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Biology, ecology and efficacy of *Lecanicillium muscarium* as a potential fungal biocontrol of the invasive hemlock woolly adelgid (*Adelges tsugae*) on eastern hemlock (*Tsuga canadensis*)

Kristen L. Wickert

Thesis submitted
to the Davis College of Agriculture, Natural Resources and Design
at West Virginia University

in partial fulfillment of the requirements for the degree of

Master of Science in
Plant Pathology

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Keywords: ***Lecanicillium*****, hemlock woolly adelgid, biocontrol, entomopathogen**
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ABSTRACT

Biology, ecology and efficacy of *Lecanicillium muscarium* as a potential fungal biocontrol of the invasive hemlock woolly adelgid (*Adelges tsugae*) on eastern hemlock (*Tsuga canadensis*)

Kristen L. Wickert

Hemlock woolly adelgid (HWA) is an exotic insect pest of eastern hemlock. The entomopathogen *Lecanicillium muscarium,* including the commercially available strain Mycotal®, is a potential candidate for fungal biocontrol. There are many factors to consider when using a fungal biocontrol such as ecology and genetic variation of candidate strains and interactions with other fungi and life stages of the target insect pest. Efforts of this study focused on: 1) sampling for reservoirs for *L. muscarium* and other *Lecanicillium* spp., 2) elucidating interactions between *Lecanicillium* and other fungi present in hemlock tissues and 3) characterizing genetic diversity of *Lecanicillium* and subsequent entomopathogenicity against HWA. Six *Lecanicillium* isolates were recovered out of 2,954 total fungal colonies isolated across all substrates, resulting in <1% incidence. Sampling of Mycotal®-treated hemlock stands failed to recover any *Lecanicillium* isolates, which suggests that *Lecanicillium* does not persist in these environments. To help explain low incidence of *Lecanicillium* recovery, common fungal community members recovered from these same hemlock tissues were co-plated with *Lecanicillium* to evaluate inhibitory effects. These frequently recovered fungi included *Colletotrichum*, *Epicoccum*, *Pestalotiopsis*, *Rhizosphaera* and an undescribed Leotiomycete. The Leotiomycete was shown to have inhibitory effects on several species of *Lecanicillium*. Since the Leotiomycete fungus is present 17% of the time on average, this could be a significant factor influencing the persistence of *Lecanicillium* in the environment. To further understand relationships among *Lecanicillium,* multi-gene phylogenetic analyses were conducted. Six separate phylogenetic analyses, with data partitioned by individual genes produced some complementary results and supported the monophyly of *Lecanicillium sensu strictu* and close relationships among *L. muscarium* and *L. longisporum as well as* uncovered novel linages of *Lecanicillium*. The phylogenetic trees informed selection of a diverse set of isolates used in entomopathogenicity testing. All isolates used were found to be pathogenic against HWA but virulence among fungal species and isolates varied. Mycotal® utilizes a virulent strain for an inundative augmentative approach to bolster naturally low population of *Lecanicillium* present in hemlock stands. However, its low infection rate on egg masses (33%) could indicate that other *Lecanicillium* isolates used in this study, especially North American strains, might be a better candidate for widespread application against HWA in the eastern United States.

Dedication

This thesis is dedicated to my mother, Tamra Paxton, who is the hardest working person I know. Without her love and admiration I would never be the person I am today. Thank you for everything.

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Most importantly, I would like to thank my advisor, Dr. Matthew T. Kasson. Looking back to when we first met, I would never have guessed I would have been lucky enough to be your first graduate student. I am so fortunate to have the opportunity of having an academic advisor that values my opinion, experience, and friendship. Our lab works hard because you work hard, Dr. Kasson. You are exemplary of a true leader.

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Chapter 1

Literature Review

Eastern hemlock (*Tsuga Canadensis* L. Carr.)

Eastern hemlock is one of the four native hemlock species belonging to the genus *Tsuga* in the United States. The continuous range of eastern hemlock extends from Nova Scotia south to northern Alabama and west to northeastern Minnesota and eastern Kentucky (Godman and Lancaster 1990). Disjunct satellite populations of eastern hemlock are also known in extreme western Alabama, western Ohio, and southern Indiana as well as east of the Appalachians mainly in Virginia. Eastern hemlock is commonly planted as a tree, shrub, or hedge in ornamental landscapes. There are at least 274 cultivars of eastern hemlock important to the landscaping industry (Godman and Lancaster 1990).

Eastern hemlock can grow in pure stands and is on occasion an associate in a mixture of species, which are generally also shade tolerant. Four forest cover types include eastern hemlock as an important component, those being northern hardwood, Allegheny hardwood, Appalachian mixed hardwood, and bottomland mixed hardwood (Godman and Lancaster 1990). Common associates include sugar maple (*Acer saccharum*), black cherry (*Prunus serotina*), eastern white pine (*Pinus strobus*), red spruce (*Picea rubens*), hickories (*Carya* spp.), oaks (*Quercus* spp.), yellow birch (*Betula alleghaniensis*), black birch (*Betula lenta)* and red maple (*Acer rubrum*) Bormann 1954). Pure stands of hemlock tend to develop distinct microclimates because of their dense canopy, shading, deep duff layer, subsequent retention of moisture and uniformly low temperatures. Habitat of hemlock includes riparian areas or in bogs near water but also on xeric hill sides which have poorer soils, such as spodisols. Eastern hemlock is generally restricted to regions with cool humid climates. The drop of naturally acidic hemlock needles perpetuate the high pH state of understory soil and promote a closed area of growth suitable primarily for hemlock and other adapted species. This is a quality of a climax species in that hemlock maintains and supports the late-successional forests it inhabits. As a slow growing and highly shade tolerant softwood species, hemlocks can be very long lived. It is not uncommon to find trees aged 200 years. This gymnosperm can take 200-350 years just to reach maturity and can exceed 800 years of age in extreme conditions (Hough 1960, Godman and Lancaster 1990).

