.1. Sampling

Through the duration of trapping, 132 specimens were trapped fortnightly from forested sites throughout the State of Alabama from August 2014 to February 2015. These sites include two National Forests (Tuskegee and Talladega) as well as a site at Auburn University's Solon Dixon Center. In each forest locale, 33 black cross vane panel insect traps (Forestry Distributing Inc., Boulder, CO, U.S.A.) were used for trapping and were placed at each site Tuskegee (August 2014- March 2016), Talladega (Oakmulgee Ranger District) National Forests (February 2015-March 2016) and at Auburn University's Solon Dixon Center in Andalusia, (February 2015-March 2016). Collection cups were attached at the bottom of each trap to catch insects that were lured to the traps. These collection cups were filled with 250 mL of a 30% propylene glycol solution to preserve specimens. Traps were baited using 8 mL glass vials filled with a mixture of 70% α-Pinene and 30% β-Pinene, previously determined by Simpson and McQilkin (1976) to be optimal for attracting *S. noctilio*. This collection method, for surveying woodwasp populations, was undertaken in accordance with Barnes et al. (2014). An additional 36 samples were collected in 2014 from traps placed on the same field sites in the Talladega National Forest, as a part of another study with these traps being baited using turpentine and ethanol.

For live specimen collections during the expected *S. noctilio* flight season (August-January) additional insect traps were deployed in both 2014 and 2015 at three additional localities: 1) a small forest located around School of Forestry and Wildlife Sciences, Auburn University, 2) Mary Olive Thomas Demonstration Forest (MOT) and 3) Louise Kreher Forest

Ecology Preserve and Nature Center in Auburn, Alabama. These traps were suitable to capture live females as they had a paper towel crumpled as a means to detain live wasps instead of the usual propylene glycol mixture in collection cups used in the other traps. Traps were checked every other day at all these additional localities. Live specimens were first killed by brushing with ethyl acetate, and then were processed using the same protocol as dead captured specimens. Additional traps were set up in order to capture live females, to obtain cultures of *Amylostereum* spp.

3.3.2. Dissection

Nematode samples were collected from eggs of the female woodwasps. Captured live *Sirex* spp. specimens were stored in the laboratory at 4° C until time of processing. All woodwasp specimens were morphologically identified to species level using the morphological key of Schiff et al. (2006). During the wasp dissection, mycangia (Fig 3.1) were removed and plated on potato dextrose agar (PDA) plates with streptomycin for fungal culturing. Dissection techniques were in accordance with Thomsen and Harding (2011). Eggs, mycangium, and leg samples were all placed in individual glass vials of 95% ethanol for storage and shipment and used for molecular analyses.

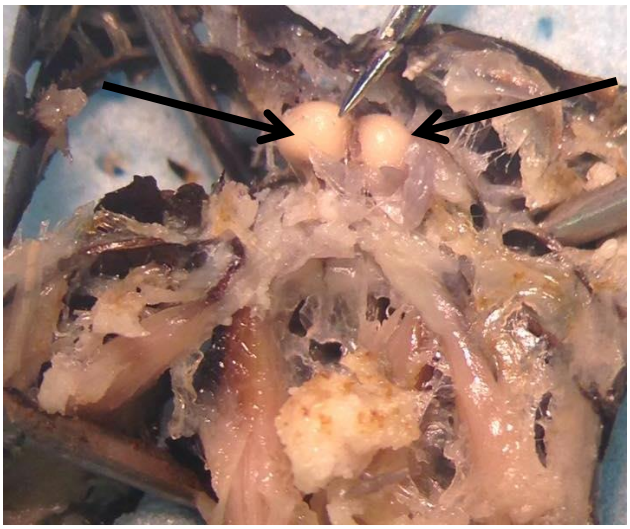

Figure 3.1. Dissected female *S. noctilio*, with arrows pointed at internal mycangia containing *Amylostereum* spp. spores.

3.3.3. Molecular Analyses

For nematode samples, a modified version (Katrin Fitza, pers. comm.) of the DNA extraction protocol from Wilhelm et al. (1992) was used. Identification of the samples were conducted using the COI primers published by Morris et al. (2013), amplifying part of the cytochrome oxidase 1 gene region, as well as the TW81 and AB28 primers published by Morris et al. (2013) to amplify the internal transcribed spacer region (ITS rDNA). The entire extracted DNA was then used for PCR reactions. PCR reactions for both primers were made to a total volume of 21.5 µl, 5 µl of MyTaq^{TM} Reaction Buffer (Bioline USA, Tauton, Massachusetts), 0.1 µg of both COIF and COIR and 0.5 µl of MyTaq^{TM} DNA polymerase (Bioline USA, Tauton, Massachusetts). The following PCR conditions were applied to both COI and ITS: preincubation of 95°C 4 min, 35 cycles of 95°C for 45 s, 56°C for 30 s and 72°C for 1 min, ending with a final extension of 72°C for 10 min. Sequencing was performed on the amplicons using the ABI PrismTM 3500xL automated DNA sequencer (Applied Biosysytems USA, Foster City, California).

To verify the *Amylostereum* isolates DNA was extracted from mycangia stored in 95% ethanol. Each mycangia was added to 50 µl of PrepMan®Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA) and homogenized with a ball mill (Retsch MM301) at 30 shakes/ second frequency for 3 minutes. The samples were spun down and heated for 10 min at 100°C in a heating block. The primers used for analysis were the mtSSU rDNA primer pair MS1 and MS2 (White et al., 1990) amplifying a portion of the mitochondrial small subunit, and the internal transcribed spacer region (ITS rDNA) primer pair ITS1 and ITS4 (White et al., 1990). PCR reactions for both primers included around 0.1 µg of the template DNA, 5 µl of MyTaq™ Reaction Buffer (Bioline USA, Tauton, Massachusetts), 0.1 µg of forward and reverse primer and 0.5 µl of MyTaq™ DNA polymerase (Bioline USA, Tauton, Massachusetts). The following PCR protocol was used for MS primers: 95°C for 3 minutes, followed by 35 cycles of 95°C for 45 seconds, 58°C for 30 seconds, 72°C for one minute, one cycle of 72°C for 10 minutes and holding at 10°C. The protocol for ITS is as follows: 94°C for 7 minutes, followed by 35 cycles of 94°C for one minute, 48°C for 1 minute, 72°C for 2 minutes, one cycle of 72°C for 10 minutes and holding at 10°C. All the amplicons were sequenced following above mentioned protocol.

Single legs of *Sirex* specimens were cut using micro-scissors and homogenized with a ball mill (Retsch MM301) at 30/s frequency for 3 min. The ZyGEM DNA extraction protocol was followed using the *prep*Gem™Insect (ZyGEM, Hamilton, New Zealand) kit. The barcoding cytochrome oxidase (cox 1) primers LCO1490 and HCO2198 designed by Folmer at al. (1994) were applied to identify the specimens. The PCR protocol described by Dittrich-Schröder et al., (2012) was followed: 95°C for 7 minutes, 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 2 minutes, followed by one cycle of 72°C for 2 minutes, then holding at 10°C.

The 658bp fragment was then sequences as described above.

3.3.4. Phylogenetic analyses

Raw sequences were edited using the software program 4 Peaks and AliView, where they were aligned using Muscle. Sequences were submitted to GenBank (Accession numbers pending). All trees were drawn in Mega 6 (Tamura et al, 2011). Neighbor-joining and Maximum-liklihood trees using 1000 bootstraps were drawn for *Amylostereum* and *Deladenus* samples. Other isolates used in comparison were accessed from GenBank and Fitza et al. (2016).

3. 4. Results

Phylogenetic relationships of *Deladenus* spp. samples were determined by sequencing on the Cytochrome Oxidase I gene. In some cases, the ITS region also was sequenced to provide greater reliability of identification (Table 3.1). Three distinct species of nematodes were molecularly identified from samples in this study (Figs 3.1, 3.2). These were identified as *Deladenus proximus, D. siricidicola* and the third species is thought to be the previously described *D. wilsoni*. From the total of nine nematode samples, obtained from dissected *S. nigricornis* eggs *D. siricidicola* and *D. proximus* were identified. This study found two other woodwasps species to be hosting *D. siricidicola*; *S. nigricornis* and *T. columba*. A total of 131 woodwasps were initially captured in an earlier survey (Wahl, Chapter 2). Out of those, 21.9% were infested with some species of nematode.

Out of the 47 *S. nigricornis* mycangium sampled, 15 specimens were confirmed to be *A. areolatum*, and 20 were confirmed as *A. chailletii* (Table 3.2, Figs. 3.3, 3.4). The mycangia

sampled from the singular *T. columba* specimen yielded a result of *C. unicolor* (Fig. 3.4).

Table 3.1. Species determinations of *Amylostereum* and *Deladenus* associated with woodwasp specimens that were infested with nematodes.

Specimen ID	Woodwasp Species	Deladenus species	Gene region(s)	Fungal species	Gene region(s)	National Forest Locale	Date Collected
AL2	*S. nigricornis*	*?*	COI	*A .areolatum*	MS2	Oakmulgee	10/16/15
AL 11	*T. columba*	*D. siricidicola*	COI, ITS	*C. unicolor*	MS2	Oakmulgee	11/13/15
AL 23	*S. nigricornis*	*D. proximus*	COI			Tuskegee	10/30/14
AL 24	*S. nigricornis*	*D. proximus*	COI			Tuskegee	10/30/14
AL 47	*S. nigricornis*	*D. siricidicola*	COI	*A. chailletii*	MS2	Tuskegee	10/30/14
AL79	*S. nigricornis*	*D. proximus*	COI, ITS	*A.areolatum*	ITS2	Tuskegee	11/24/15
AL 81	*S. nigricornis*	*D. siricidicola*	COI			Tuskegee	12/11/14
AL 92	*S. nigricornis*	*D. siricidicola*	COI, ITS			Tuskegee	12/11/14
AL94	*S. nigricornis*	*D. proximus*	COI			Tuskegee	12/11/14

Table 3.2. Species determinations of *Amylostereum*, along with gene region sequenced.

Specimen ID	Woodwasp spp.	Fungal spp.	Gene Region(s)	National Forest	Date Collected
AL 3	*S. nigricornis*	*A. areolatum*	MS2	Oakmulgee	11/29/15
AL7	*S. nigricornis*	*A. areolatum*	MS2	Tuskegee	10/14/15
AL8	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/10/15
AL9	*S. nigricornis*	*A. chailletii*	MS2	Oakmulgee	11/29/15
AL18	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	10/30/14
AL29	*S. nigricornis*	*??*	MS2	Tuskegee	11/12/14
AL 30	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	11/24/14
AL 33	*S. nigricornis*	*A .areolatum*	ITS2	Tuskegee	12/11/14
AL37	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL39	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL40	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	11/25/14
AL 41	*S. nigricornis*	*A. chailletii*	ITS2	Tuskegee	11/25/14
AL42	*S. nigricornis*	*A. areolatum*	MS2, ITS2	Tuskegee	11/12/14
AL 43	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	11/12/14
AL 44	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	11/12/14
AL 45	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	11/12/14
AL 49	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	10/30/14
AL53	*S. nigricornis*	*A. areolatum*	MS2	Tuskegee	10/30/14
AL 54	*S. nigricornis*	*A. chailletii*	ITS2	Tuskegee	10/30/14
AL59	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	11/12/14
AL 61	*S. nigricornis*	*A. areolatum*	MS2	Tuskegee	11/25/14
AL 65	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	10/30/14
AL 66	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	10/30/14

AL 68	*S. nigricornis*	*A. chailletii*	ITS2	Tuskegee	12/11/14
AL 69	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 70	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/16/14
AL 71	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 73	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	11/25/14
AL 74	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 75	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 77	*S. nigricornis*	*A. areolatum*	ITS4	Tuskegee	12/11/14
AL 78	*S. nigricornis*	*A. chailletii*	ITS4	Tuskegee	12/11/14
AL 79	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	11/25/14
AL 81	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 88	*S. nigricornis*	*A. chailletii*	ITS4	Tuskegee	10/30/14
AL 93	*S. nigricornis*	*A. chailletii*	ITS4	Tuskegee	12/11/14
AL 100	*S. nigricornis*	*A .areolatum*	ITS2	Tuskegee	12/11/14
AL 108	*S. nigricornis*	*A .areolatum*	ITS2	Tuskegee	12/11/14
AL 109	*S. nigricornis*	*A. chailletii*	ITS2	Tuskegee	12/11/14
AL 116	*S. nigricornis*	*A. areolatum*	ITS2	Tuskegee	12/11/14
AL 122	*S. nigricornis*	*A .areolatum*	MS2	Tuskegee	12/11/14
AL 123	*S. nigricornis*	*A. chailletii*	MS2	Tuskegee	12/11/14
AL 128	*S. nigricornis*	*A. chailletii*	MS2	Oakmulgee	12/9/14
AL 129	*S. nigricornis*	*A. areolatum*	MS2	Oakmulgee	12/9/14
AL 136	*S. nigricornis*	*A. areolatum*	MS2	Oakmulgee	12/9/14
AL 135	*S. nigricornis*	*A. chailletii*	ITS4	Oakmulgee	12/9/14
AL 145	*S. nigricornis*	*A .areolatum*	ITS2	Oakmulgee	12/9/14
AL 153	*S. nigricornis*	*A. chailletii*	ITS2	Oakmulgee	12/9/14
AL 156	*S. nigricornis*	*A. areolatum*	ITS2	Oakmulgee	12/9/14

AL S1	*S. nigricornis*	*A. chailletii*	MS2	MOT	10/22/15
AL 15B	*S. nigricornis*	*A. chailletii*	MS2	MOT	10/22/15

3.5. Discussion

The relationship observed between *S. nigricornis* and *D. siricidicola* in the present studies is previously unreported. *Deladenus siricidicola* had previously only been associated with *S. noctilio*, even in areas where populations of *S. nigricornis* overlap (Hartshorn et al., 2015). It is still unclear as to whether this strain of *D. siricidicola* is capable of sterilizing eggs, as visual inspection of eggs, prior to sampling, revealed nematodes only present on the outside of egg cases.

Our findings from this study provide new insight as to the relationship between *Sirex* spp. and *Deladenus* spp. Previous studies found that there was strong host specificity between *D. siricidicola* and *S. noctilio* (Table 3.1, Figs. 3.1, 3.2). A horizontal transfer of fungal associates in other areas where the two *Sirex* spp. overlap has been hypothesized by Wooding et al. (2013), so it is conceivable that nematode parasites also might be transferred in a similar manner. The tree feasibly could have been attacked by another siricid carrying *D. siricidicola*. An interesting aspect of this study, however, is that there have been no reports of *S. noctilio* in this region of the country.

The association observed between *T. columba* and *D. siricidicola* also is a new finding. *Tremex columba* does not typically colonize the same trees as *Sirex* spp. Even though the preferred hosts of both of these species are found together in the plots surveyed, some form of

direct contact would have to be made if *D. siricidicola* did not naturally coexist with *T. columba*. Although the capture of the *T. columba* specimen was within the same time period as *S. nigricornis* flight in Alabama, the flight season of *T. columba* is longer than that of *S. nigricornis*. A previous study showed that *T. columba* can emerge as early as June, well before typical *S. nigricornis* flight (Wahl, Chapter 2). The earlier emergence period observed for *T. columba* might suggest that this species could overlap temporally with *S. noctilio* if it were to be found in the same locality.

Finding that *S. nigricornis* in Alabama is associated with both *A. areolatum* and *A. chailletii* are in accordance with Wooding et al. (2013) and Olantinwo et al. (2013). This finding is unsurprising, but is significant because it supports the possibility that *D. siricidicola* was originally passed to *S. nigricornis* from a *S. noctilio* in the area of the United States where their populations overlap.

One interesting aspect of *S. nigricornis* carrying either *A. areolatum* or *A. chailletti* is the implication that *D. siricidicola* and *D. proximus* may lack fidelity to respective fungal species, as previously believed. Prior reports (Bedding and Akhurst, 1978; Hajeck and Morris, 2014) state that *D. proximus* cannot feed on *A. areolatum.* These sources also claim that *D. siricidicola* cannot subsist on *A. chailletii*. The fungal and nematode relationships determined for specimens 47 and 79 are not in accordance with the previous studies' findings. The ITS tree drawn (Fig. 3.2) suggests that Specimen 79 is *D. proximus*. Further genes need to be sequenced for this specimen, as the COI tree (Fig. 3.1) suggests that it may group with a different species, where no known reference specimens are available.

3.6. Conclusions

This link between *S. nigricornis* and *D. siricidicola* is previously unreported, and has the potential to impact how *Sirex* control methods are implemented in commercial forestry. The fact that *D. siricidicola* is not genus specific, much less species specific, may warrant more research looking at how this nematode is used as a biological control agent. Lesser amounts of research have gone into studying *S. nigricornis* and its symbiotic relationships than the invasive pest *S. noctilio*. If the *D. siricidicola* found in *S. nigricornis* samples in Alabama are capable of sterilization, this has implications of damaging a population of native species in an environment in which they act harmlessly as a vector of decomposing fungi.