Hemlock is an excellent example of the versatile benefits of a riparian species and the economic, ecological and intrinsic value it provides. Economically hemlock is important because it produces valuable lumber. This versatile softwood is exceptional for light framing, subflooring, boxes, crates, pallets and general millwork (Brisbin 1970). A portion of the leathering industry that chooses not to use synthetics use tannins produced in the hemlock bark for processing. Much of the present production is used in paper pulping for newsprint and wrapping papers.

Recreation traffic and revenue increases with the presence of hemlocks in state parks due to their ability to create shaded cool escape areas in the summer and warmer windbreaks in the winter. The dense canopy of hemlocks is known for keeping stream waters cool for sensitive trout and other aquatic life. These aquatic species are important ecologically and economically in that they create a large amount of revenue from fishing enthusiasts. As a riparian species hemlock provides vastly important ecosystem services to humans through the mitigation of flood waters, food chain support, and water quality protection. These ecosystem services can be hard to quantify and set a specific value to, however it is estimated that a forest with high evergreen and minimal deciduous components can provide around $2,173 per acre per year. This estimation considers biodiversity, carbon sequestering, cultural aspects and watershed benefits (Texas Forest Service, 2015).

Hemlock is important ecologically as a resource for wildlife. Co-dominant hemlock help maintain microenvironments important to native organisms in hardwood forests. Eastern hemlock stands are considered essential for shelter and bedding of white-tailed deer, ruffed grouse, turkeys and many other animals (Godman and Lancaster 1990). In the southern Appalachians, there are greater than 240 known insect species associated with eastern hemlock. These insects encompass a diversity of lifestyles including hematophage, herbivore, fungivore, parasitoid, predator, scavenger, and transients (Dilling et al. 2007). Native insect communities can alter drastically with the introduction of invasive pests like the elongated hemlock scale and the hemlock woolly adelgid (Buck 2004). Since many insects are at the bottom of the food chain, the introduction of an invasive pest such as hemlock woolly adelgid can impact not only the insects that previously held hemlock woolly adelgid’s niche, but the animals that subsist on the native insects in hemlock canopies in a cascading effect through the food chain.

Hemlock woolly adelgid (*Adelges tsugae*)

Hemlock woolly adelgid (HWA, *Adelges tsugae* Annand) is a hemipteran insect in the suborder Sternorrhyncha, native to East Asia and in the superfamily Phylloxeroidea in the family Adelgidae. This insect is a specialist and can survive mainly on hemlock species. Hemlock woolly adelgid have piercing and sucking mouthparts. Unlike closely related insects that feed on nutrients in sap, HWA feeds primarily intracellularly on stored starches in the xylem ray parenchyma (Young 1995). These starch reserves are critical to the tree's growth and long-term survival.

The hemlock woolly adelgid is parthenogenetic with all individuals being females utilizing asexual reproduction. There are three stages of development. Life begins in an egg and development continues through four nymphal instars until reaching adulthood (Salom et al. 2002). Adelgid populations complete two generations a year on hemlock. The winter generation, called sistens, developes from early June to March of the following year. The spring generation, called progrediens, developes from March to June (Figure 1-1). The generations overlap in mid to late spring. The ovisacs of the winter generation contain up to 300 eggs, while the spring generation ovisacs contain between 20 and 75 eggs (Chowdhury 2002). Depending on spring temperatures, eggs hatch from April to June. After hatching the first instar nymphs, called crawlers, search for suitable feeding sites on the twigs at the base of hemlock needles and prefer new growth. The crawlers are the only mobile stage of HWA. This is also the most vulnerable stage of HWA, due to a lack of protective woolly covering. Once settled at the base of a hemlock needle, the nymphs begin feeding on the young twig tissue and remain at that location throughout the remainder of their life. The development time from progrediens to adult ranges from 52 days in warmer temperatures to 147 days in colder temperatures (Salom et al. 2002). The hemlock woolly adelgid enters a period of dormancy, or diapause, during the hot summer months. The reasons for this are unknown and is the continued focus for several researchers. During diapause, the nymphs have a tiny halo of woolly wax surrounding their bodies, but lack a complete covering like the later months. The adelgids begin to feed once cooler temperatures return, usually in October, and continue throughout the winter months. Temperature and photoperiods influence the length of diapause for hemlock woolly adelgid.

Many adelgids fail to break diapause and the reasons are still unknown, however this may be due to temperature changes (Salom et al. 2001). However, adelgid populations are known to be self-regulating, so egg laying numbers will compensate for the loss (McClure 1991). Once diapause is broken and the adelgid matures, it produces a covering of wool-like wax filaments to protect itself and its eggs from natural enemies (Skinner 2003). The wax also prevents the adelgid from becoming desiccated. Additionally, the waxy coating contains anthraquinones, antifeedant compounds which are thought to act as a chemical defense against predation (Jones 2014). The white wool that covers the ovisacs is the most conspicuous when the adelgid is mature and laying eggs from late fall to early summer on the underside of the outermost branch tips of hemlock trees.

Another unique facet to the HWA life cycle is that it involves two species of host trees. The progrediens have two forms, a wingless form that remains on the hemlock and a winged form, called sexupara, which flies in search of a suitable spruce tree host upon which to start a sexual reproductive cycle (McClure 1999). Throughout much of the range of the spreading HWA in North America, there are no suitable spruce hosts meaning the sexual life stage is unable to be completed. Most of the adults of the spring progrediens generation are wingless and remain on the hemlock tree feeding and producing eggs protected by woolly masses just like the overwintering generation, but during June to July (Figure 1-1). Their offspring hatch into crawlers, and repeat the previously described developmental cycle. These nymphs become the next overwintering generation called sistens.

Dispersal and movement of HWA occur primarily during the first instar crawler stage as a result of wind and by birds, deer, and other forest-dwelling mammals that come in contact with the sticky ovisacs and crawlers. Although adelgid appear more commonly in the upper canopy they can be found in all sections of the tree.

Impacts of hemlock woolly adelgid

Hemlock woolly adelgid are of little to no concern in its native range of East Asia. The coevolution of HWA and its east Asian plant hosts has resulted in resistance and a discontinuous distribution of infested trees in Asian forests (Havill 2006). Generally only single or small groups of trees dieback and succumb as the insect develops within its native range in eastern Asia. Hemlock woolly adelgid was first reported in the Western United States in the early 1920s

and can be found today in northern California to southeastern Alaska (Chowdhury 2002). In 1951 HWA was introduced again into the Eastern United States near Richmond, Virginia accidentally from Osaka, Japan (Ward 2004). In North America the exotic nature of the HWA-hemlock relationship results in a widespread infestation and accelerated mortality of entire stands. As of 2014, HWA is present in most states contained within the native range of hemlock and occupying about half of the continuous range of hemlocks in the east (Evans 2007).

Hemlock woolly adelgid feed on all four species of native hemlock in the United States but populations of mountain and western hemlock (*T. mertensiana* and *T. heterophylla* respectively) along the west coast are not as susceptible as the native Eastern species. The two western hemlock species have maintained healthy populations even with HWA being present for almost 100 years. It is suspected that the two western *Tsuga* species have a thicker cuticle wax that inhibits the stylet insertion of HWA to some degree (Oten et al. 2012). This has led to significantly less mortality in the western species. Within HWAs range in the eastern U.S., HWA feeds on both eastern and Carolina hemlock (*Tsuga caroliniana*). Carolina hemlock is more restricted to higher elevation sites between 2,300–3,900 ft in the southern Appalachian Mountains between southwest Virginia and Northern Georgia and has a much smaller population than eastern hemlock (Coladonato 1993). The two eastern species of hemlock in particular are vastly more susceptible to hemlock woolly adelgid infestation and are experiencing major mortality. Areas of extensive tree mortality and decline are found throughout the infested region, but the impact has been most severe in some areas of Virginia, New Jersey, Pennsylvania, Tennessee, and Connecticut. Many of the eastern satellite populations remain uninfested presumably due to geographic isolation. According to the Ohio Department of Natural Resources, as of 2015, HWA is spreading through West Virginia and into eastern Ohio where seven counties are newly infested. Outlying infestations around the advancing front, are also known. HWA is slow moving compared to more recently introduced insect pests such as emerald ash borer, presumably due to the lack of mobility of the adelgids themselves.

The initial symptoms from HWA infestation are chlorosis and needle drop, followed by branch desiccation and an overall lack of vigor indicated by crown thinning (Hale 2004). Individual trees weakened by HWA may likewise become predisposed to further decline through continued stress contributing to eventual death. Hemlock stressors including drought, poor site conditions,

and other insect pests and diseases such as elongate hemlock scale (*Fiorinia externa*) (Hemiptera: Diaspididae), hemlock looper (*Lambdina fiscellaria*) (Lepidoptera: Geometridae), spruce spider mite (*Oligonychus ununguis*) (Trombidiformes: Tetranychidae), hemlock borer *(Melanophila fulvogutta*) (Coleoptera: Buprestidae), *Armillaria* root rot disease (*Armillaria mellea*), and hemlock needle rust (*Melampsora parlowii*) likely accelerate the rate and extent of hemlock mortality. The greatest impacts of HWA are an increase in hemlock mortality and the associated ecosystem changes that follow, such as increased water temperatures, decreased wildlife habitat and increased loads of coarse woody debris (Quimby 1996). Hemlock decline and mortality typically occur within 4 to 10 years of infestation in the insect's northern range, but can occur in as little as 3 to 6 years in its southern range due to differences in temperature (Paradis 2007). All life stages of hemlock are impacted. HWA can be seen on regeneration in the understory, although it is suggested that HWA prefer more mature hosts. Disturbance created by HWA in hemlock stands removes hemlock and opens gaps for other less desirable species. Black birch will readily recruit small gaps created by the hemlock woolly adelgid disturbance (Black and Abrams 2005). Birch proportions will decline as sugar maple or other tolerant species move in, resulting in extreme changes in species composition and ecological function. Stands of hemlock that suffer massive mortality are susceptible to being overtaken by invasive species such a tree-of-heaven (*Ailanthus altissima*), winged euonymus (*Euonymus alatus*), common reed (*Phragmites australis*), mile-a-minute (*Polygonum perfoliatum*), Japanese hops (*Humulus japonicas*) and autumn olive (*Elaeagnus umbellata*) (Eichelberger and Perles 2009).

Management of hemlock woolly adelgid

Eradicating HWA is no longer a realistic goal due to the widespread dissemination and proliferation of this invasive pest. Many land managers understand this limitation and aim to instead mitigate the spread and the populations already present in parks and forests. Cultural, regulatory, chemical and biological controls can reduce the rate of spread of HWA. Protection of individual trees is possible, but ecosystem-level management has not been realized. Actions such as removing isolated infested trees from a woodlot and state quarantines can help prevent further infestations.