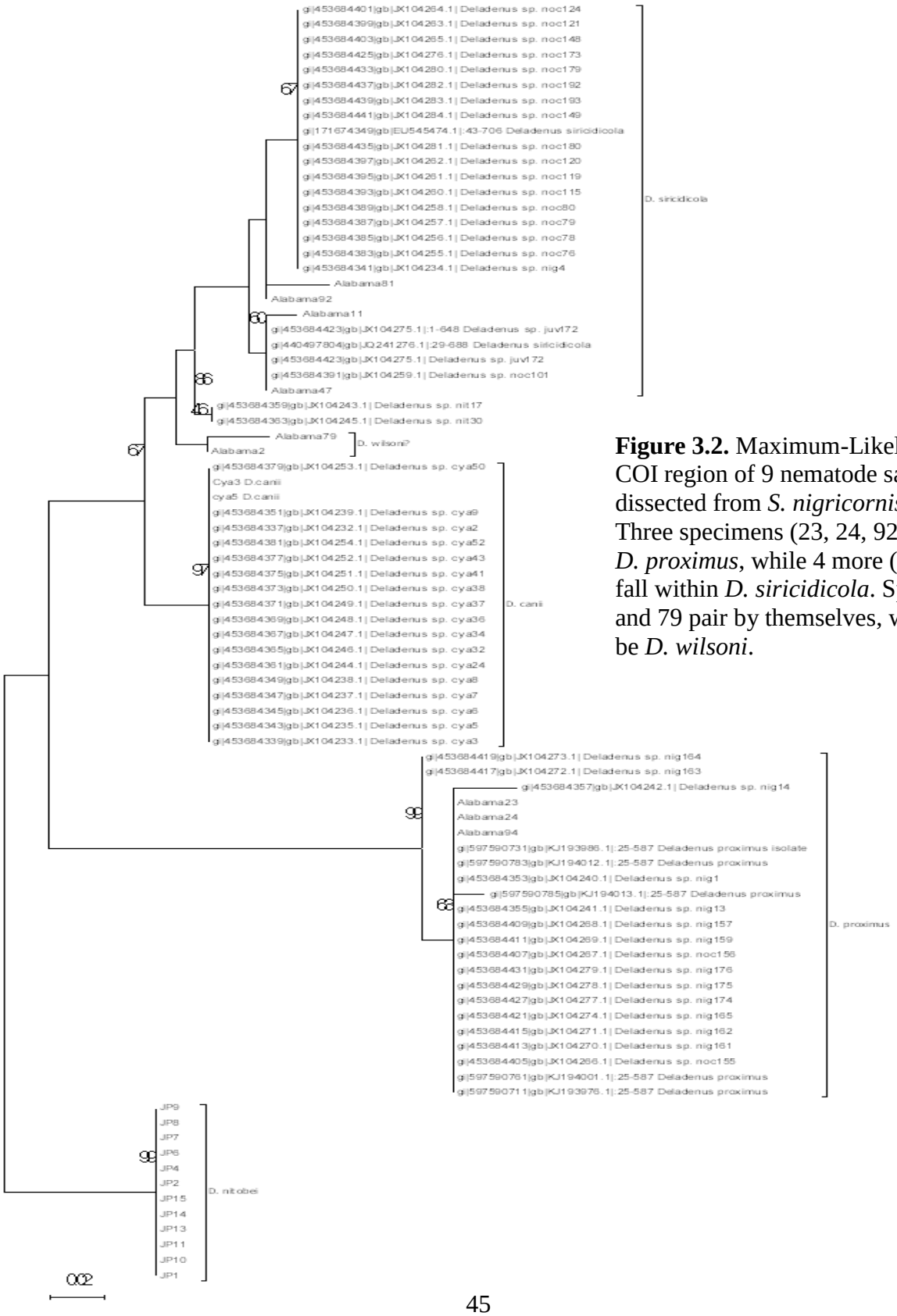

Figure 3.2. Maximum-Likelihood tree of COI region of 9 nematode samples dissected from *S. nigricornis* in Alabama. Three specimens (23, 24, 92) fall within *D. proximus*, while 4 more (11,47,81,92) fall within *D. siricidicola*. Specimens 2 and 79 pair by themselves, which could be *D. wilsoni*.

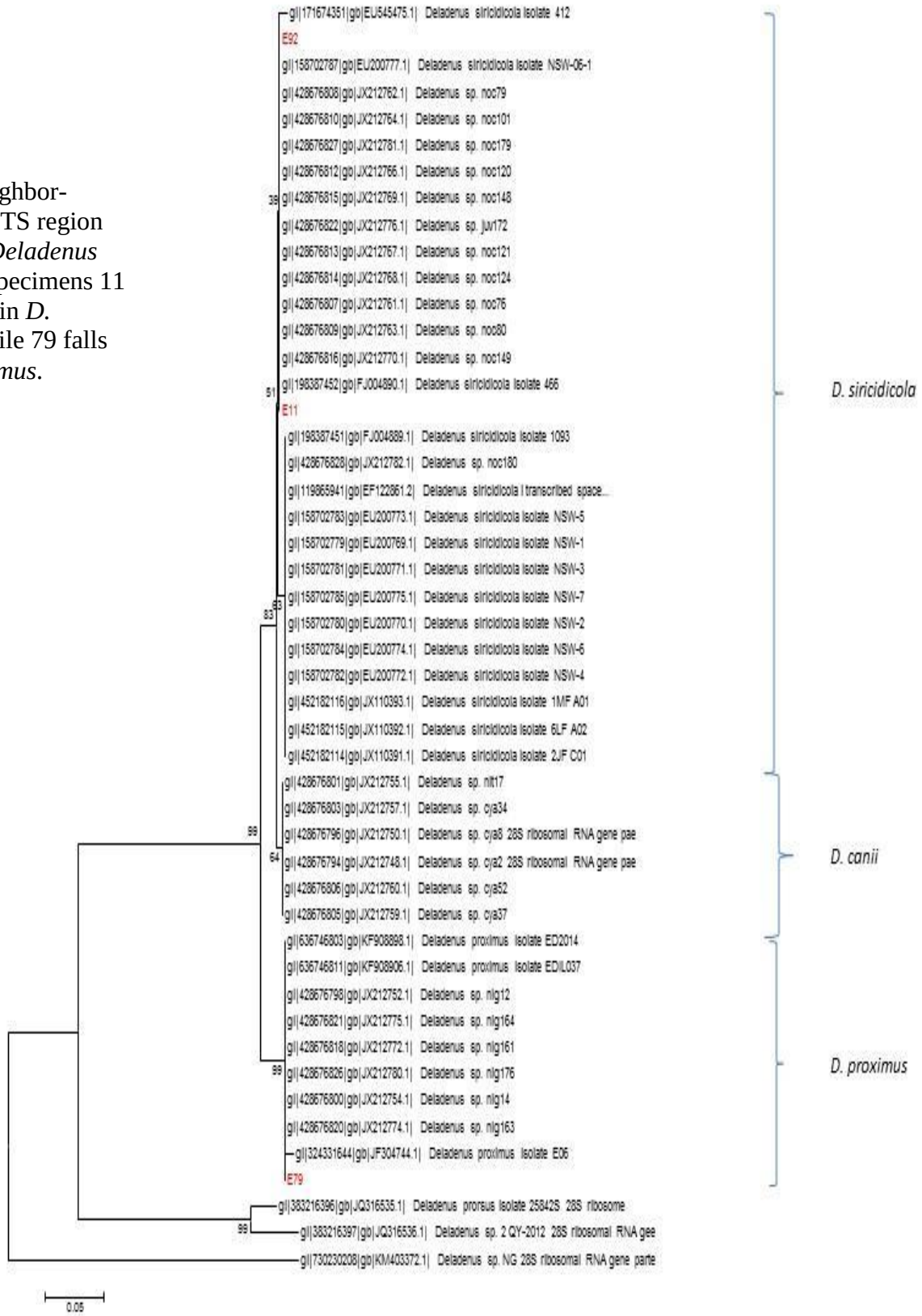

Figure 3.3. Neighbor-Joining tree of ITS region sequenced for *Deladenus* spp. samples. Specimens 11 and 92 fall within *D. siricidicola*, while 79 falls within *D. proximus*.

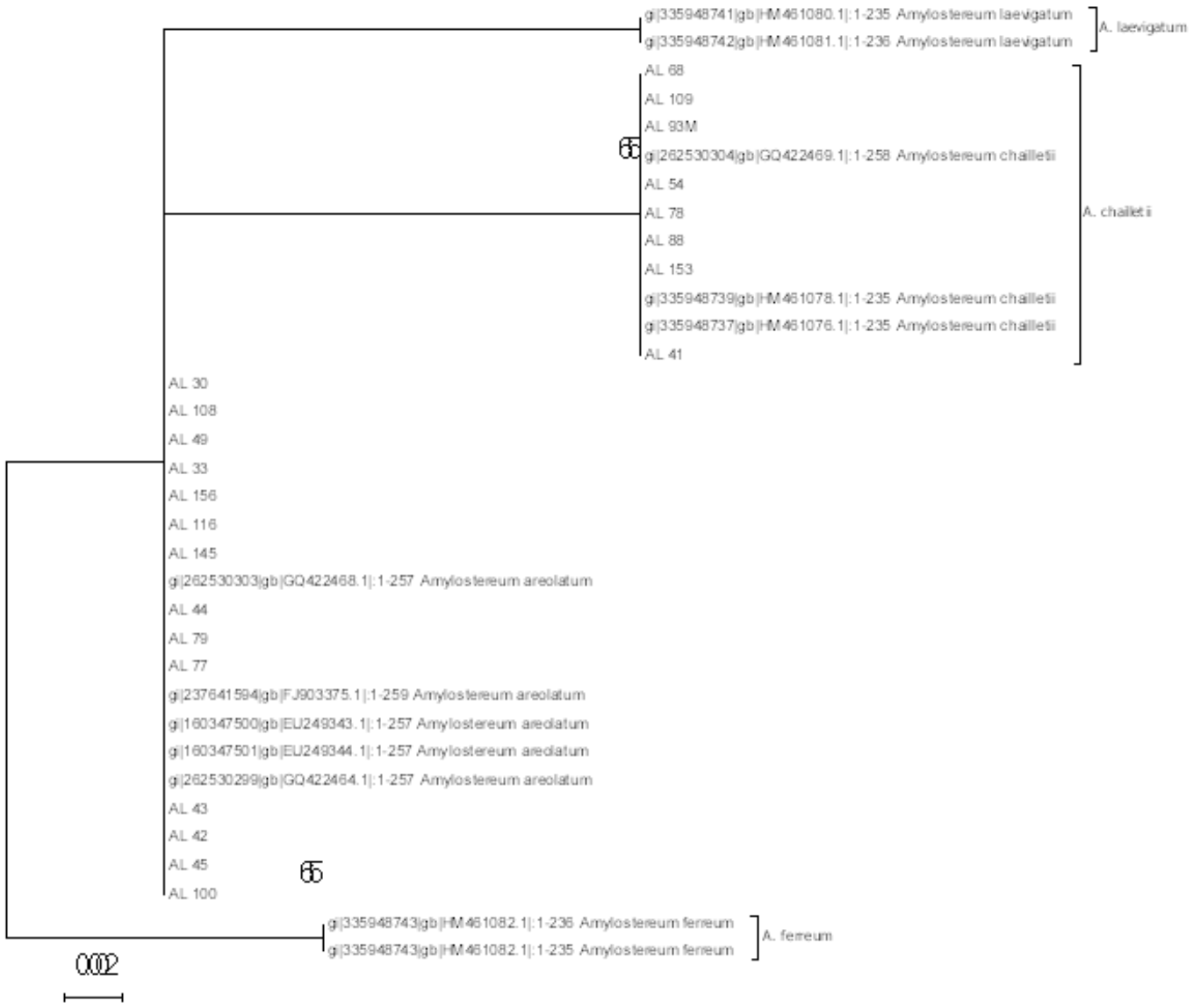

Figure 3.4. Maximum-Likelihood ITS tree of *Amylostereum* spp. samples, showing Alabama isolates grouping as *A. areolatum* (n= 14) and *A. chailletii* (n=8).

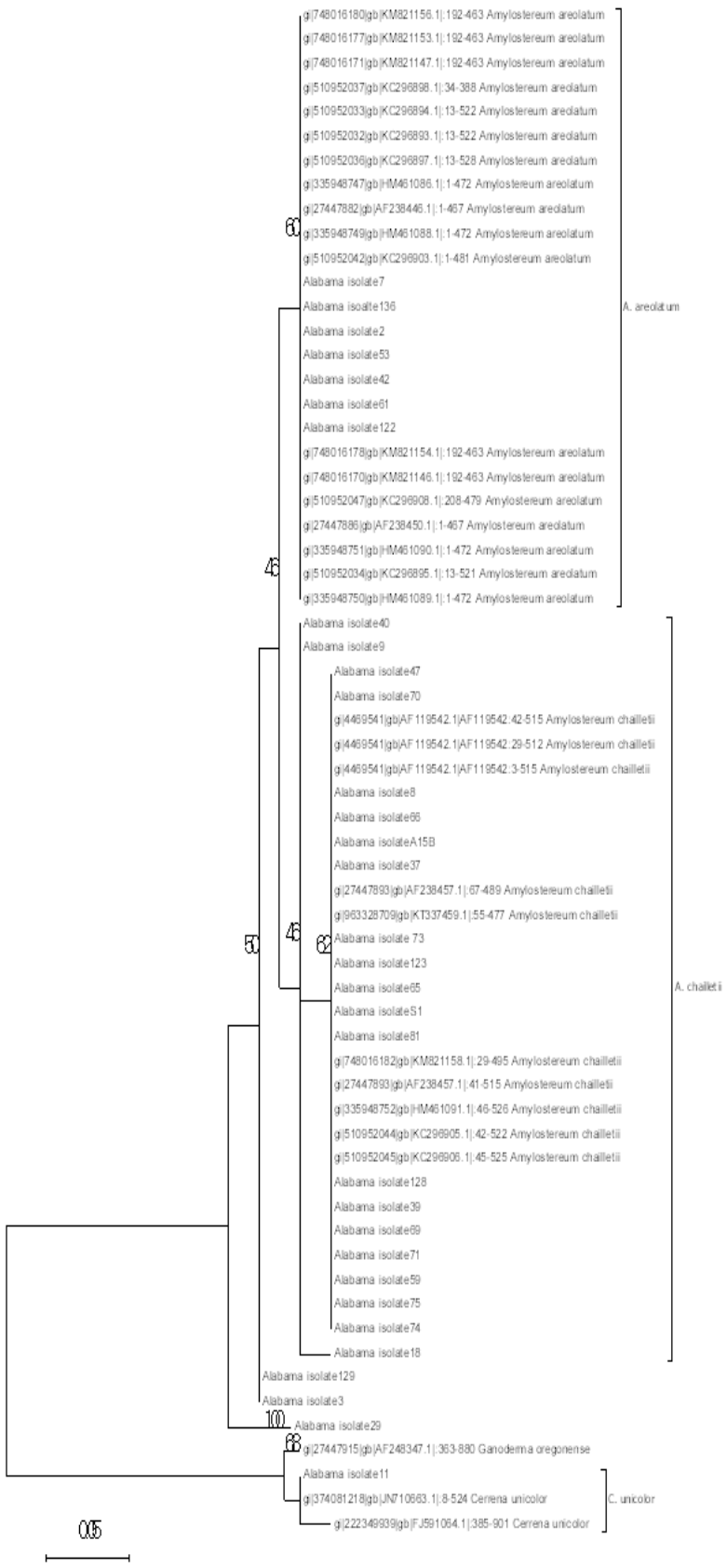

Figure 3.5. Maximum-Likelihood MS tree of 32 fungal samples isolated from woodwasps. Isolate 11 groups with *C. unicolor*. Other isolates are determined to be *A. areolatum* (n=7) and *A. chailletii* (n=21). Three isolates do not fall into a distinct species (3, 29, and 129).

Chapter 4

Effect of growth rate on *Amylostereum* spp. fungus by terpenes

4.1. Abstract

Sirex noctilio is a species of woodwasp native to Europe that has been identified as invasive in Australia, South Africa, South America, New Zealand, and the northeastern United States. This pest has caused significant economic and ecological damage, and in some cases mortality of previously healthy trees. Females attack *Pinus* spp. by drilling into the xylem to oviposit eggs, venom, and a mutualistic fungus, causing trees to begin to die within days of inoculation. Certain chemicals emitted by stressed pines have been observed to serve as chemical attractants to the wasps. As a means of exploring pine resistance to *Sirex* associated fungi, the effect of these mentioned host plant secondary metabolites on the growth of these fungi were tested. Eighteen isolates of *Amylostereum* spp. were grown in saturated atmospheres or in direct contact with pure monoterpenes for 7 days. Fungal growth in the saturated atmosphere was measured on day 7 while the tactile experiment was measured at 3, 5, and 7 days. These experiments showed that certain metabolites such as 4AA, α-Phellandrene, (+) Camphene, and (-) Limonene significantly reduced growth of isolates compared to control treatments. Conversely, α-Pinene and β-Pinene treatments tended to increase growth rates of the fungal isolates. A difference in growth rates between isolates from the northern hemisphere and southern hemisphere also was observed. The treatments (+) α- Pinene and β- Myrcene resulted in the

highest percentage of fungal growth for all isolates tested when comparing fungal growth as a percent area relative to the controls.