Numerous abiotic and biotic factors can influence adelgid populations. Temperature plays a large role in the success of adelgid populations. Lab-reared populations of adelgid under

artificially stabilized temperature regimes showed considerable differences in development. Adelgid in 4°C took 147 days for progrediens nymphs to develop from 1st instar to adult, whereas adelgids in 22°C took 52 days to develop (Salom et al. 2002). First instar development is not affected by temperature as much as the development of the other instars. Some adelgids can survive temperatures as low as -30°C for periods up to 24 hours. There seems to be a low temperature threshold of -3.8°C and a high temperature threshold for progrediens which will not complete development at a constant temp of 27°C (Salom et al. 2002). These temperature effects were exemplified with the recent polar vortex winter of 2013-2014 which accounted for up to 75% reduction in adelgid populations throughout the Mid-Atlantic. Specific sites further north experienced mortality rates as high as 99.4% due to the 2013-2014 winter temperatures (Whitmore 2015).

Although there are natural enemies that are native to Eastern North America that feed on hemlock woolly adelgid they are not effective at reducing populations to prevent tree mortality. There are no known parasitoids of HWA. Predatory insects of HWA include native generalists such as *Harmonia axyridis* (Coleoptera: Coccinellidae), lacewings (Neuroptera: Chrysopidae and Hemerobiidae), and gall gnats (Diptera: Cecidomyiidae). All of these predatory species are non-specific feeders that are associated with density and are negligible as regulators (Wallace and Hain 1998).

There are several effective chemical methods that can be used to protect against HWA on small scales and high value singular trees. These chemical control methods are foliar sprays of insecticides, horticultural oils and insecticidal soaps. Trees must be fully saturated with these methods in order to prove effective. Foliar sprays are not feasible in forests, particularly when large numbers of trees are infested, due to cost and chemical application limitations. Two main utilization strategies of chemical control on larger forest scales involve soil trenching and chemical stem injection with systemic insecticides. Merit® is a systemic neonicotinoid insecticide that utilizes Imidacloprid that has proven to be very successful in HWA control. The mode of action is through ingestion when adelgid uptake the chemical through their stylet. Success rates of using chemicals vary with many factors, such as temperature and drought in that they slow the uptake and translocation of the systemic chemical (Bennet 1957). In an 18-26 month study evaluations of adelgid populations after chemical insecticide application determined

that fall and spring application timing did not significantly differ (Cowles et. al. 2005). Mode of delivery of insecticide also seems to be an important determinant of HWA population control. Trunk injections of Merit resulted in 100% decline of HWA populations whereas soil applications of systemic insecticides resulted in 79% suppression and increased to 98.5% in the following year (Cowles et. al. 2005). Yet other factors seem to have little effect on success.

Chemical control is limited to individual tree treatments that are in readily accessible and in non-environmentally sensitive areas. These insecticides are toxic to aquatic organisms which limits their application along stream beds, which unfortunately is the main ecotype for hemlocks (Cowles et al. 2006). Chemical treatments offer a short-term solution, and applications may need to be repeated in subsequent years. Crawler stages seem to be the most impacted by chemical treatments, so aligning treatments with the adelgid lifecycle is key.

Several predator insects that are known to feed exclusively on adelgids have been imported from China, Japan and Northwestern and Southeastern North America as a form of biological control. Some of these released insects are slowly becoming established throughout the infested region. Primary biocontrol agents include *Laricobius nigrinus* (Coleoptera: Derodontidae), *L. rubidus* (Coleoptera: Derodontidae), *L. osakensis* (Coleoptera: Derodontidae), *Scymnus coniferarum* (Coleoptera: Coccinellidae), *Leucopis argenticollis* (Diptera: Chamaemyiidae), *L. piniperda* (Diptera: Chamaemyiidae), and *Sasajiscymnus tsugae* (Coleoptera: Coccinellidae) (Cheah 2011, Wallin Unpublished). Currently, *Laricobius* spp. appear to be the most efficient insect biocontrols of HWA. The efficacy of *L. osakensis* and *L. nigrinus* against HWA is promising as both the larvae and adults feed on all life stages of HWA (Zilahi-Balogh et al. 2002). Furthermore, studies show that *Laricobius spp.* can only complete development on HWA which means *Laricobius* species are specialists (Vieira et al. 2011). Early emergence of *Laricobius* at a time when there are no adelgid for them to consume is a perennial problem. This is problematic in that they emerge at a time there are no adelgid for them to consume (Zilahi-Balogh 2003). Options of alternate naturally occurring food sources for predatory beetles are being looked into such as the pine bark adelgid (*Pineus strobi*) and balsam woolly adelgid (*Adelges piceae*) until the HWA break diapause (Zilahi 2001). However, only adults can survive on consuming these alternate hosts, meaning they only add to the diet but cannot be a replacement for the diet. At the same time, high HWA mortality due to the polar

vortex across the east coast in the winter of 2013-2014 contributed to HWA population crashes and scarce resources available for feeding beetle populations in rearing labs. Demand of field collected predatory beetles can also cause population crashes in their native environment and limit their efficacy. It will likely take a complex of natural predators and traditional chemical methods to maintain HWA populations below damaging levels. Efforts to locate, evaluate, and establish other natural predators continue.

Fungal biocontrol of Insects

Fungal biocontrol of insect have shown much promise in the agricultural business, especially in the greenhouse setting. *Beauvaria bassiana*, *Metarhizium* spp., *Paecilomycetes fumosorosues*, *Purpureocillium*, *Trichoderma* and *Lecanicillium* spp.; all have been successfully used as fungal biocontrols of various insect pests including whiteflies, thrips. In the United States products such as BotaniGard®, which is a manufactured *Beauvaria bassiana*, are EPA approved and used in greenhouses mainly against whiteflies and aphids. Green Muscle® is a successfully commercialized fungal biocontol of grasshoppers and locusts (Douthwaite 2001). Green Muscle® can control grasshopper and locust swarms with a single spraying of an extremely virulent strain of *Metarhizium anisopliae.* This shows that there are differences not only between different entomopathogenic species of fungi, but there are differences in strains of a single species in strength against specific hosts coupled with other factors (Douthwaite 2001). Fungal biocontrols are a more environmentally friendly option than chemical insecticides, but commercially they are more expensive.

Lecanicillium spp. are a promising group of generalist fungal entomopathogens with a cosmopolitan distribution. To date, *Lecanicillium* in many cases are being exploited as biological controls. *Lecanicillium* spp. can be frequently isolated from soil and plant materials. Like most entomopathogenic fungi, spores of *Lecanicillium* attach to the exoskeleton of an insect, germinate there and then penetrate the cuticle via the production of an appressorium, which uses mechanical pressure to gain ingress into the host (St. Leger 1989). Once inside the insect body the fungal hyphae lose the need for a cell wall and will proliferate the internal cavity of the insect until all nutrients in the hemolmpyh are utilized (Hajek 1994). Although the duration of infection leading up to their ultimate death may vary, all insects eventually succumb. Death of the insect is caused by tissue destruction and sometimes toxins produced by the entomopathogen (Sujeetha

2014). External sporulation will then occur and the use of passive dissemination by wind will distribute the spores onto the next host (Parker et al. 2004).

Like most generalist entomopathogens, *Lecanicillium* fungi can be successfully used in certain environments coupled with the correct environmental factors (Shipp 2003). For example *Lecanicillium lecanni* has shown 85-100% mortality when used as a biocontrol for *Coccus hesperidium* (Hemiptera: Coccidae), a common greenhouse and indoor plant pest (Samsinakova and Kalalova 1975). A variety of biological, ecological, and behavioral factors serve to limit their effects on non-target insect species. Very important factors such as sufficient moisture and a strict temperature range are regularly required for infection to occur (Reddy and Bhat 1989).

Lecanicillium muscarium (formerly *Lecanicillium lecanii*) has been isolated from white fly (*Trialeurodes vaporariorum*) (Hemiptera: Aleyrodidae) and is marketed in the Netherlands as a fungal biocontrol product called Mycotal® distributed by Koppert B.V. Mycotal® consists of 16.1% *Lecanicillium muscarium* as the active ingredient and 83.9% inert ingredients. Mycotal® is attractive as a biocontrol in that there is limited evidence that suggests that Mycotal® has negligible effects on commercially available natural enemies in greenhouses and therefore it can be used in conjunction with other biocontrol measures such as predators and parasitoids. This disclaimer on Mycotal® packaging can be supported with previous fungal isolations from live *L. nigrinus,* predatory beetles of HWA, which revealed that *Lecanicillium* propagules were abundant (Table 1-1). Not only does this show that fungal propagules can come in contact with predatory insects of HWA in the field and not kill them, but it also suggests these beetles may serve as inadvertent vectors for entomopathogenic fungus (Kasson, Martin, and Wickert, unpublished data). This management technique of coupling two biocontrols is not an unfamiliar method and could add to the success of *L. muscarium* (Down et al. 2009). Interestingly, *L. nigrinus* spends part of its lifecycle in the soil, where previous studies have shown *Lecanicillium* spp. among other important entomopathogens to be abundant (Kasson, unpublished data). In this way, *Laricobius* species could further disseminate Mycotal® propagules and increase the efficacy of Mycotal® as a fungal biocontrol method for HWA.

Taxonomy and Phylogenetics of *Lecanicillium*

The genus *Lecanicillium* was introduced to accommodate entomogenous and fungicolous *Verticillium*-like anamorphs in the Claviciptaceae family previously classified in *Verticillium*

sect. *Prostrata* including *V. lecanii,* characterized by its ellipsoidal-cylindrical conidia and *V. psalliotae,* characterized by fusiform-falcate conidia (Zare and Gams 2001). Species within *Lecanicillium* generally form slender aculeate phialides, mostly with procumbent or postrate aerial hyhae, singly or in terminal and intercalary whorls. Conidia are generally elongate adhering in heads or fascicles at the tips of phialides, often at right angles to the phialide, a morphological feature exclusive to *Lecanicillium* (Zare and Gams 2001).

Lecanicillium resides in the Clavicipitaceae family within the order Hypocreales. Members of the Clavicipitaceae include pathogens of arthropods (e.g., *Cordyceps*, *Hypocrella*, and *Torrubiella*), parasites of truffles (e.g., *Elaphocordyceps*), and endophytes and epiphytes of the grass family (e.g., *Claviceps*, *Balansia*, and *Epichloe*) (Sung et al. 2007). Previous subfamilial classification was based on host affinity as a diagnostic character; Clavicipitoideae includes all species of grass symbionts (e.g., *Claviceps*, *Balansia*, and *Epichloe*) and Cordycipitoideae and Oomycetoideae contain all of the pathogens of arthropods and fungi (e.g., *Cordyceps*, *Hypocrella*, and *Torrubiella*). However, recent multi-gene phylogenetic analyses were conducted to address the evolution of Clavicipitaceae (Ascomycota) which revealed the subfamily Cordycipitoideae is not monophyletic (Sung et al. 2007) (Figure 1-2). In particular, species of the genus *Cordyceps*, which are pathogens of arthropods and truffles, are found in all three clavicipitaceous clades. Clavicipitaceae clade C, which includes *Lecanicillium*, consists of two major subclades: a strongly supported asexual lineage, which includes three species of the asexual genus *Simplicillium* and are primarily isolated as parasites of fungi and are not linked to any sexually reproducing species of Clavicipitaceae. Subclade C2 which includes the members of genera *Cordyceps* and *Torrubiella* as well as several members of asexual genera (e.g., *Beauveria*, *Isaria*, and *Lecanicillium*) with known links to *Cordyceps* and *Torrubiella* (Sung et al. 2007).