4.2. Introduction

Different species of *Amylostereum* fungi are associated with multiple *Sirex* (Hymenoptera: Siricidae) species from around the world, including *A. areolatum* (Chaillet) Boidin, *A. chailletii* (Pers.: Fries) Boidin, *A. ferreum* (Berk. & Curt.) Boidin & Lanquetin, and *A. laevigatum* (Fries) Boidin (Hurley et al., 2007; Slippers et al., 2003). *Amylostereum chailletti* is generally thought to be the species most often associated with the North American native *S. nigricornis* Fabricius, while *A. areolatum* is found in accordance with *S. noctilio* Fabricius. (Nielson et al., 2009). Worldwide, there are 100 species in the family Siricidae. Twenty three species of woodwasps are currently found in North America, including invasive species (Schiff et al., 2006). *Sirex noctilio* is an aggressively invasive species that has been associated with the symbiotic fungus *Amylostereum*, which along with phytotoxic venom causes wood of affected trees to rot (Bordeaux et al., 2014; Hurley et al., 2007). Adult females oviposit *Amylostereum* into the xylem tissue of the tree where it secretes cellulases, giving the siricid larvae a substrate to feed upon (Ayres et al., 2009; Kukor and Martin, 1983b).

Once a tree has been attacked by a *Sirex* spp. wasp, the tree begins to exhibit defensive behavior. This behavior includes the production of a series of commonly produced terpenes by southern pines. Stress chemicals such as α-Pinene and β-Pinene are given off when a tree is under stress, and defense chemicals such as α-Phellandrene, and 4-allylanisole (4-AA), emitted

by healthy pines, and are found in higher concentrations when trees are not under stress (Eckhardt et al., 2009). These chemicals, oleoresins, are found in different concentrations in each species of the *Pinus* genus. When produced, oleoresins of different chemistries have been shown to be capable of both inhibiting and encouraging fungal colonization, depending on species of fungus and chemical compound (Eckhardt et al., 2009; Himejima et al., 1992; Gershenzon and Dudareva, 2007). Paine et al. (1987) states that Phellandrene and Myrcene both have been shown to significantly impact the biota of microorganisms living within the host tree.

Chemicals such as those mentioned above may be artificially used as lure to bait live siricids to traps, indicating that these chemicals are attracting adult siricids to oviposit in stressed pine stands (Barnes et al., 2014). While previous studies have determined the interaction of terpenes with other plant parasitic fungi, there is still a lack of understanding how defense and stress chemicals found in pine trees affect fungal species associated with the invasive species *S. noctilio*. Since this species is rapidly spreading through pine stands in the southern hemisphere, and has been documented in the northeastern United States, a greater understanding of this fungal genus is warranted (Nielson et al., 2009). The primary objective of this study was to determine how different terpenes commonly produced by southern pine trees affect the growth of *Amylostereum* spp. fungi isolated from several locations worldwide.

4.3. Materials and Methods

4.3.1. Isolation

Standard protocol has been established for the dissection of siricids to isolate fungal samples. Thomsen and Harding (2011) describe in detail these standard procedures. They were

kept in a cooler in glass vials from the time of collection to the time of dissection. Wasps were killed with ethyl acetate, and then the exoskeleton was surface sterilized by brushing with 95% ethanol. Wasps were pinned and placed under a dissection microscope. Curved bladed micro-scissors were sterilized, and then inserted between the last tergal and sternal plate on either side of the ovipositor. The abdomen of the wasps was opened and pinned so that the dissector could remove eggs and body contents lying on top of the mycangia and venom sac. The mycangia were removed, gently brushed with 95% ethanol, and plated on a potato dextrose agar (PDA) plate. The mycangia were gently prodded until the spore mass is exuded on the plate. The spore mass was then transferred to another PDA plate. Thomsen and Harding (2011) suggested culturing two to three plates from each mycangia of each wasp dissected. This methodology was followed to obtain the two isolates of fungus from Alabama. The other isolates were obtained from the culture collection at the University of Pretoria, Forest and Agricultural Biotechnology Institute, South Africa.

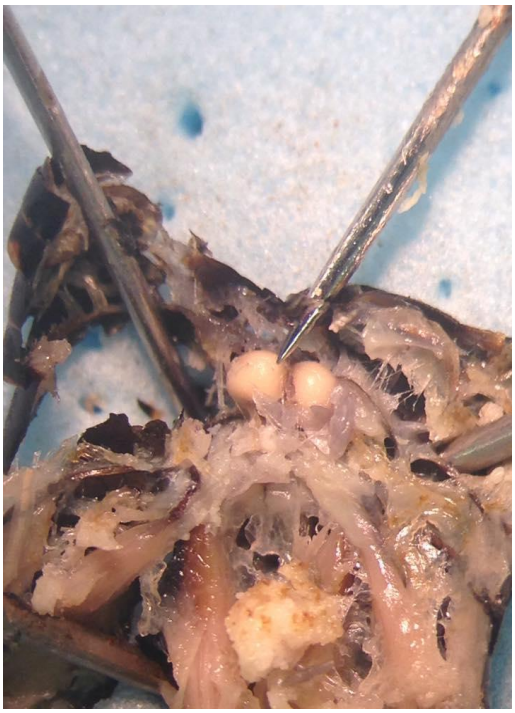

Figure 4.1 Dissected abdomen of a female *S. noctilio* from South Africa that was preserved in 95% ethanol. The pointer draws attention to the two mycangia that house *Amylostereum* spp. spores.

4.3.2. Project design

Inoculation methods and chamber set up are in accordance with Eckhardt et al. (2009). Terpenes utilized included commonly produced stress and defense chemicals in pine trees: (+) α-Pinene, (-) α-Pinene, (+/-) α-Pinene, (-) β-Pinene, β-Myrcene, (+) Camphene, (+) Limonene, (-) Limonene, α-Phellandrene, and 4-AA (Sigma-Aldrich, St. Louis, MO, USA). Two studies were conducted to test how the terpenes affected the growth rates of the fungus via both direct (tactile) and indirect (volatile) contact. For the tactile study, 1 mL of terpene treatment was pipetted onto each PDA (potato-dextrose agar – Difco, Voigt Global Distribution, Lawrence, KS, USA) plate and was allowed to dry before a 4 mm inoculum punched with a cork borer was placed in the center. Isolates used for inoculation can be found in Table 1. The growth of inoculum was traced

onto transparencies at three, five, and seven days after inoculation. The growth of the leading edge of each tracing was measured in millimeters with a digital planimeter (Lasico 1281-12; Lasico, Los Angeles, CA, USA). Six replications of each of the terpene treatments plus six replications of control plates with 1 mL of deionized water added were performed.

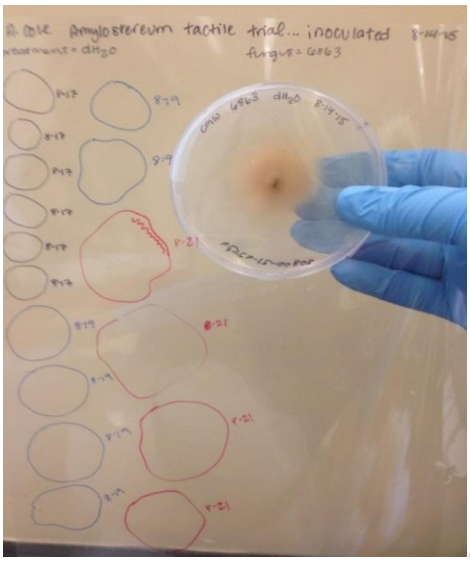

Figure 4.2. Traced hyphae of *Amylostereum areolatum* culture during the tactile trial. The leading edge of the hyphae was traced onto transparency every other day, and was later measured using a digital planimeter

The second study included setting up vapor chambers to test how volatilized chemicals affected the inoculum without direct contact. Glass petri dishes poured with PDA were inoculated with the same isolates from the tactile study, tops removed, and stacked inside a standard paint can (Fig. 4.3a) (Freund Container #1800T01; Freund Container and Supply, Lisle, IL, USA) under sterile conditions. A smaller petri dish containing 2 mL of the tested terpene was placed at the bottom of the paint can without a lid to allow volatilization. Two different control methods were used, one set of cans with 2mL-deionized water, and one set of cans with no added liquid. Cans were then sealed (Fig. 4.3b). Seven days after inoculation, the plates were removed from the sealed cans where the growth was traced onto transparencies and measured with the digital planimeter (Fig 4.3c). Six replications of plates were carried out, arranged so that three

paint can chambers of each treatment were created. The chambers were arranged so that there were two plates of each inoculum randomly placed in each can.

Figure 4.3. Glass plates inoculated with *Amylostereum* spp. were stacked inside paint cans (A) to create atmospheric chambers (B). After the 7 day growth period was completed, plates were removed from paint can chambers (C). The leading edge of hyphae was traced after the seventh day of treatment.

4.3.3. Statistical Analyses

Post- Hoc analysis of growth rate between fungal and chemical treatments was conducted using a Tukey HSD [Honest Significant Difference] test. All statistical analyses were run using the SAS program (SAS 9.4, 2013) and graphs were drawn using STATISTICA (version 13.0, 2015).

Table 4.1 Sources of fungal isolates used in Tactile and Vapor Study, obtained from the culture collection at the FABI, University of Pretoria, South Africa.

Isolate Number	Species	Origin of isolate
CMW 3045	*Amylostereum ferreum*	Brazil
CMW 3309	*A. areolatum*	Unknown
CMW 3310	*A. areolatum*	France
CMW 6863	*A. areolatum*	Australia
CMW 8898	*A. areolatum*	Brazil
CMW 8902	*A. areolatum*	Russia
CMW 13827	*A. areolatum*	South Africa (1)
CMW 15102	*A. chailletii*	Sweden
CMW 15117	*A. laevigatum*	Sweden
CMW 27326	*A. chailletii*	Czech Republic
CMW 27371	*A. areolatum*	Czech Republic
CMW 37116	*A. areolatum*	New Zealand
CMW 37414	*A. areolatum*	South Africa (2)
CMW 37416	*A. areolatum*	South Africa (3)
CMW 40565	*A. areolatum*	Spain
CMW 40874	*A. areolatum*	South Africa (4)
S1	*A. chailletii*	Alabama, USA (1)
N1	*A. chailletii*	Alabama, USA (2)

4.4. Results

The isolates collected from the Northern Hemisphere were slower growing compared to the fungal isolates collected from the Southern Hemisphere in both trials. Alabama isolates of *A. chailletii* (Fig. 4.1, Fig. 4.4) performed similarly to the *A. areolatum* isolates (Figs. 4.2, 4.3, 4.5, 4.6). Chemical treatments 4-AA, (+) Camphene, (-) Limonene, and α- Phellandrene significantly reduced the growth of isolates compared to the control and pinenes. The exception was that β-Myrcene was found to significantly increase growth of fungal isolates for the tactile trial (Figs.4.7, 4.8, 4.9), this trend did not occur in the atmospheric study.

In the atmospheric trial, (+) α- Pinene and β- Myrcene resulted in the highest percentage

of fungal growth compared the control for all isolates of *Amylostereum* spp. (Fig. 4.6). The defense chemical terpenes α- Phellandrene and 4-AA resulted in the greatest reduction of growth of *A. areolatum* in this trial (Figs. 4.6, 4.7). The enantiomers of α- Pinene and β- Pinene showed a greater increase in growth in the atmospheric trial compared to the tactile trial.

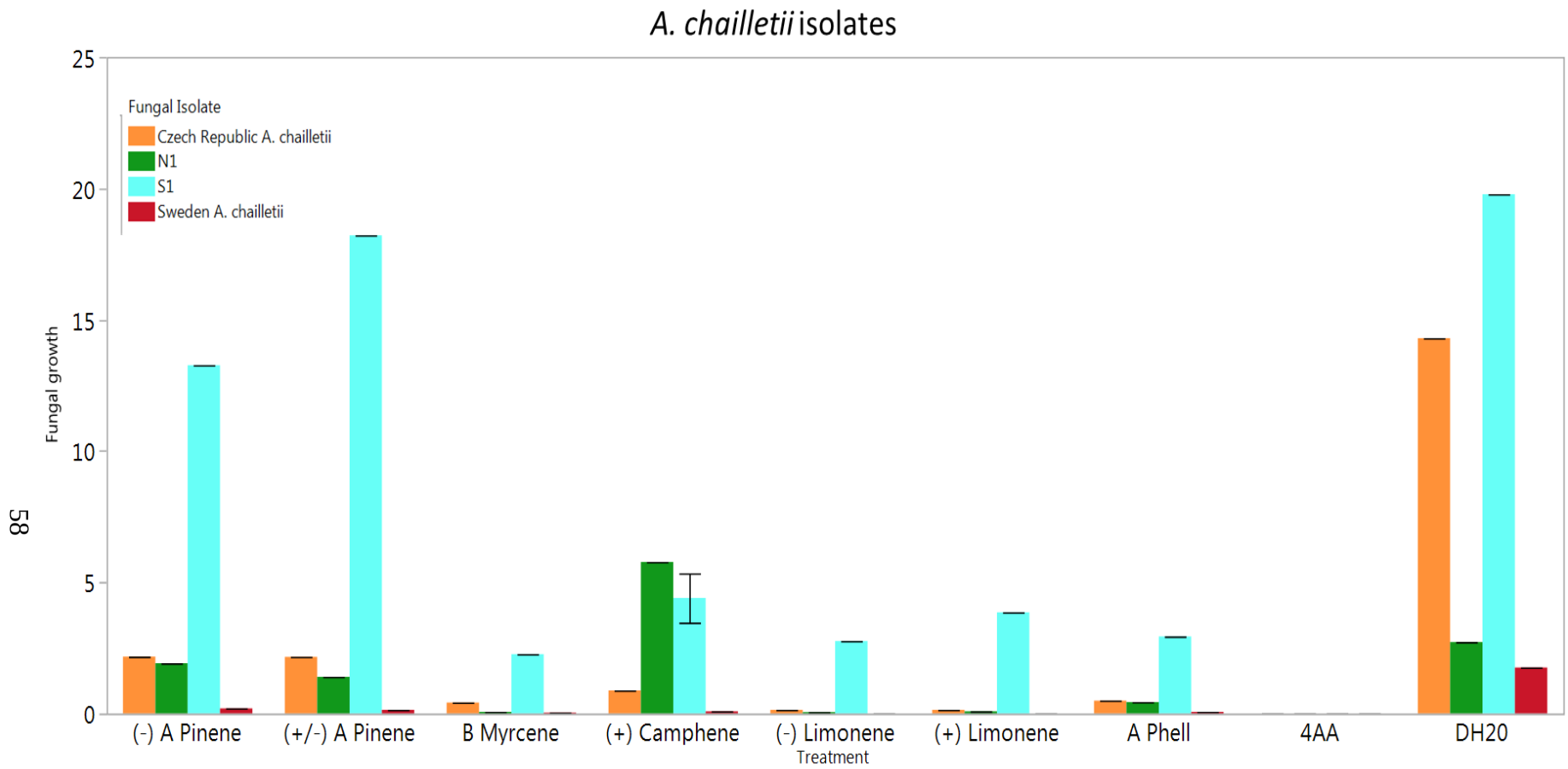

Figure 4.4. Effects of the atmospheric study on unknown isolates from *Sirex nigricornis* trapped in Tuskegee, Alabama. Alabama 1 is isolate S1, and Alabama 2 is isolate N1, both *A. chailletii*.

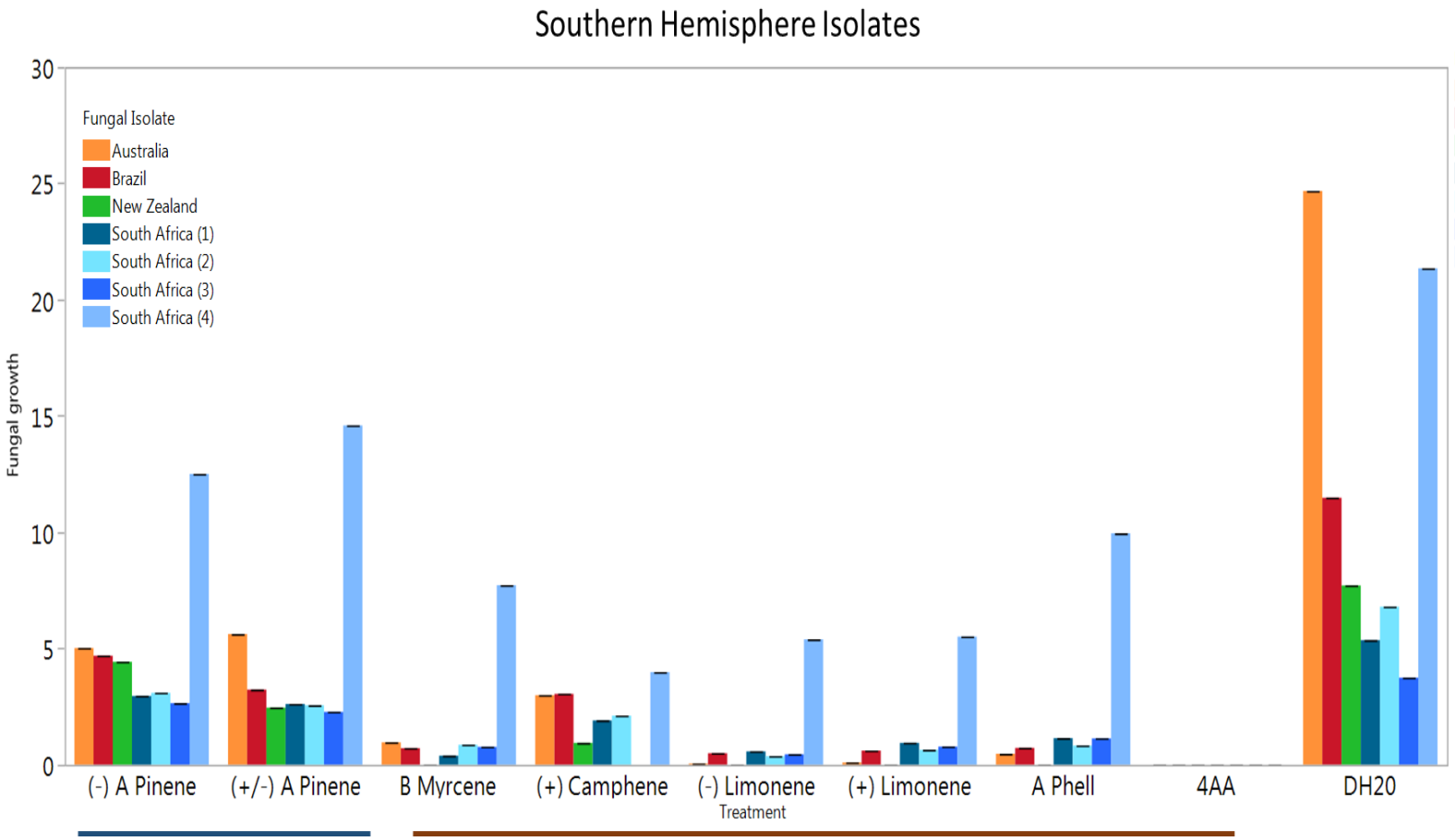

Figure 4.5. Effects of the atmospheric study on isolates of *Amylostereum areolatum* from the Southern Hemisphere. Blue bar on left indicates stress chemicals, while maroon bar on right indicates defense chemicals. B-Myrcene and 4-AA treatments significantly reduced the growth of hyphae in comparison to the dH_2O control.

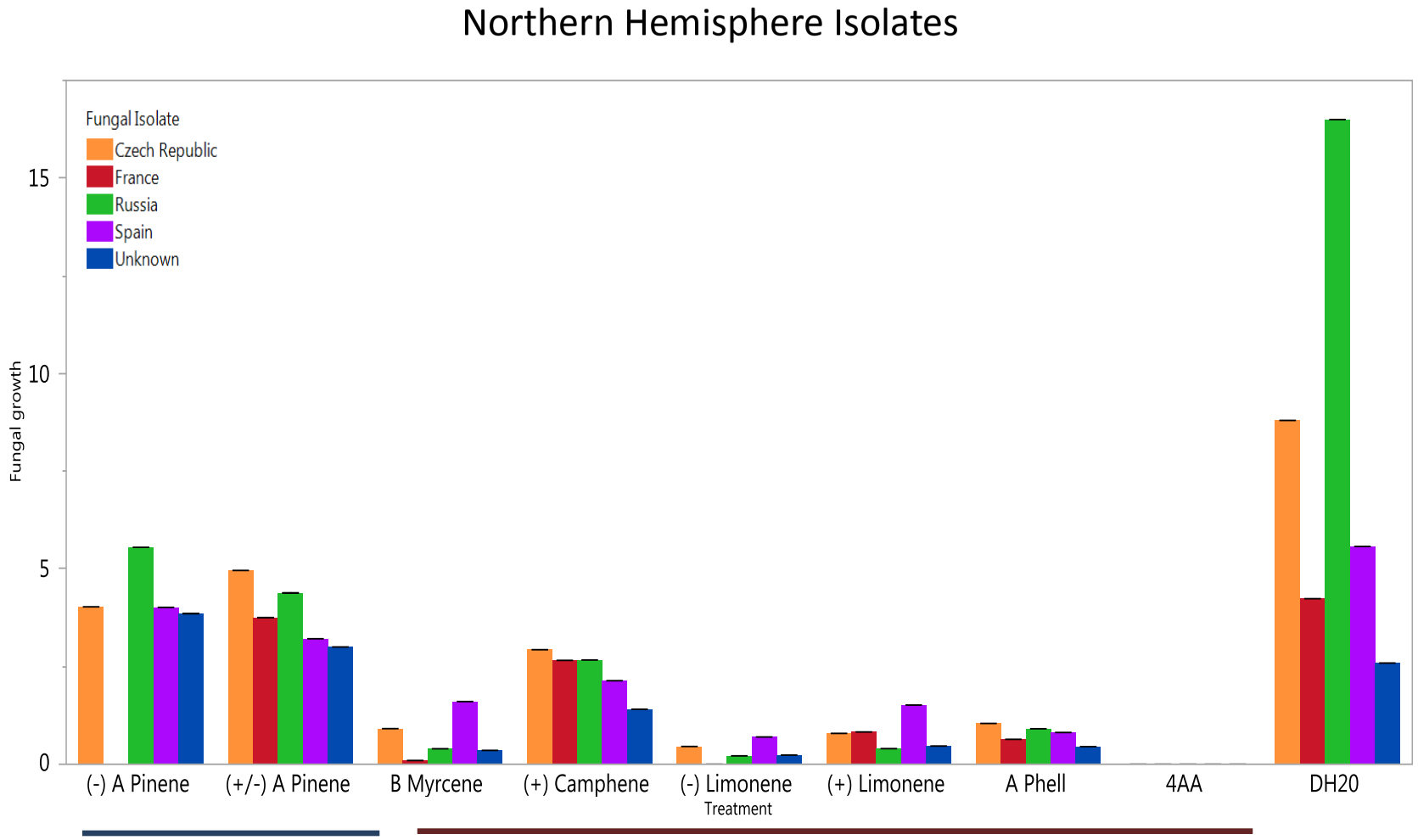

Figure 4.6. Effects of the atmospheric study of *A. areolatum* isolates from the Northern Hemisphere. Isolates grow slower than Southern Hemisphere isolates. Blue bar on left indicates stress chemicals, while maroon bar on right indicates defense chemicals. B-Myrcene and 4-AA treatments significantly reduced the growth of hyphae in comparison to the dH_2O control.

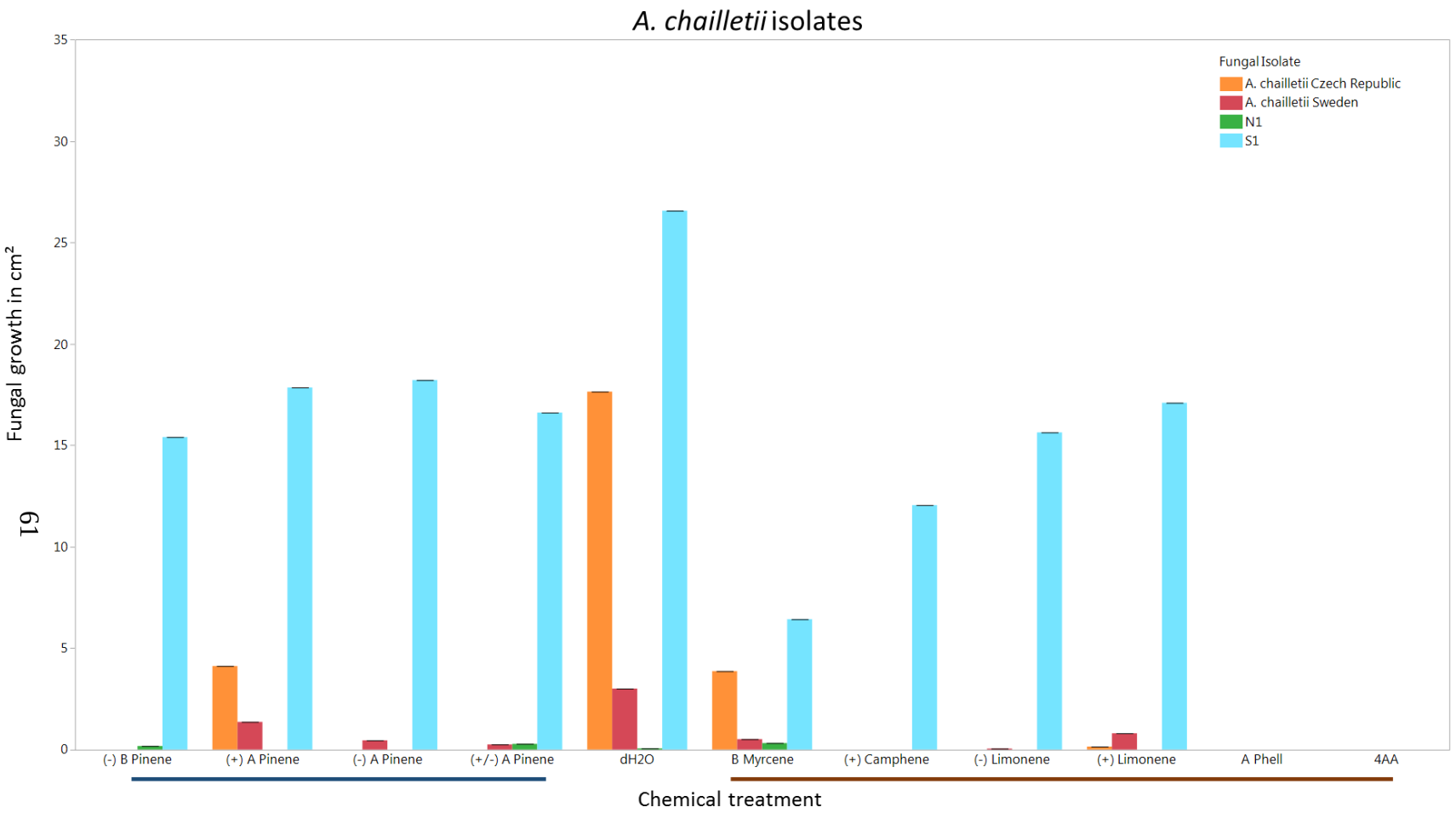

Fig. 4.7 Effects of the tactile study on unknown isolates from *S. nigricornis* trapped in Tuskegee, Alabama. Alabama 1 represents isolate S1, Alabama 2 represents isolate N1. Treatments α-Phellandrene and 4-AA significantly reduced growth in both isolates.

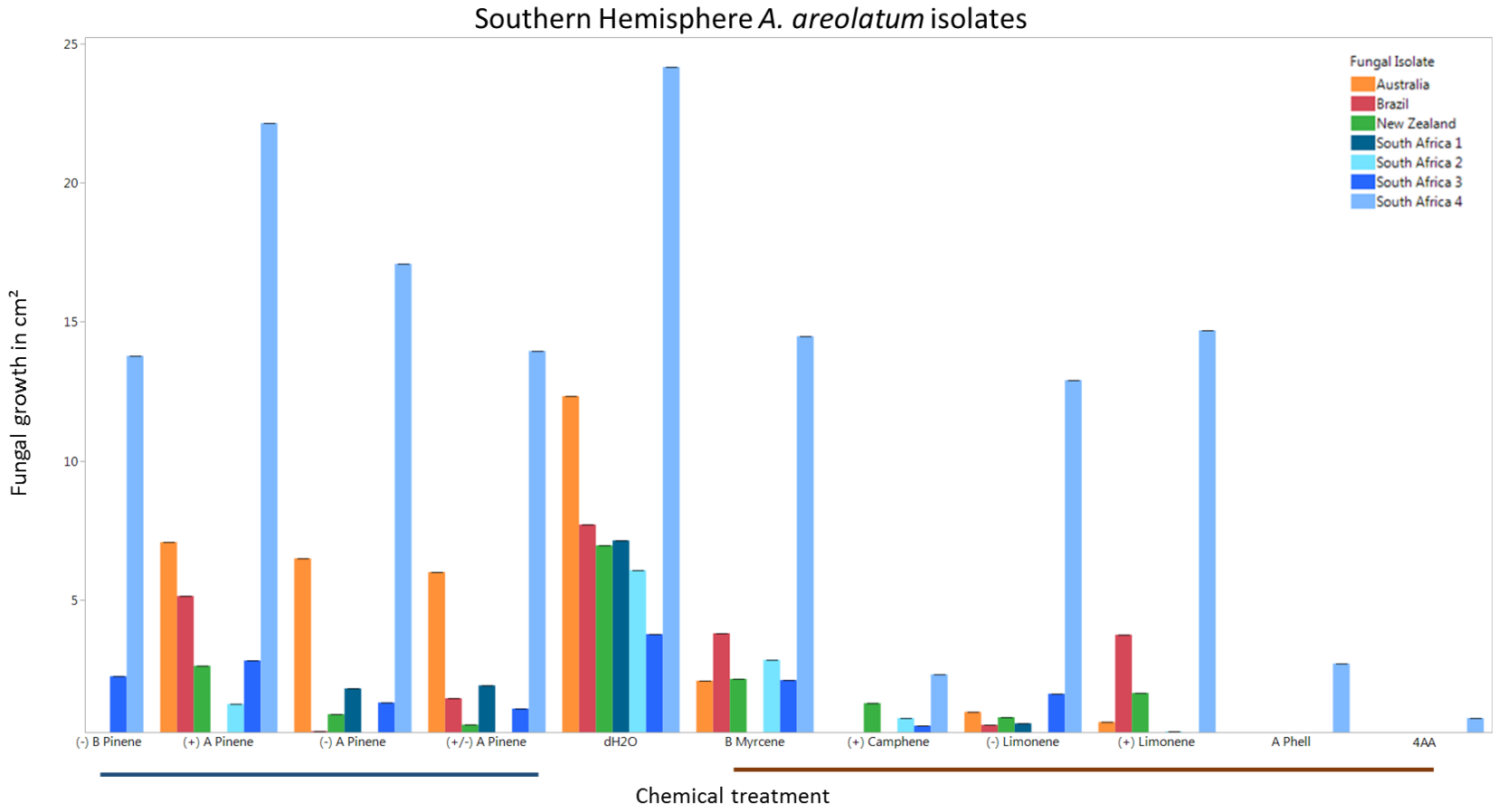

Figure 4.8. Effects of the tactile study of *A. areolatum* isolates from the Southern Hemisphere. Blue bar on left indicates stress chemicals, while maroon bar on right indicates defense chemicals. (+) Camphene, (+) Limonene, and 4-AA treatments significantly reduced the growth of hyphae in comparison to the dH_2O control.