A Framework for Biological Control of HWA

At this time best option for managing further spread of HWA in forests is biological control mixed with some utilization of chemical stem injections on high value areas. The ultimate goals are to reduce losses of hemlock and regenerate healthy hemlock in infested and non-infested sites. Research on characterization, resistance, impacts, and possible management of HWA has been described above.

Table 1-1: Fungal propagules isolated from *Laricobius nigrinus* predatory beetles in a recapture program of beetles that were previously released and recaptured after one year in hemlock forests infested with hemlock woolly adelgid. Accession numbers and their % similarity relate to GenBank.

Plate ID	BLAST ID	Accession	% Similarity
D1	*Cordyceps confragosa*	AB111495	99
D2	*Isaria farinosa* isolate HK7	KC768083	99
D3	*Cordyceps confragosa*	AB111495	98
D5	*Microdiplodia* sp. G16A	EF432267	99
D6	*Simplicillium lamellicola*	AB214656	99
D7	*Isaria farinosa* isolate HK7	KC768083	100
D8	*Simplicillium lamellicola*	AB214656	99

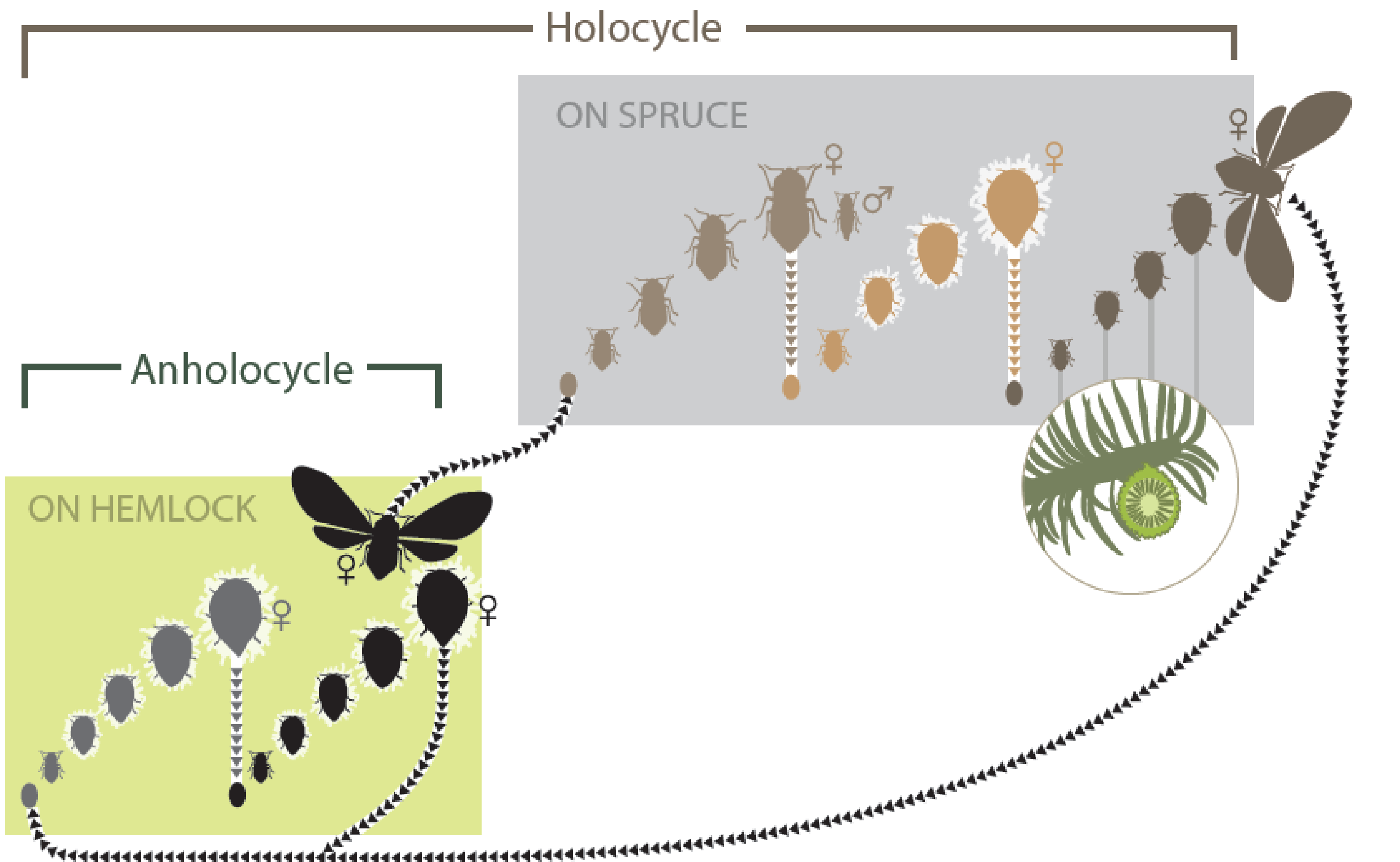
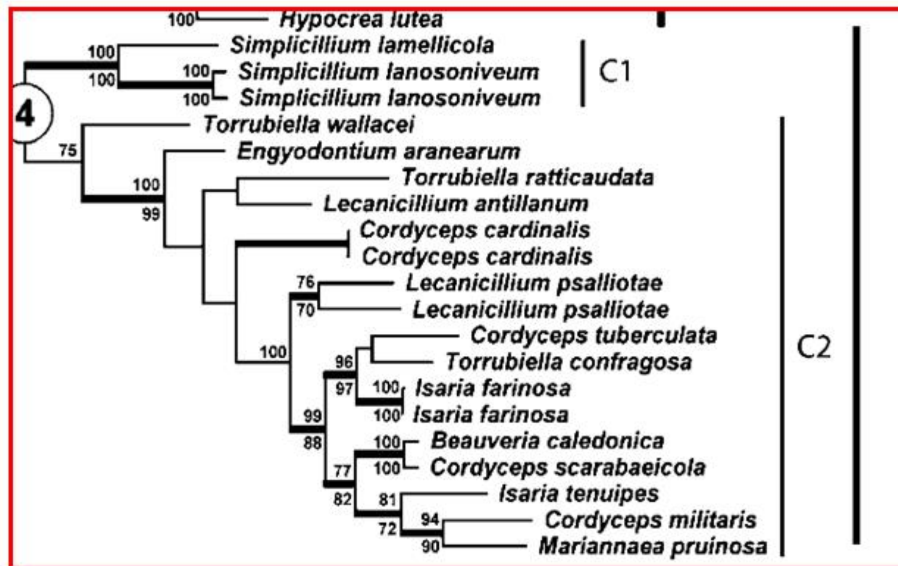