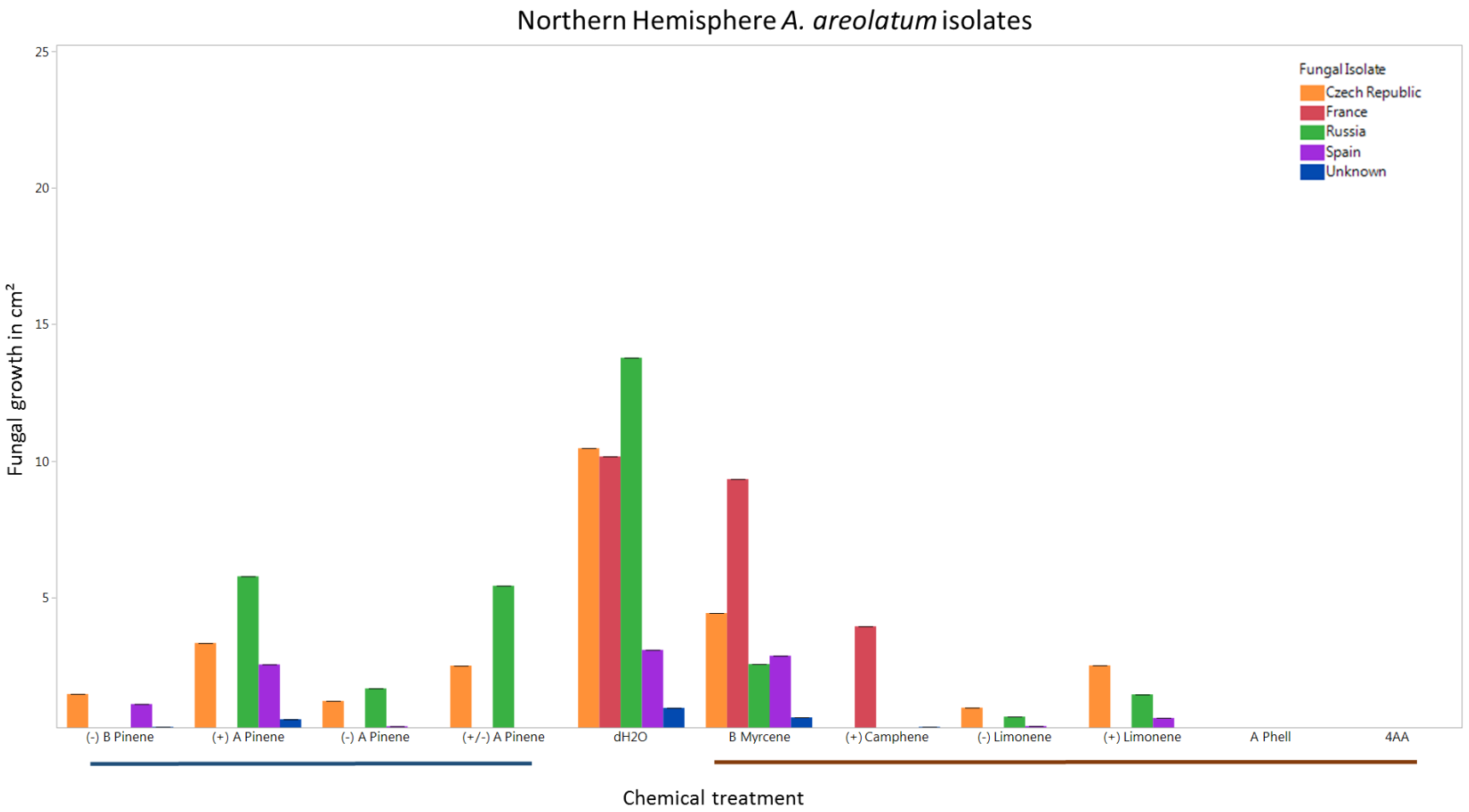

Figure 4.9. Effects of the tactile study on isolates of *A. areolatum* from the Northern Hemisphere. Blue bar on left indicates stress chemicals, while maroon bar on right indicates defense chemicals. (+) Camphene, (+) Limonene, α-Phellendrene, and 4-AA treatments significantly reduced the growth of hyphae in comparison to the dH_2O control.

4.5. Discussion

For the majority of the results, the volatile and tactile trial results were concurrent. The exception to these findings was the way that the fungus responded to β -Myrcene. In this study, growth of all *Amylostereum* isolates were significantly reduced by the terpenes deemed defense chemicals, especially 4-AA and α-Phellandrene. The terpenes deemed stress chemicals, such as both enantiomers of α- Pinene and (-) β- Pinene, tended to increase the growth of the fungal cultures when compared to the control treatment in the volatile trial. Eckhardt et al. (2009) also identified these same chemicals as reducing and stimulating fungal growth, respectively. Conversely, Klepzieg (1994) found that the presence of α- Pinene could be linked to reduced growth rates of fungal isolates. Those findings are more in line with the results of the tactile trial, which yielded a reduced growth of fungus on α- Pinene and (-) β- Pinene. Further studies are warranted in looking at how these chemicals directly affect fungal growth. It is hypothesized that this is partially due to the direct contact with these chemicals, as they were harsh enough to melt the plastic petri dishes. If this experiment were to be performed again, glass petri dishes may be recommended.

This study yielded results that the same terpenes that are known to be emitted when trees are already under stress and are known to draw insects into stressed stands are the same compounds that increase growth rates of the fungal species associated with the genus *Sirex*. The terpenes α- Pinene and β- Pinene are commonly used to trap and bait woodwasps (Barnes et al., 2014), and are the same treatments that allowed for the highest percentage of fungal growth in relation to the control treatments. This is an unfortunate cycle, which leads to attracting more woodwasps to the area to colonize the stressed trees. These trees are then a hospitable

environment for the fungal mycelia to colonize. Conversely, defense chemicals found in higher concentrations in healthy trees significantly reduced the growth of the same isolates of *Amylostereum* than the stress terpenes found in higher concentrations in stressed trees.

4.6. Conclusions

It could be hypothesized that certain terpenes, such as 4-AA and α-Phellandrene could be used to control growth rates of *Amylostereum* species. It seems as though these chemicals would be naturally suppressing the growth of this fungus when inoculated into a pine tree, if the tree is healthy and not stressed. It would most likely not be feasible to try to use these chemicals to control growth rates if applied artificially, as these chemicals are very expensive. Identifying chemicals that could control growth of *Amylostereum* spp. is substantial because this pathogen rots the affected trees from within, degrading the tissues of the tree, and can eventually lead to the death of the tree (Coutts, 1969). Currently, the procedure to control the *Sirex / Amylostereum* complex is to control wasp populations with various biological control agents, including inoculating infested stands of pine trees with *Deladenus* spp. nematodes (Ryan et al., 2012a).

Chapter 5

Competitiveness of *Amylostereum* spp. fungi against *Leptographium* spp. fungi

5.1. Abstract

Amylostereum spp. are basidiomycetes, which cause white rot fungi of pine trees. Spores of these fungi are vectored by *Sirex* spp. woodwasps, who infect host trees by ovipositing eggs into the xylem of affected trees. The invasive complex associated with *Sirex noctilio* and *Amylostereum areolatum* (native to Europe and Northern Africa) has been devastating to planted non-native pine forests in the Southern Hemisphere, but has not been problematic in North America where *S. noctilio* also has been introduced. While there is currently no evidence that *S. noctilio* is in the southeastern United States, studies are being carried out to determine how its symbiont, *A. areolatum*, might interact with other fungi that are already present within the southern pine ecosystem. For this study two species of *Leptographium* were chosen, since they are commonly found in industrial pine stands in Alabama. Both are ascomycetous root pathogens that behave differently than *Amylostereum* spp., and also would affect the overall tree vigor if inoculated into the same substrate. Isolates of *Amylostereum* spp. from around the world were plated on petri dishes with isolates of *Leptographium terebrantis* and *Leptographium procerum*. Growth rates were determined by measuring the leading edge of mycelia with a planimeter every other day for two weeks after inoculation. In most cases, *Leptographium* isolates outcompeted

Amylostereum isolates, and in some cases completely overgrew them. This study suggests that *Amylostereum* spp. likely would not outcompete *Leptographium* spp. in a forest setting, although further studies need to be undertaken to see how the two fungi would compete in situ.

5.2. Introduction

Amylostereum is a genus of white rot fungi that affects *Pinus* spp. This genera is associated with *Sirex* spp. woodwasps, the obligate symbiont that vectors *Amylostereum* spores from tree to tree. This relationship between wasp and fungi is crucial to the development of the siricid larvae. *Amylostereum* hyphae decreases the moisture content of the tree, rotting the wood from within, until it is hospitable for larval development. Historically, *A. chailletii* (Pers.) Boidin has been linked with *S. nigricornis*, the species of woodwasp native to the southeastern United States. Both insect and pathogen are associated with declining stands, normally attracted to dead or dying wood. More recently, studies have shown that *S. nigricornis* F. might now be associated with *A. areolatum* (Chaillet ex. Fr) Boidin, a pathogen associated with the invasive *S. noctilio* F.. The pairing of *S. noctilio* and *A. areolatum* are different than other woodwasp-fungal associations. The two species colonize fairly healthy trees, eventually causing mortality if the infestation is heavy.

Since commercial pine plantations are a sizable industry in the southeastern United States, many independent collection efforts have been waged to try to better understand woodwasp populations in this region. Recent studies (Wahl, Chapter 2; Barnes et al., 2014; Johnson et al., 2013) have surveyed southeastern United States forests, finding mostly native siricids. No known captures of *S. noctilio* have been reported in the area, but this species has been identified in the northeast United States since 2004 (Hoebeke et al., 2005). Other non-

native siricids have been identified in the United States, specifically *Eriotremex formanosanus* Matsumura in the Southeast (Smith et al., 1996).

One objective of this study is to determine how isolates of native and non-native *Amylostereum* might compete with common root pathogens found in the Southeast (*Leptographium terebrantis* (Kendrick) Wingfield and *L. procerum* S.J. Barras & T.J. Perry). These and similar root pathogens are factors in a phenomenon known as loblolly pine decline (Eckhardt et al., 2007). The fungi that are part of this syndrome are vectored by root feeding beetles, such as *Hylastes* spp., *Hylobious pales* (Herbst), and *Pachylobious picivorous* Germar in the southeast (Matusick and Eckhardt, 2010), as well as phoretic mites (Hofstetter and Moser, 2014).

These bark and ambrosia beetles are commonly found in the same forest ecosystem that siricids inhabit, and have the potential to be attracted to the same sort of pine material as female *Sirex* spp. (Ryan et al., 2012a). Both bark beetles and woodwasps tend to be attracted to volatilized chemicals given off by stressed trees (Franceschi et al., 2005; Böröczky et al., 2012). These chemicals not only give the insects an idea of which trees might be suitable substrate, but also have been shown to affect the growth of the associated fungi the insects inoculate the tree with (Eckhardt et al., 2009; Wahl, Chapter 4). Gaining a better understanding of how these complexes overlap in ecosystems is crucial to better manage pine forest systems.

An additional objective of this study is to determine the relationships between isolates of *Amylostereum* used in the competition assay. A method of determining relationships between isolates of fungi in the same species is testing for Vegetative Compatibility Groupings (VCGs). This is a test where multiple inoculums of the same species are grown on the same plate to

determine how hyphae interact with each other. This is a useful method for determining relationships of isolates, as clones form the VCGs. Recognizing that multiple isolates are clones can allow pathways of fungal introduction to be traced back to its origins (Vasiliauskas et al., 1998).

5.3. Materials and Methods

5.3.1. Competition Study Inoculation

Amylostereum areolatum and *A. chailletii* isolates from around the world were obtained from the culture collection at the Forestry and Agricultural Biotechnology Institute at the University of Pretoria, South Africa. Two isolates of *A. chailletii* were isolated from live female *S. nigricornis* wasps in Auburn, Alabama, United States. *Leptographium* isolates were from the culture collection of the Forest Health Dynamics Lab, Auburn University, isolated in previous chapters of this thesis.

Isolates of *Amylostereum* were inoculated onto plates of Potato Dextrose Agar (PDA) four days prior to adding the *Leptographium* inoculums to allow the *Amylostereum* isolates time to begin to grow in accordance with the methods of Ryan et al. (2011). *Leptographium* isolates were plated directly across from the growing *Amylostereum* isolate at the edge of the plate, so that they would have to grow towards each other over the duration of the study (Fig. 5.1).

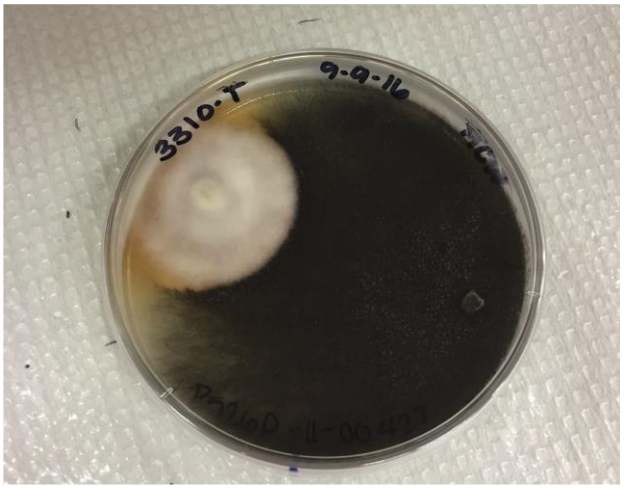

Figure 5.1 *Amylostereum areolatum* isolate from France (white hyphae) plated with *Leptographium terebrantis* isolated in Alabama (green hyphae).

5.3.2. Competition Study Measurement

Plates were kept in dark cabinets in a temperature controlled laboratory, to mimic the growth environment within the bole of a pine tree. The hyphal growth was traced onto transparencies, starting two days after the inoculation of the *Amylostereum* isolates. The plates were traced every other day for two weeks, or until the plate was completely grown over by one of the isolates. The surface area (cm^2) was computed by measuring the traced hyphal growth with a digital planimeter (Lasico 1281-12; Lasico, Los Angeles, CA, USA) as in Eckhardt et al., (2009).

5.3.3. Statistical Analyses

Statistical analyses were performed on the competition plates using SAS version 9.3 (2010; SAS Institute, Inc., Cary, NC). A repeated measures one-way ANOVA was performed on

Amylostereum isolates plated against each of the *Leptographium* spp. An ANOVA between isolates of *Amylostereum* spp. also was performed. A post hoc Tukey's Standardized range Test, and a Paired T-tests were performed.

5.3.4. Vegetative Compatibility Grouping Study

The same *Amylostereum* spp. isolates used in the competition assay were plated in a similar fashion as the previously described plate study, this time placing different isolates of the same species on the same plate. Only isolates of the same species were plated together, in the cases where the species were determined before the assay. VCGs were scored 0 if a barrier formed between the hyphae of the two isolates, or 1 if the hyphae grew into each other. If a score of 1 was given, it was assumed that the two isolates were clonal. Isolates N1, S1, and 15B from Alabama were tested against each of the other isolates, regardless of species.

5.4. Results

5.4.1. Competition Study

Amylostereum isolate growth rates differed significantly from each other. Certain isolates of *Amylostereum* such as 15102 (*A. chailletii*, Sweden) had no significant growth over the duration of the study ($P=0.2790$), whereas 15B (*A. chailletii* from Alabama) grew significantly at each of the measurements ($P<0.0001$) (Fig. 5.2). As expected, *Leptographium* growth was more uniform, and did not differ significantly from plate to plate, but did differ significantly over time (Fig 5.3).

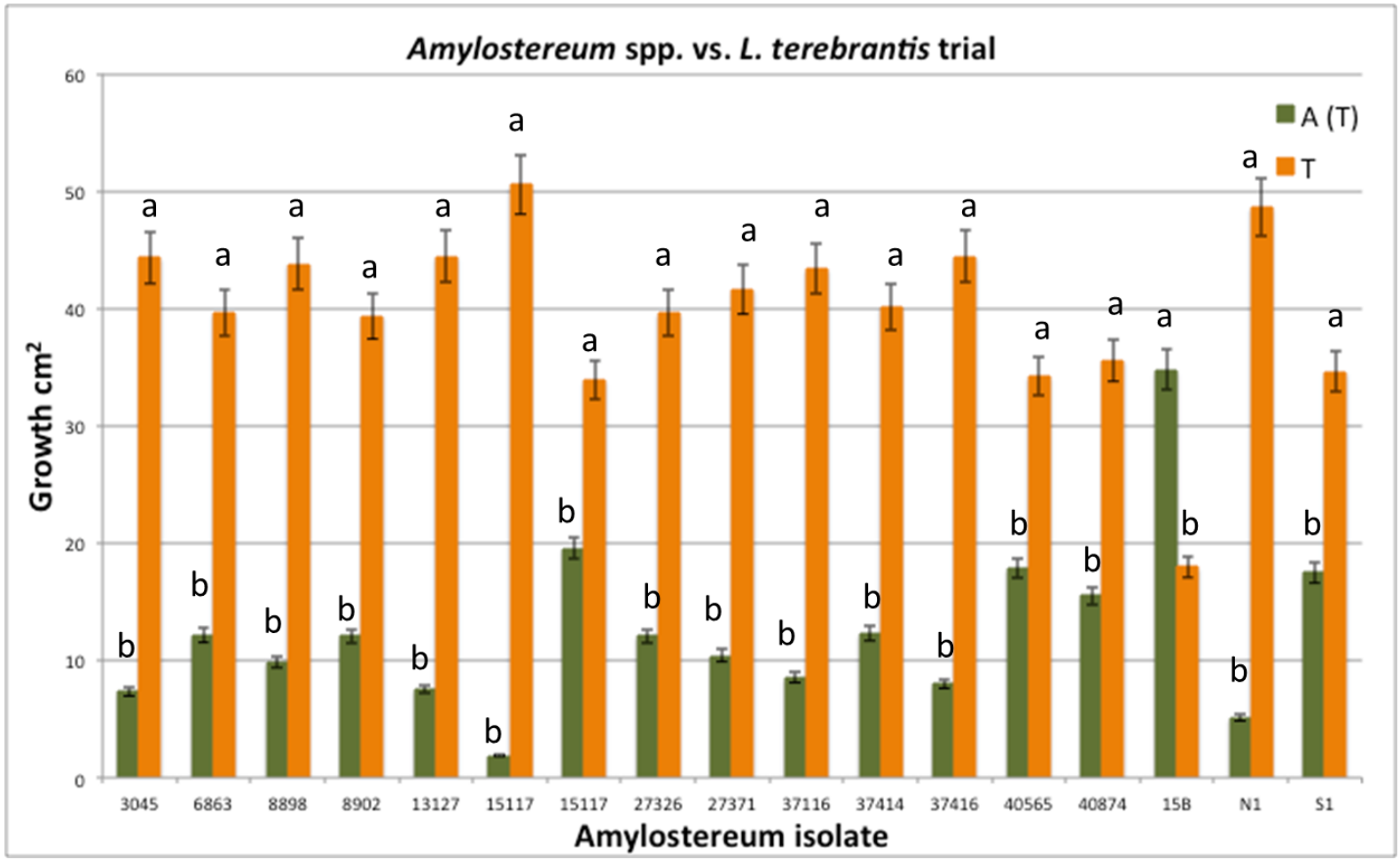

Figure 5.2. *Amylostereum* spp. growth in cm² plated against *Leptographium terebrantis.* Isolate 15B (*A. chailletii*) has a significantly higher growth rate through the duration of the study.

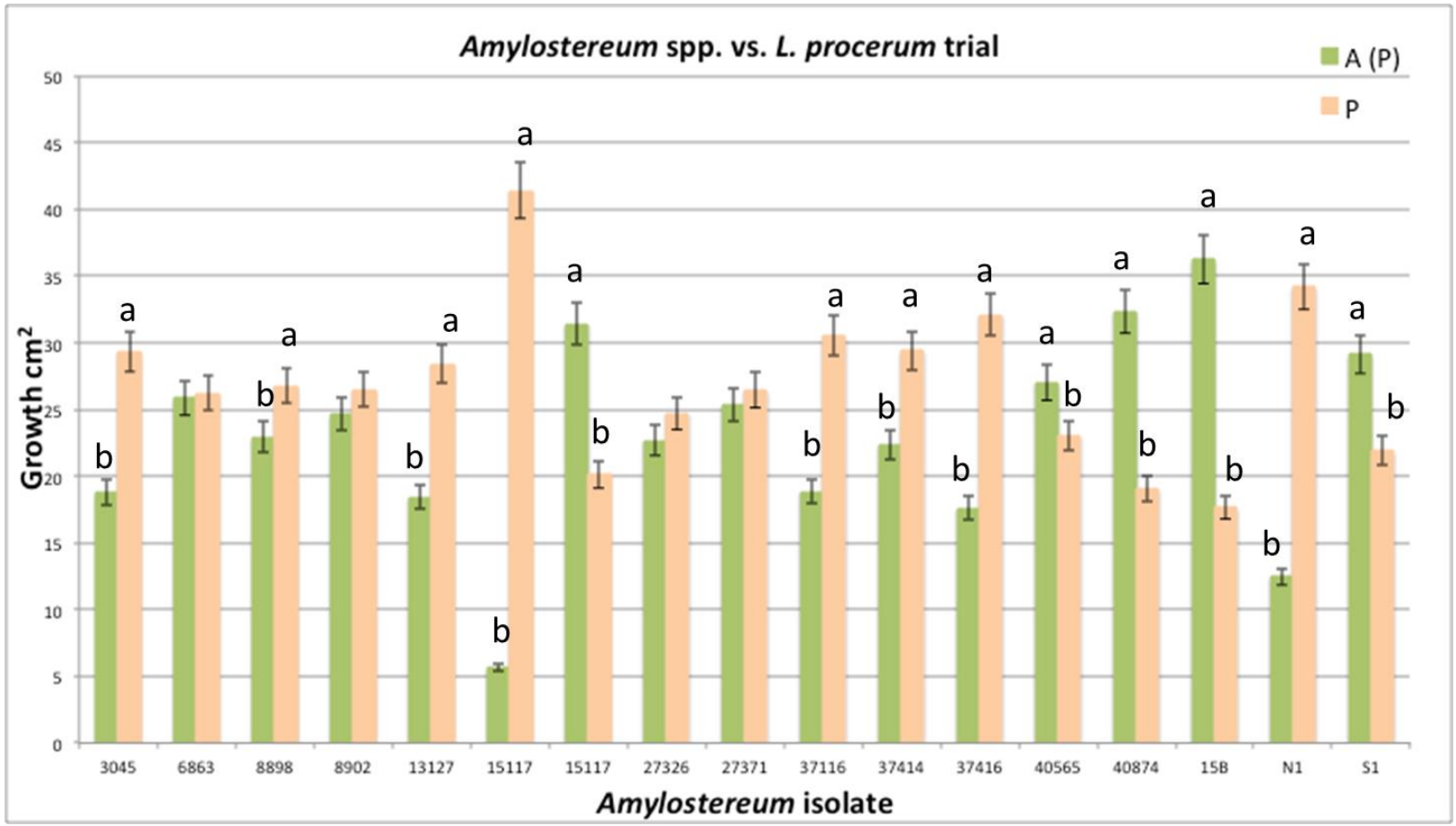

Figure 5.3. *Amylostereum* spp. growth in cm² plated against *Leptographium procerum.* Isolate growth rates differ significantly in response to their competitors.

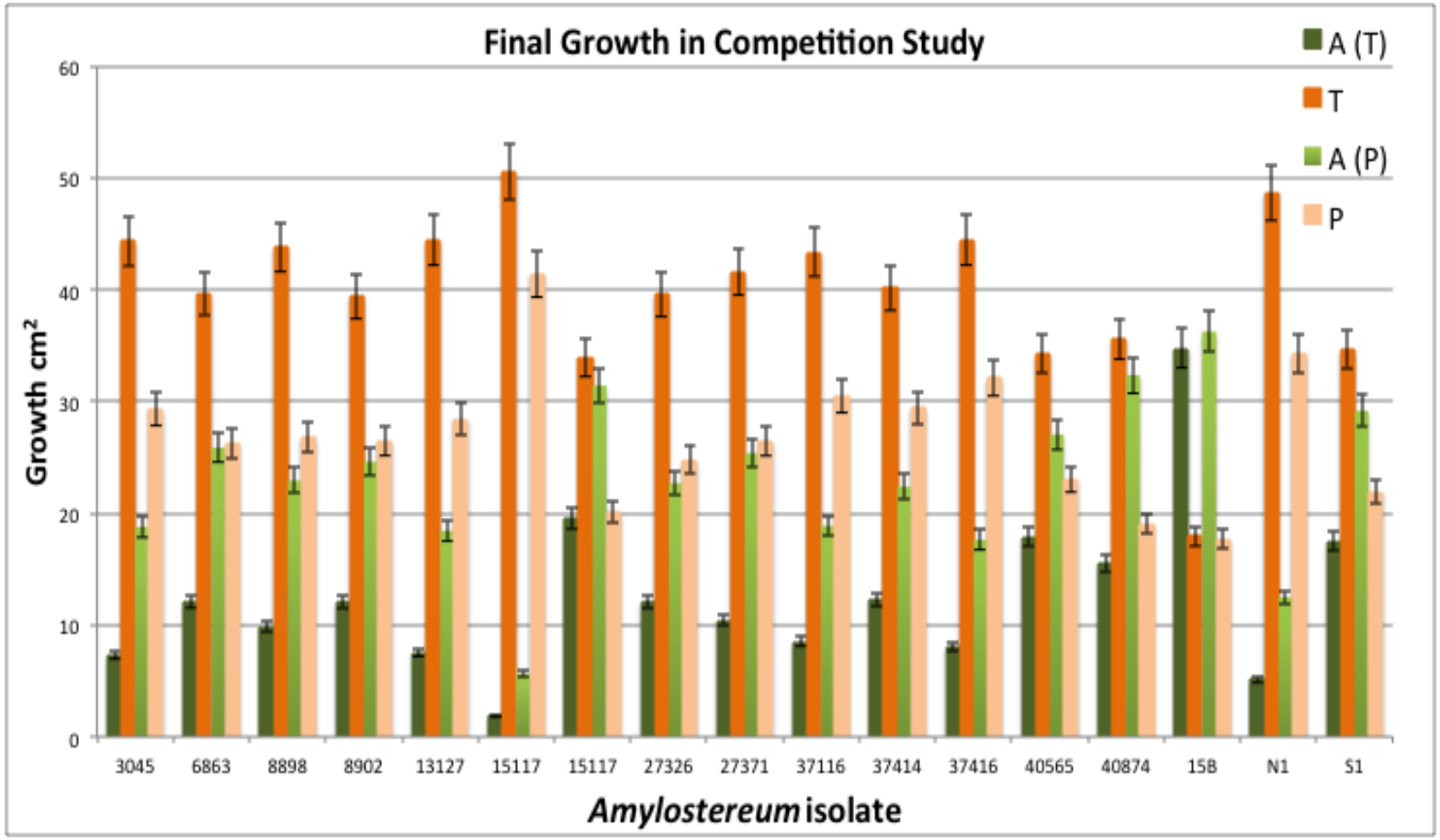

Figure 5.4. Overall results from competition study. Treatment effect from *Leptographium* spp. can be seen.

5.4.2. Vegetative Compatibility Groupings

Table 5.1. Results from the VCG plate study. 1 represents that the two isolates paired together as a VCG, suggesting they are clones. 0 represents that the two isolates did not form a VCG, and therefore are not clonal.

	3309	3310	6863	8898	8902	13827	27371	37116	37414	37416	40565	40874	S1	N1	15B	15102	27326
3309	--																
3310	0	--															
6863	0	1	-														
8898	0	1	1	--													
8902	0	0	0	0	--												
13827	1	0	0	0		--											
27371	0	0	0	0	1	0	--										
37116	0	0	0	0	0	0	0	--									
37414	0	0	0	0	0	0	0	1	--								
37416	0	0	0	0	0	0	0	1	1	--							
40565	0	0	0	0	0	0	0	0	1	1	--						
40874	0	0	0	0	0	0	0	0	1	1	1	--					
S1	0	0	0	0	0	0	0	0	0	0	0	0	--				
N1	0	0	0	0	0	0	0	0	0	0	0	0	1	--			
15B	0	0	0	0	0	0	0	0	0	0	0	0	0	0	--		
15102	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	--	
27326	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	--

Isolates of *A. areolatum* formed three compatibility groups. All isolates from Alabama, *A. chailletii* (S1 and 15B), and the unknown N1 were not in the same vegetative compatibility groups as other isolates used.

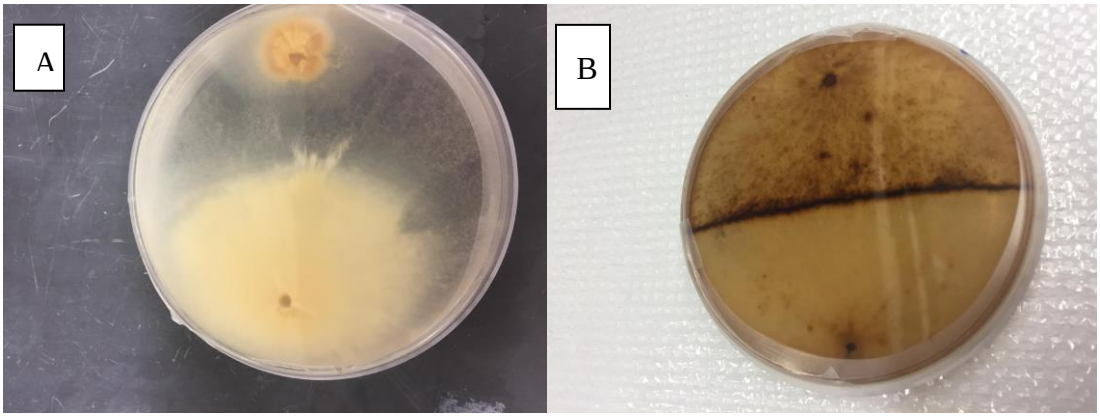

Figure 5.5. VCG trial results. (A) Isolates 37414 and 37416 form a VCG, suggesting they are clonal. (B) Isolates 6863 and 8902 have boundary separating hyphae, and do not form a VCG, suggesting they are not clonal.

5.5. Discussion

In most cases, *Leptographium* isolates significantly outcompeted *Amylostereum* isolates, and in some cases completely overgrew them. *Leptographium* spp. performed in a more uniform manner, which is to be expected, as only one isolate of *L. terebrantis* and *L. procerum* were used throughout the duration of the study. Growth rates of *Leptographium* isolates were significantly higher overall than *Amylostereum* competitors during the first two growth measurements ($p=0.0003$, 0.0009 respectively). In some cases, *Leptographium* isolates overgrew their

Amylostereum competitors, causing the *Amylostereum* surface area to decrease near the end of the study.

The isolate of *L. terebrantis* had significantly higher growth rates than most isolates of *Amylostereum* (all except 15B), while the results from the *L. procerum* trial varied much more (Fig. 5.4). Certain isolates of *A. areolatum* (3310, 8898, 8902, 13127, 37116, 37414, 37416) did not differ significantly overall in growth from the *L. procerum* isolate (Fig. 5.4). Interestingly, these are from three different VCG groupings. Isolate 13127 and 3309 are from the same VCG, yet did not behave in the same means when paired with *L. procerum*. This is unsurprising, as *L. procerum* does not tend to be as severe a pathogen (Eckhardt et al., 2004). In previous studies, *L. procerum* was found to be a less virulent competitor than *L. terebrantis* (Wingfield, 1986).

One isolate of *Amylostereum chailletii*, 15B isolated from a female *S. nigricornis* in Auburn, Alabama outcompeted both the *L. terebrantis* ($p<0.0001$) and *L. procerum* ($p<0.0001$) in all replications of the study. The performance of this isolate was an outlier from the normal behavior of *Amylostereum* isolates used in this study, as well as the findings of Ryan et al. (2011). This growth pattern differed even from the other *A. chailletii* isolates from the same area. This difference observed could be attributed to the fact that these two isolates were found in the same area, in arguably similar environmental conditions.

In the VCG study several distinct compatibility groupings were determined (Fig. 5.5). Unfortunately, this test was inconclusive to try to trace isolates of *A. chailletii* from Alabama back to a point of origin. This uniqueness would suggest that these isolates are endemic to the area where they were naturally located. Interestingly, the two isolates from Alabama that are molecularly confirmed as *A. chailletii*, 15A and S1, (Wahl, Chapter 3) did not form a VCG.

However, isolate S1 did form a VCG with N1, the unknown isolate, which suggests that this is the same clone of *A. chailletii.* This theory supports the different reactions of isolate 15B versus S1 and N1 in the *Leptographium* trials.

5.6. Conclusion

This study shows that non-native isolates of *Amylostereum* spp. fungi tend to be poor competitors to *Leptographium* spp. that are found in the southern pine ecosystem. One isolate of *A. chailletii* from Alabama significantly outcompeted both of the *Leptographium* spp. isolates. This was especially surprising, as *Amylostereum* spp. are generally thought of as slow growing decomposers in a system. The more economically damaging *A. areolatum* when coupled with the invasive *S. noctilio* has been shown to cause eventual mortality of trees, but that is typically not the role of *A. chailleti* in the southern pine ecosystem.

Isolates of *A. chailleti* from Alabama did not all form a vegetative compatibility group, suggesting that the three isolates are not clonal. This is further supported by the difference in growth rates when in competition with a *Leptographium* spp. inoculum.

Chapter 6

Summary and Conclusions

6.1. Siricids in the Southeast

Trapping for siricids in various sites throughout the State of Alabama yielded three species of woodwasps: *S. nigricornis*, *T. columba*, and *U. cressoni*. The flight seasons observed tended to be fairly uniform with what was previously reported in the literature (Haavik et al., 2013; Hartshorn et al., 2015). Bark and ambrosia beetle bycatch from siricid traps were kept and cataloged, to determine if these beetles could compete with siricids for material to lay eggs in. Bark and ambrosia beetle populations tended to peak earlier in spring and fall, before the flight season of mature *S. nigricornis*. To greater understand flight phenology of *S. nigricornis* in Alabama, further trapping should be conducted in a wider variety of habitats. These surveys also should be conducted for more than one or two flight seasons to gain a true understanding of *S. nigricornis* population patterns.

No invasive *S. noctilio* were captured through the duration of the surveys. This does not necessarily prove that *S. noctilio* is not currently found in the southeast, but suggests that if it is in the area, population levels are still low. The idea that *S. noctilio* could be established in Alabama, but have low enough populations to remain undetected could be explained by the

hypothesis of high levels of competition for substrate for larvae to develop in.

If *S. noctilio* were to be found in Alabama, they would likely attack healthy trees, as they have been seen to do in other ecosystems where they have been introduced. Once attacked, the trees would become stressed, and therefore open to further secondary beetle and *S. nigricornis* attacks in the bole of the tree.

Further work could be done to determine how *S. noctilio* larvae could be affected by other woodwasp or beetle larvae in the same area of the tree, or how different associated fungi would affect growth of larvae. *Sirex noctilio* might be able to colonize healthy trees in either a natural forest or planted pine habitat, but then inevitably a secondary colonization would occur by *S. nigricornis* and beetle species. Future experiments could be undertaken to rear *S. noctilio* larvae in wood material that has been infected with *Leptographium* spp, *Ophiostoma* spp., and other fungal pathogens associated with the bark and ambrosia beetles found in the southeast.

6.2. *Amylostereum* spp. and their role in Southeastern forests

Two species were confirmed to be in association with *S. nigricornis* throughout this study, *A. areolatum* and *A. chailletii*. These relationships are in accordance with Wooding et al. (2013) and Olantinwo et al. (2013). One further species was determined through molecular analysis, *C. unicolor* isolated from *T. columba*. Multiple isolates from around the world of both species of *Amylostereum* were utilized in growth studies: how hyphal growth is affected by terpenes, how *Amylostereum* spp. compete with *Leptographium* spp., and how

these isolates form vegetative compatibility groupings.