Figure 1-2: Life cycle of hemlock woolly adelgid (*Adelges tsugea*). In Asia, hemlock woolly adelgid completes its life cycle on two host species, hemlock and spruce. In the United States, there is only one of the two necessary hosts to complete the sexual life cycle. The Anholocycle on hemlock produces three life stages, an egg, four nymphal stages, and an adult. This cycle continues due to Parthenogenesis.



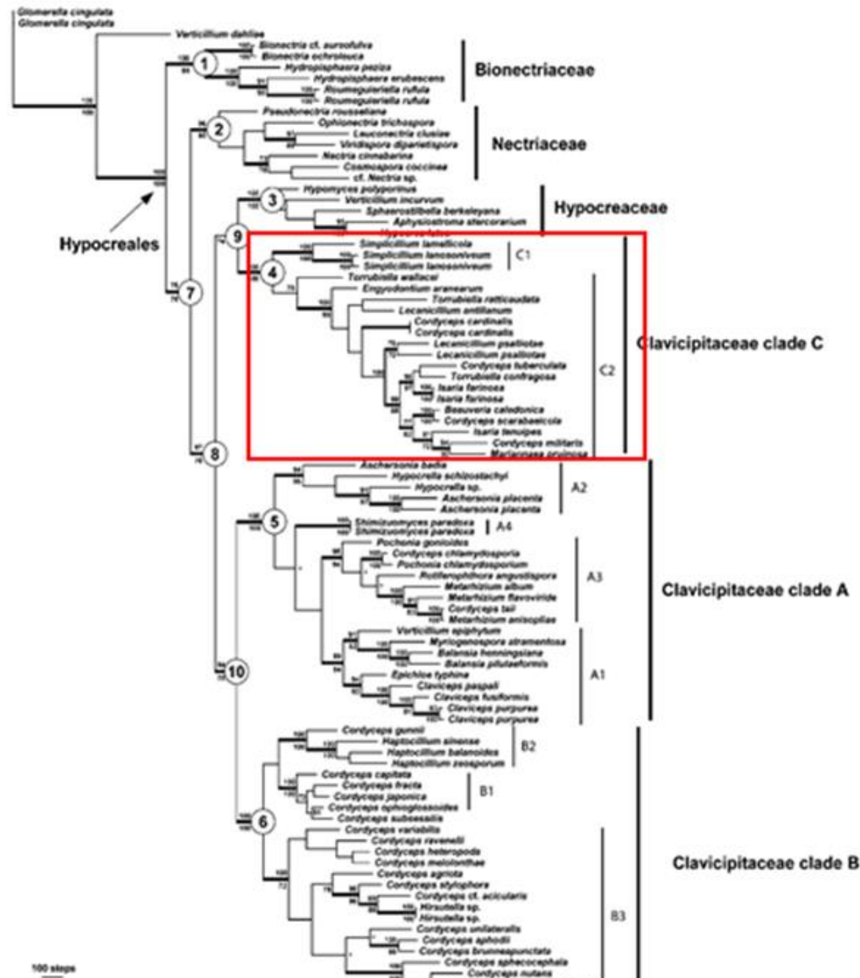


Figure 1-2: Clavicipitaceae phylogenetic tree with emphasis on Clavicipitaceae clade C which includes *Lecanicillium* and *Cordyceps* genera (Sung et al. 2007).

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Chapter 2

Persistence of entomopathogenic *Lecanicillium* (Clavicipitaceae, Ascomycota) in hemlock stands

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Abstract

Lecanicillium species are generalist fungal entomopathogens native to the United States and are a main component in the product Mycotal®. Mycotal® is a commercially formulated *Lecanicillium muscarium* biopesticide commonly used in greenhouses in the Netherlands. In determining the efficacy of a *Lecanicillium muscarium* based fungal biocontrol method against hemlock woolly adelgid (HWA), it is necessary to understand other fungi present in the hemlock environment. This is imperative because of the ability of other already-present fungal community members to inhibit *L. muscarium.* In order to find preexisting fungi in hemlock ecosystems, hemlock tissues, HWA and soil were sampled in a standardized method for varying sites. Five sites were established in the range of eastern hemlock with varying levels of HWA infestation and management types to contain and mitigate HWA populations. One site in Tennessee was included in the study due to a pilot study of Mycotal® in 2009 and 2010 which allowed observance of persistence of Mycotal® in the environment five years post inoculation. Five fungal taxa were frequently isolated from surface disinfested hemlock tissues and HWA in MD, OH, PA, TN and WV including *Colletotrichum fioriniae*, *Epicoccum nigrum*, *Pestalotiopsis microspora*, *Rhizosphaera macrospora* and a potentially undescribed Leotiomycete. The Leotiomycete, inhibited other fungi in the community and several species of *Lecanicillium*. Since the Leotiomycete fungus was present 17% of the time on average across all five sites, this fungus could be a significant factor against the persistence of *Lecanicillium* in the environment. In inhibition assays, *Lecanicillium* species/strains were inhibited 35% of the time by the Leotiomycete fungus. However, different fungal strains and isolates of both the Leotiomycete and *Lecanicillium* had different responses to each other. The *L. muscarium* strain in Mycotal® was not inhibited by the Leotiomycete. A total of six isolates were recovered of *Lecanicillium* out of 2,954 total fungal colonies across all substrates. *Lecanicillium* species were recovered from a chlorotic hemlock needle, an insect pupating within chlorotic/ necrotic hemlock needles, hemlock needles in the soil duff and soil samples. However the target entomopathogen was recovered in extremely low incidence and appears to be locally rare. In the 2015 fall/winter