An effect of growth rate on *Amylostereum* spp. hyphae was observed when put into direct or in direct contact with terpenes emitted by pines. Certain defense chemicals like (+) Camphene, α-Phellendrene and 4-AA were shown to significantly reduce growth rates of hyphae in comparison to a dH_2O control. Certain stress chemicals, such as α –Pinene and α – Pinene seemed to have a positive effect on hyphae growth rates, although no significant difference was detected. β- Myrcene affected growth of hyphae differently when in direct or indirect contact. Further studies could be conducted to determine why β- Myrcene affects hyphae differently when directly in contact with hyphae, as opposed to an atmospheric environment.

Amylostereum spp. tend to be less competitive than *Leptographium* spp. when plated in direct contact. One exception was *A. chailletii* isolate 15B from Alabama, which significantly outgrew both the *L. terebrantis* and *L. procerum* isolates. This isolate is from the same ecosystem that the *Leptographium* isolates were found. Being isolated from the same general area alone could not explain this difference, as two other isolates of *A. chailletii* from Alabama were poor competitors with the *Leptographium* isolates. These other two *A. chailletii* isolates (N1 and S1) did not form a VCG with isolate 15B.

Further work on this clone (15B) of *A. chailletii* needs to be conducted to determine if it could become a potential problematic pathogen in the southern pine ecosystem. More samples of *A. chailletii* should be obtained from the area, and another VCG test should be run, to determine if any form a VCG with isolate 15B. Isolate 15B also should be used in an inoculation trial against other bole infecting fungi inside living trees. An inoculation trial to

further study competition between *Amylostereum* spp. isolates and *Leptographium* spp. isolates could be conducted in both seedlings and mature trees.

6.3. *Deladenus* spp. in the Southeast

A novel relationship between *S. nigricornis* and *D. siricidicola* was observed in this study. The most logical explanation of why *D. siricidicola* is now found to affect other species and genera than its documented host, *S. noctilio* (Morris et al., 2013) is that the nematode was transferred horizontally in the tree setting. If both *S. noctilio* and *S. nigricornis* females ovipositted in the same pine material, it is feasible that *Deladenus* spp. could swap hosts on larvae while they developed inside the tree. These wasps would then carry whichever species of nematode as an adult that they were subjected to as larvae. Since *S. nigricornis* in Alabama were seen to be infested with *D. siricidicola*, it would seem that either *S. noctilio* is actually in the southeast, but population numbers are so low that it has yet to be detected, or this transfer happened and *S. nigricornis* populations from the northeast overlap with southern populations enough to transfer the nematode species. An alternate explanation to this finding is that *D. siricidicola* has always colonized *S. nigricornis*, but has yet to be reported. Since it was once thought to affect only *S. noctilio*, clearly a greater body of knowledge is required to fully understand the complete host range of *D. siricidicola*.

Further studies are warranted to better understand which strains of *D. siricidicola* are found in *S. nigricornis* populations in the southeast. More work needs to be done to determine whether or not the *D. siricidicola* associated with *S. nigricornis* is capable of sterilization,

because if sterilization is occurring, native populations of the non-pest host could be affected.

More research also should be directed at *T. columba,* and its relationship with *D. siricidicola*. More T. Columba specimens should be collected throughout the southeast in order to gain a greater body of knowledge on their flight phenology, as well as species of nematodes that might be infesting their populations. If more specimens were collected, phylogenetic analyses could be conducted to ensure that the findings of this thesis were not unique, and were reproducible.

6.4. Overall Conclusions

After exploring many facets of a potential establishment of *S. noctilio* in the southeastern United States, it is unlikely that this species would cause the same economic and ecological damage that has been seen in the Southern Hemisphere. It seems that the insect itself would have plenty of competitors for oviposition substrate, and that larvae would have competitors for resources while developing within the tree. The associated fungal species within the *Amylostereum* genus seem to be poor competitors to already established pathogens found in the southern pine ecosystem. Within well managed pine stands in the area, many steps are taken to keep insect and pathogen loads to a minimum. These efforts also would contribute to keeping *S. noctilio* populations below the economic threshold of damage, even if the species was able to become established in the area.

References

Adams, A.S. Jordan, M.S., Adams, S.M., Suen, G., Goodwin, L.A., Davenport, K.W., Currie, C.R., Raffa, K.F. 2011. Cellulose- degrading bacteria associated with the invasive woodwasp *Sirex noctilio*. *International Society for Microbial Ecology Journal*. 5: 1323-1331.

Alabama State Port Authority: Port Facts (Alabama State Port Authority: Port Facts)

http://www.asdd.com/portfacts.html

Ayres, M., Sullivan, J., Harrison, T., Lombardero, M., 2009. Diagnosing the presence of *Sirex noctilio* from examination of dead and dying pine trees. Report for USDA APHIS.

Bain, J., 2005. *Sirex noctilio* (Hymenoptera: Siricidae) - the New Zealand experience. In K. W. Gottschalk (Ed.), Proceedings of the 16th USDA Interagency Research Forum on Gypsy Moth and Other Invasive Species, Annapolis, MD, 18-21January 2005. (p. 2.). Gen. Tech. Rep. NE-337. Newtown Square, PA: U.S. Department of Agriculture, Forest Service, Northeastern Research Station.

Baker, J.B., and Langdon, O.G. 1990. *Pinus taeda* L. loblolly pine. In: Burns, R.M., Honkalatech, B.H. (eds) Silvics of North America: 1. Conifers. Agriculture handbook 654. U.S. Department of Agriculture, Forest Service, Washington, D.C.

Barnes, B.F., Meeker, J.R., Johnson, W., Asaro, C., Miller, D.R. and Gandhi, K.J., 2014. Trapping techniques for siricids and their parasitoids (Hymenoptera: Siricidae and Ibaliidae) in the southeastern United States. *Annals of the Entomological Society of America*, 107(1), pp.119-127.

Bashford, R. 2008. The development of static trapping systems to monitor for wood-boring insects in forestry plantations. *Aust. For.* 71: 236-241.

Bedding, R.A. and Akhurst, R.J., 1974. Use of the nematode Deladenus siridicola in the biological control of *Sirex noctilio* in Australia. *Austral Entomology*, 13(2), pp.129-135.

Bedding, R.A., Akhurst. R.J. 1978. Geographical distribution and host preferences of Deladenus species (Nematoda: Neotylenchidae) parasitic in siricid woodwasps and associated hymenopterous parasitoids. *Nematologica.* 24: 286- 294.

Bedding, R.A. 2009. Controlling the pine-killing woodwasp, *Sirex noctilio*, with nematodes. In: Hajek, A.E., Glare, T.R., O'Callaghan, M. (Eds.), Use of microbes for control of invasive arthropods. Springer, Amsterdam, The Netherlands. 213-235.

Bedding, R.A., Iede, E.T., 2005. Application of Beddingia siricidicola for Sirex woodwasp control. In: Grewal, P.S., Ehlers, R., Shapiro-Ilan, D.I. (Eds.), Nematodes as Biocontrol Agents. CAB International, Wallingford, U.K, 453–470.

Boidin, J. 1958. Heterobasidiomycetes saphrophytes et Homobasidiomycetes resupines: V- Essai sur le genre Stereum Pers. Ex S.F. Gray. *Rev. Mycol*. 23: 318-346.

Boidin, J. and Lanquetin, P. 1984. Le genre *Amylostereum* (Basidiomycetes) intercompatibilites partielles entre especes allopartriques. *Bull Soc. Mycol. France*. 100: 211-236.

Bordeaux, J., Lorenz, W., Johnson, D., Badgett, M., Glushka, J., Orlando, R., Dean, J. 2014. Noctilisin, a Venom Glycopeptide of *Sirex noctilio* (Hymenoptera: Siricidae), Causes Needle Wilt and Defense Gene Responses in Pines. *Journal of Molecular Entomology*. 107(5): 1931-1945.

Böröczky, K., Crook D.J., Jones, T.H., Kenny, J.C., Zylstra, K.E., Mastro, V.C., Tumlinson, J.H. 2009. Monoalkenes as contact sex pheromone components of the woodwasp *Sirex noctilio*. *Journal of Chemical Ecology*. 35: 1202-1211.

Böröczky, K., Zylstra, K.E., McCartney, N.B., Mastro, V.C., Tumlinson, J.H. 2012. Volatile profile differences and the associated Sirex noctilio activity in two host tree species in the northeastern United States. *Journal of chemical ecology*, *38*(2), 213-221.

Burnip, G.M., Voice, D. and Brockerhoff, E.G., 2010. Interceptions and incursions of exotic Sirex species and other siricids (Hymenoptera: Siricidae). NZJ For. Sci, 40, pp.133-40.

Campbell, W.G. 1932. The chemistry of the white rots of wood. *Biochem*. 26: 1829-1838.

Carnegie, A.J., Klasmer, P., Sun, J., Iede, E., Ahumada, R., Matsuki, M., Haugen, D.A., Hurley, B.P. 2006. Predicting the potential distribution of *Sirex noctilio* (Hymenoptera: Siricidae), a significant exotic pest of *Pinus* plantations. *Annals of Forest Science*. 63: 119- 128.

Chase, K.D., Gandhi, K.J.K, Riggins, J.J. 2014. Effects of Forest Type and Management on Native Wood Wasp Abundance (Hymenoptera: Siricidae) in Mississippi, United States. *J. Econ. Entomol.* 107(3): 1142-1149.

Ciesla, W.M. 2003. European woodwasp: a potential threat to North America's conifer forests. *J. For*. 101: 18-23.

Coutts, M. P., Dolezal, J.E. 1965. *Sirex noctilio*, its associated fungus, and some aspects of wood moisture content. *Australian Forest Research*. 1: 3-13.

Coutts, M.P., 1969. The mechanism of pathogenicity of *Sirex noctilio* on *Pinus radiata*: Effects of *S. noctilio* mucus. Australian Journal of Biological Science. 22, 1153-1161.

Coyle, D., Pfammatter, J., Journey, A., Pahs, T., Cervenka, V., Koch ,R. 2012. Community composition and phenology of native Siricidae (Hymenoptera) attracted to semiochemicals in Minnesota. *Environmental Entomology* 41: 91–97.

Crook, D.J., Böröczky, K., Zylastra, K.F., Mastro, V.C., Tumlinson, J.H. 2012. The Chemical Ecology of *Sirex noctilio*. The Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest (ed. By B. Slippers, P. de Groot, and M.J. Wingfield), pp 149-158. Springer, The Netherlands.

Chrystal, R.N. 1928. The *Sirex* wood-wasps and their importance in forestry. *Bulletin of Entomological Research*, 19: 219-247.

de Groot, P., and Turgeon, J.J. 1998. Insect-pine interactions. *Ecology and Biodiversity of Pinus* (ed. By D.M. Richardson), pp. 354- 380. Cambridge University Press, U.K.

Dittritch-SchrÖder G.U., Wingfield M.J., Klein H., Slippers, B. 2012. DNA extraction techniques for DNA barcoding of minute gall-inhabiting wasps. *Molecular ecology resources*.12(1):109-15.

Dodds, K.J. and de Groot, P. 2012. Sirex, surveys and management: challenges of having *Sirex noctilio* in North America. The Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest (ed. By B. Slippers, P. de Groot, and M.J. Wingfield), pp 265-286. Springer, The Netherlands.

Eckhardt, L.G., Jones, J.P., Klepzig, K.D. 2004. Pathogenicity of *Leptographium species* associated with Loblolly pine decline. *Plant Dis.*, 88, 1174–1178.

Eckhardt, L., Weber, A., Menard, R., Jones, J., Hess, N. 2007. Insect- Fungal Complex Associated with Loblolly Pine Decline in Central Alabama. *Forest Science*, 53(1): 84-92.

Eckhardt, L.G., Menard, R.D., Gray, E.D. 2009. Effects of oleoresins and monoterpenes on *in vitro* growth of fungi associated with pine decline in the Southern United States. *Forest Pathology*. 39: 157-167.

Edmonds, R.L., Agee, J.K., Gara, R. I. 2011. Forest Health and Protection, Waveland Press, Inc.

Enebak, Scott A., and Robert A. Blanchette. Canker formation and decay in sugar maple and paper birch infected by *Cerrena unicolor*. *Canadian Journal of Forest Research* 19.2 (1989): 225-231.

Fitza, K. N., Tabata, M., Kanzaki, N., Kimura, K., Garnas, J., Slippers, B. 2016. Host specificity and diversity of *Amylostereum* associated with Japanese siricids. *Fungal Ecology*, *24*, 76-81.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R.1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, 294-299.

Franceschi, V.R., Krokene, P., Christiansen, E., Krekling, T. 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytologist*, *167*(2), 353-376.

Gandhi, K.J., Herms, D.A. 2009. Direct and indirect effects of invasive exotic insect herbivores

on ecological processes and interactions in forests of eastern North America. *Biol. Invasions*. 12: 389- 405.

Gaut, I.P.C. 1970. Studies of siricids and their fungal symbionts. Ph.D. thesis, University of Adelaide, Australia.

Gershenzon, J. and Dudareva, N. 2007. The function of terpene natural products in the natural world. *Nature Chemical Biology*. 3(7), 408-414.

Goulet, H. 2012. *Sirex* Systematics; Problems and Solutions. The Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest (ed. By B. Slippers, P. de Groot, and M.J. Wingfield), pp 1-14. Springer, The Netherlands.

Haavik LJ, Meeker JR, Johnson W, Ryan K, Turgeon JJ, Allison JD. 2013. Predicting *Sirex noctilio* and *S. nigricornis* emergence using degree days. *Entomologia Experimentalis et Applicata* 149: 177–184.

Haavik, L.J., Batista, E., Dodds, K.J., Johnson, W., Meeker, J.R., Scarr, T.A., Allison, J.D. 2014. Type of intercept trap not important for capturing female *Sirex noctilio* and *S. nigricornis* (Hymenoptera: Siricidae) in North America. *J. Econ. Entomol*. 107(3): 1295-1298.

Haack, R.A., Britton, K.O., Brockerhoff, E.G., Cavey, J.F., Garrett, L.J., Kimberley, M., Lowenstein, F., Nuding, A., Olson, L.J., Turner, J. Vasilaky, K.N. 2014. Effectiveness of the International Phytosanitary Standard ISPM No. 15 on reducing wood borer infestation rates in wood packaging material entering the United States. *PLoS One*, *9*(5), p.e96611.

Hajek, A.E., Nielson, C., Kepler, R.M., Long, S.J., Castrillo, L.A. 2013. Fidelity among *Sirex* woodwasps and their fungal symbiont. *Microb. Ecol*. 65, 753- 762.

Hajek, A.E., Morris, E.E. 2014. Sirex woodwasp (*Sirex noctilio* Fabricius) (Hymenoptera: Siricidae). Chapter XXXII (331-346) *in* The Use of Classical Biological Control to Preserve Forests in North America (R. Van Driesche & R. Reardon, eds.) USDA Forest Service Publication FHET-2013-2.

Hanson, H.S. 1939. Ecological notes on the *Sirex* wood wasps and their parasites. *Bull ent. Res*. 30: 27-76.

Hartshorn JA, Fisher DM, Galligan LD, Stephen FM. 2015. Seasonal phenology of *Sirex nigricornis* (Hymenoptera: Siricidae) in Arkansas with implications for management of *Sirex noctilio*. *Florida Entomologist* 98: 933–938.

Hartshorn JA, Haavik LJ, Allison JD, Meeker JR, Johnson W, Galligan LD, Chase KD, Riggins JJ, Stephen FM. 2016. Emergence of adult females *Sirex nigricornis* F. and *Sirex noctilio* F. (Hymenoptera: Siricidae) coincides with a decrease in daily minimum and maximum temperature. *Agricultural and Forest Entomology* 18: 206-213.

Haugen, D.A. 1990. Control procedures for *Sirex noctilio* in the Green Triangle; review from

detection to severe outbreak (1977-87). *Australian Forestry*, 53: 24-32.

Haugen, D.A., and Hoebeke, E.R. 2005. Pest alert: Sirex woodwasp- *Sirex noctilio* F. (Hymenoptera: Siricidae). U.S. Department of Agriculture, Forest service, Newtown Square, PA NA-PR-07-05.

Himejima, M., Hobson, K.R., Otsuka, T., Wood, D.L., and Kubo, I. 1992. Antimicrobial terpenes from oleoresin of Ponderosa Pine tree *Pinus ponderosa*: A defense mechanism against microbial invasion. *Journal of Chemical Ecology*. 18(10), 1809-1818.

Hoebeke ER, Haugen DA, Haack RA. 2005 *Sirex noctilio*: discovery of a Palearctic siricid woodwasp in New York. *Newsletter of the Michigan Entomological Society* 50: 24–25.

Hofstetter, R.W. and Moser, J.C., 2014. The role of mites in insect-fungus associations. Annual review of entomology, 59, pp.537-557.

Hurley, B.P., Slippers, B., Wingfield, M.J. 2007. A comparison of control results for the alien invasive woodwasp, *Sirex noctilio*, in the southern hemisphere. *Agric. For. Entomol*. 9, 159-171.

Hurley, B. P., Slippers, B., Croft, P. K., Hatting, H. J., van der Linde, M., Morris, A. R., Wingfield, M. J. 2008. Factors influencing parasitism of Sirex noctilio (Hymenoptera: Siricidae) by the nematode Deladenus siricidicola (Nematoda: Neotylenchidae) in summer rainfall areas of South Africa. *Biological Control*, *45*(3), 450-459.

Hurley, B.P., Hatting, H.J., Wingfield, M.J., Klepzig, K.D., Slippers, B. 2012. The influence of *Amylostereum areolatum* diversity and competitive interactions on the fitness of *Sirex* parasitic nematode *Deladenus siricidicola*. *Biol. Control*. 61: 207-214.

Hurley, B.P., Garnas, J., Cooperband, M.F. 2015. Assessing trap and lure effectiveness for the monitoring of *Sirex noctilio*. *Ag. and For. Entomol*. 17: 64-70.

Ismail, R. and Mutanga, O., 2011. Discriminating the early stages of *Sirex noctilio* infestation using classification tree ensembles and shortwave infrared bands. I*nternational journal of remote sensing*, 32(15), pp.4249-4266.

Johnson, C.W., Meeker, J.R., Ross, W.G., Petty, S.D., Bruce, B., Steiner, C. 2013. Detection and seasonal abundance of *Sirex nigricornis* and *Eriotremex formosanus* (Hymenoptera: Siricidae) using various lures and trap trees in central Louisiana, *U.S. J. Entomol. Sci.* 48(3): 173-183.

Keeler, D., 2012. Flight period and species composition of Sirex (Hymenoptera: Siricidae) and associated Deladenus (Nematoda: Neotylenchidae) within Arkansas pine forests.

Klepzig, K.D. 1994. Interactions of stress, plant chemical defenses and subcortical insect-fungal complexes in red pine decline. PhD dissertation. University of Wisconsin-Madison. 246 pp.

Kroll SA, Hajek AE, Morris EE, Long SJ. 2013. Parasitism of *Sirex noctilio* by non-sterilizing *Deladenus siricidicola* in northeastern North America. *Biological Control* 67: 203-

211.

Kukor, J.J., and Martin, M.M. 1983a. Acquisition of Digestive Enzymes by Siricid Woodwasps from Their Fungal Symbiont. *Science*. 220, 1161-1163.

Kukor, J.J. and Martin, M.M., 1983b. Siricid wood wasps acquire digestive enzymes from their fungal symbiont. *Science*, 220, pp.1161-1163.

Leung, B., Springborn, M.R., Turner, J.A. Brockerhoff, E.G. 2014. Pathway level risk analysis: the net present value of an invasive species policy in the US. *Frontiers in Ecology and the Environment*, *12*(5), 273-279.

Lindgren, B.S.1983. A multiple funnel trap for scolytid beetles (Coleoptera). *Can. Entomol*. 115: 299- 302.

Lovett, G.M., Weiss, M., Liebhold, A.M., Holmes, T.P., Leung, B., Lambert, K.F., Orwig, D.A., Campbell, F.T., Rosenthal, J., McCullough, D.G. Wildova, R. 2016. Nonnative forest insects and pathogens in the United States: Impacts and policy options. *Ecological Applications*, *26*(5), 1437-1455.

Madden, J.L.1968a. Behavioural responses of parasites to the symbiotic fungus associated with *Sirex noctilio* F. *Nature*. 218: 189-190.

Madden, J.L.1968b. Physiological aspects of host tree favourability for the woodwasp, *Sirex noctilio* F. *Proceedings of the Ecological Society of Australia*. 3: 147-149.

Madden, John L. 1968c. *Characterization of Deladenus siricidicola (Tylenchida: Neotylenchidae) associated with Sirex noctilio (Hymenoptera: Siricidae) in Canada*. "Behavioural Responses of Parasites to the Symbiotic Fungus associated with *Sirex noctilio* F." *Nature* 218 (1968): 189-190.

Madden, J.L. 1974. Oviposition Behaviour of the Woodwasp, *Sirex noctilio* F. *Australian Journal of Zoology*. 22: 341-351.

Madden, J.L. 1981. Egg and Larval Development in the Woodwasp, *Sirex noctilio* F. *Australian Journal of Zoology*. 29: 493-506.

Madden, J.L. 1988. *Sirex in Australasia Dynamics of Forest Insect Populations Patterns, Causes, Implications* (ed. By A.A. Berryman), pp. 407-429. Plenum Press, New York, New York.

Madden J.L., and Coutts M.P. 1979. The role of fungi in the biology and ecology of woodwasps (Hymenoptera: Siricidae). In: Insect-Fungus Symbiosis (ed Batra LR), pp. 165-174. Allanheld, Osmun & Co., Montclair.

Martínez, A.S., Villacide, J., Ajó, A.A.F., Martinson, S.J., Corely, J.C., 2014. *Sirex noctilio* flight behavior: toward improving current monitoring techniques. *Entomologia Experimentalis et Applicata* 152: 135-140.

Matusick, G. and Eckhardt, L.G. 2010. The pathogenicity and virulence of four Ophiostomatoid

fungi on young Longleaf pine trees. *Canadian Journal of Plant Pathology*, *32*(2), 170-176.

Miller, D.R., and Asaro, C. 2005. Ipsenol and ipsdienol attract Monochamus titllator (Coloptera: Cerambycidae) and associated large pine woodborers in southeastern United States. *J. Econ. Entomol.* 98: 2033-2040.

Miller, D.R., Asaro, C., and Berisford, C.W. 2005. Attraction of southern pine engravers and associated bark beetles (Coleoptera: Scolytidae) to ipsenol, ipsdienol, and lanierone in southeastern United States. *J. Econ. Entomol*. 98: 2058-2066.

Morgan, F.D. 1968. Bionomics of siricidae. *Annu. Rev. Entomol*. 13: 239-256.

Morgan, F. D., and Stewart, N. C. 1966. The biology and behaviour of the woodwasp *Sirex noctilio* F. in New Zealand. Trans. roy. Soc. N. Z., Zool. 7:195–204.

Morris, E.E., Kepler, R.M., Long, S.J., Williams, D.W., Hajek, A.E. 2013. Phylogenetic analysis of *Deladenus* nematodes parasitizing northeastern North American *Sirex* species. *J. Invertebr. Pathol.* 113, 177-183.

Morris, E.E., Hajek, A.E., Zieman, E., Williams, D.W. 2014. *Deladenus* (Tylenchida: Neotylenchidae) reproduction on species and strains of the white rot fungus *Amylostereum*. *Biological Control*. 73: 50-58.

Myers, S.W., Zylastra, K.E., Francese, J.A., Borchert, D.M., Bailey, S.M. 2014. Phenology and flight periodicity of *Sirex noctilio* (Hymenoptera: Siricidae) in central New York, U.S.A. *Ag. and For. Entomol.* 16: 129-135.

Nielsen, C., D. W. Williams and A. E. Hajek. 2009. Putative source of the invasive *Sirex noctilio* fungal symbiont, *Amylostereum areolatum*, in the eastern United States and its association with native siricid woodwasps. *Mycological Research.* 113, 1242-1253.

Neumann, F.G., Morey, J.L., McKimm, R.J. 1987. The *Sirex* wasp in Victoria, Victoria bulletin no. 29. Lands and Forests Division, Department of Conservation, Forests and Lands, Melbourne.

Olatinwo R, Allison J, Meeker J, Johnson W, Streett D, Aime MC, Carlton C. 2013. Detection and Identification of *Amylostereum areolatum* (Russulales: Amylostereacea) in the Mycangia of *Sirex nigricornis* (Hymenoptera: Siricidae) in Central Louisiana. *Environmental Entomology* 42: 1246-1256.

Paine, T. D.; Blanche, C. A.; Nebeker, T. E.; Stephen, F. M., 1987: Composition of loblolly pine resin defenses: Comparison of monoterpenes from induced lesion and sapwood resin. *Can. J. For. Res.* 17, 1202–1206.

Pažoutová, S., Šrůtka, P., Holuša, J., Chudíčková, M. and Kolařík, M., 2010. Diversity of xylariaceous symbionts in Xiphydria woodwasps: role of vector and a host tree. *Fungal Ecology*, *3*(4), pp.392-401.

Pimentel, D., Zuniga, R., Morrison, D. 2005. Update on the environmental and economic costs

associated with alien-invasive species in the United States. *Ecol. Econ*. 52: 273-288.

Rawlings, G.B. 1955. Epidemics in *Pinus radiata* forests in New Zealand. *New Zealand Journal of Forestry*. 7: 53-58.

Ryan, K., Moncalvo, J. M., Groot, P. D., Smith, S. M. 2011. Interactions between the fungal symbiont of Sirex noctilio (Hymenoptera: Siricidae) and two bark beetle-vectored fungi. *The Canadian Entomologist*, *143*(3), 224-235.

Ryan, K., de Groot, P., Smith, S.M. 2012a. Evidence of interaction between *Sirex noctilio* and other species inhabiting the bole of *Pinus*. *Ag. and For. Entomol*. 14: 187- 195.

Ryan, K., de Groot, P., Nott, R.W., Drabble, S., Ochoa, I., Davis, C., Smith, S.M., and Turgeon, J.J. 2012b. Natural Enemies Associated with *Sirex noctilio* (Hymenoptera: Siricidae) and *S. nigricornis* in Ontario, Canada. *Environ. Entomol*. 41(2), 289-297.

Ryan, K. and Hurley, B.P. 2014. Life History and Biology of *Sirex noctilio*. The Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest (ed. By B. Slippers, P. de Groot, and M.J. Wingfield), pp 15-30. Springer, The Netherlands.

Schiff, N.M., Valley, S.A., LaBonte, J.R., Smith, D.R. 2006. Guide to the Siricid Woodwasps of North America, USDA Forest Service Forest Health Technology Enterprise Team.

Schiff, N., Goulet, H., Smith, D.R., Boudreault, C., Wilson, A.D., Scheffler, B.E. 2012. Siricidae (Hymenoptera: Symphyta: Siricoidea) of the Western Hemisphere. *Can. J. Arthropod Ident.* 21: 1-305.

Shultz, R. P. 1997. "Loblolly Pine – The ecology and culture of Loblolly pine (*Pinus taeda* L.) Agricultural handbook 713.

Simpson, R. F. 1976. Bioassay of pine oil components as attractants for *Sirex noctilio* (Hymenoptera: Siricidae) using electroantennogram techniques. Ent. Exp. Appl. 19:11–18.

Simpson, R.F., and McQuilkin, R.M. 1976. Identification of volatiles from felled Pinus radiate and the electroantennograms they elicit from *Sirex noctilio*. *Entomol. Exp. Appl*. 19: 205-213.

Slippers, B., Coutinho, T.A., Wingfield, B.D., Wingfield, M.J. 2003. A review of the genus *Amylostereum* and its association with woodwasps. *South African Journal of Science*. 99: 70-74.

Slippers, B., Vasiliauskas, R., Hurley, B., Stenlid, J., Wingfield, M.J. 2006. A collaborative project to better understand Siricid- Fungal symbioses. *Aktuelt fra skogforskningen*. 1: 65-68.

Slippers, B., Hurley, B.P., Mlonyeni, O., de Groot, P., Wingfield, M. J. 2012. Factors Affecting the Efficacy of *Deladenus siricidicola* in Biological Control Systems. The Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest (ed. By B. Slippers, P. de Groot, and M.J. Wingfield), pp 119-133. Springer, The Netherlands.

Slippers, B., Wingfield. M.J. 2012. Sirex research and management: future prospects. The Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest, Springer pp. 287–295

Slippers, B., Hurley, B.P. and Wingfield, M.J., 2015. Sirex woodwasp: a model for evolving management paradigms of invasive forest pests. Annual review of entomology, 60, pp.601-619.

Smith, D.R., 1996. Discovery and spread of the Asian horntail, Eriotremex formosanus (Matsumura) (Hymenoptera: Siricidae), in the United States. *Journal of Entomological Science*, *31*(2), 166-171.

Smith, D. R., Schiff, N. M. 2002. A review of the siricid woodwasps and their ibaliid parasitoids (Hymenoptera: Siricidae, Ibaliidae) in the eastern United States, with emphasis on the mid-Atlantic region.

Spradberry, J.P., and Kirk, A.A. 1978. Aspects of the ecology of siricid woodwasps (Hymenoptera: Siricidae) in Europe, North Africa and Turkey with special reference to the biological control of *Sirex noctilio* F. in Australia. *Bull. Ent. Res*. 68: 341-359.

Stillwell. M.A. 1965. Hypopleural organs of the woodwasp larva *Tremex columba* (L.) containing the fungus *Daedalea unicolor* Bull. ex Fries. Canadian Entomologist, 97, pp. 783–784.

Talbot, P.H.B. 1977. The *Sirex- Amylostereum- Pinus* association. *Annual Review of Phytopathology*. 15: 41-54.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution, 28(10), pp.2731-2739.

Taylor, K.L. 1976. The introduction and establishment of insect parasitoids for the control of *Sirex noctilio* in Australia. *Entomophaga*. 21: 429- 440.

Taylor, K.L. 1981. The *Sirex* woodwasp; ecology and control of an introduced forest insect. In: The ecological pests some Australian case histories. Melbourne, CSIRO. pp. 231- 248.

Thomsen, I.M., Harding, S. 2011. Fungal symbionts of siricid woodwasps: isolation techniques and identification. *Forest Pathology* 41: 325-333.

Tize, F.F. 1970. Some aspects of the metabolism of *Amylostereum areolatum*, the fungal symbiont of *Sirex noctilio*, in cultures on wood of *Pinus radiata*. *Aust. For. Res.* 4(4): 19-22.

(USDA-APHIS) U.S. Department of Agriculture- Animal and Plant Health Inspection Service. 2008. Proposed Program for the Control of the Woodwasp *Sirex noctilio* F. (Hymenoptera: Siricidae) in the Northeastern United States (PDF, 4.67 MB). Riverdale, MD.

van der Nest, M. A., Wingfield, B. D., Wingfield, M. J., Stenlid, J., Vasaitis, R., Slippers, B. 2012. Genetics of *Amylostereum* Species Associated with Siricid Woodwasps. The Sirex Woodwasp and its Fungal Symbiont: Research and Management of a Worldwide Invasive Pest (ed. By B. Slippers, P. de Groot, and M.J. Wingfield), pp 81-94. Springer, The Netherlands.

Vasiliauskas, R., Stenlid, J., Thomsen, I.M. 1998. Clonality and genetic variation in *Amylostereum areolatum* and *A. chailletii* from northern Europe. *New Phytol*. 139: 751- 758.

White, T. J., Bruns, T., Lee, S. J. W. T., Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR protocols: a guide to methods and applications*, *18*(1), 315-322.

Wingfield, M.J. 1986. Pathogenicity of *Leptographium procerum* and *L. terebrantis* on *Pinus strobus* seedlings and established trees. *Eur. J. For. Path*., 16, 299–308.

Wood, S. L. 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. *Great Basin Naturalist Memoirs* 6: 1-1356.

Wooding, A.L., Wingfield, M.J., Hurley, B.P., Garnas, J.R., de Groot, P., Slippers, B. 2013. Lack of fidelity revealed in an insect- fungal mutualism after invasion. *Biol. Let.* 9.

Ye, W., Giblin-Davis, R. M., Braasch, H., Morris, K., Thomas, W. K. 2007. Phylogenetic relationships among Bursaphelenchus species (Nematoda: Parasitaphelenchidae) inferred from nuclear ribosomal and mitochondrial DNA sequence data. *Molecular Phylogenetics and Evolution*, *43*(3), 1185-1197.

Yemshanov, D., Koch, F. H., McKenney, D.W., Downing, M.C., Sapio, F. 2009. Mapping Invasive Species Risks with Stochastic Models: A Cross-Border United States-Canada Application for *Sirex noctilio* Fabricius. *Risk Analysis* 29: 868-884.

Yousuf, F., Carnegie, A., Bashford, R., Bedding, R., Nicol, H. I., Gurr, G.M. 2014. Bark beetle (Ips grandicollis) disruption of woodwasp (*Sirex noctilio*) biocontrol: direct and indirect mechanisms. *For. Ecol. And Management*. 323: 98-104.

Yu, Q., Groot, P. D., Leal, I., Davis, C., Ye, W., Foord, B. 2009. Characterization of *Deladenus siricidicola* (Tylenchida: Neotylenchidae) associated with *Sirex noctilio* (Hymenoptera: Siricidae) in Canada. *International Journal of Nematology*, *19*(1), 23-32.

Zylastra, K.E., Dodds, J.K., Joseph, A.K., Mastro, V. 2010. *Sirex noctilio* in North America; the effect of stem- injection timing on the attractiveness and suitability of trap trees. *Agric. For. Entomol.* 12: 243-250.