sample season, two fungal colonies out of 2,472 recovered from hemlock needles were *Lecanicillium* species resulting in a <1% incidence across all five sites. This indicates that *Lecanicillium* is already present in the environment, at an extremely low incidence. The Mycotal® treated TN site harbored zero *Lecanicillium* isolates, which suggests that *Lecanicillium* does not persist in the environment.

Introduction

Hemlock woolly adelgid has resulted in significant mortality of eastern hemlock throughout eastern North America since its introduction near Richmond, Virginia around 1950. HWA is now widespread and deeply entrenched throughout much of hemlocks' continuous range in the eastern United States, where it continues to impact forests and ecosystem functions (Figure 2-1). In response to the massive mortality of eastern and Carolina hemlock throughout the eastern U.S. research efforts have intensified to better understand the biology, ecology, and population dynamics of HWA in areas long-affected by this invasive pest and areas along the advancing edge where efforts to slow the spread are most concentrated.

The last few decades have produced control methods that have mitigated the spread of HWA but are unable to eradicate the invasive pest. Current management methods that are most effective at reducing HWA populations include classical insecticide applications and biocontrol methods used individually and in combination. Classical insecticides include Imidacloprid (trade name Merit^{tm}) (which is a systemic neonicotinoids that have proven to be very successful in HWA control. Success rates of using chemical insecticides vary with many factors, such as temperature and drought in that they can slow the uptake and translocation and ultimately decrease the efficacy of these controls (Bennet 1957). Chemical control is limited to individual tree treatments that are in readily accessible, non-environmentally sensitive areas. Despite their effectiveness, insecticides including those most effective against HWA are harmful to a diversity of non-target insect species spanning several insect orders including many pollinators and can bioaccumulate in the environment (Blacquiere et al. 2012). These insecticides are acutely toxic to aquatic organisms limiting their application along stream beds, which unfortunately is the main ecotype for hemlocks (Cowles et al. 2006). Because of these non-target effects, their use and mode of application has been restricted in many forest cover types where hemlock fills the role of a keystone species including: White Pine-Hemlock, Eastern Hemlock, Hemlock-Yellow Birch, and Yellow-Poplar-Eastern Hemlock.

The first exploration for native natural enemies (*i.e.* classical biocontrols) of HWA in Japan began in 1992 where *Sasajiscymnus tsugae* was recovered (Cheah 2011). Shortly thereafter, in 1995, a federally funded program for biological control of HWA using non-native predators was initiated in the eastern United States, resulting in importation of predators from

Japan, China, and Canada. *Scymnus* lady beetles were recovered in China (Montgomery and Keena 2011). Native *Laricobius nigrinus* were collected from a western hemlock seed orchard in Victoria, British Columbia in 1997. Following the identification, quarantine evaluation, mass rearing, and releases of these first group of biocontrol candidates, several other additional biocontrols were introduced and include: *L. osakensis* (Coleoptera: Derodontidae)*, Scymnus coniferarum* (Coleoptera: Coccinellidae)*, Leucopis argenticollis* (Diptera: Chamaemyiidae), *L.s piniperda* (Diptera: Chamaemyiidae), and *Sasajiscymnus tsugae* (Coleoptera: Coccinellidae) (Cheah 2011). Currently, *Laricobius* spp. appear to be the most successful insect biocontrols on account of widespread establishment in HWA impacted areas throughout the eastern U.S. The efficacy of *L. osakensis* and *L. nigrinus* as controls is promising as both the larvae and adults feed on all life stages of HWA (Salom et al. 2011, Zilahi-Balogh et al. 2002). The release of insect predators as biocontrols of HWA have proven useful in more sensitive areas where chemical controls are restricted, yet the post-release results for many of these biocontrols are inconclusive. For example, some of the predatory beetles' lifecycles are asynchronous with that of HWA and therefore effective control has not been realized (Salom et al. 2012). On the other hand, since 2008 hemlocks have stopped dying in the 5,000-square mile *L. nigrinus* release area around Grandfather Mountain, NC, and regrowth of adelgid-infested hemlocks at several of the release sites has been observed (Oakes 2015). Nevertheless, when used together with chemical controls, these management methods are somewhat successful, but they are not able to eradicate HWA from the landscape or to levels that limit additional mortality.

Other avenues for control are being looked into to add to the management and hopeful eradication of HWA. Fungal biocontrol might be an appropriate option to add to the ongoing integrated pest management against HWA, especially if those controls can be used in concert with insect and chemical controls. Fungal biocontrols have proven to be a successful management method against other invasive species such as *Entomophaga maimaiga* on gypsy moth (*Lymantria dispar*) (Lepidoptera: Erebidae) and *Beauvaria bassiana* on aphids and other greenhouse insect pests (Hajek et al. 1996, Hong and Kim 2007). Green Muscle®, a successfully commercialized and extremely virulent strain of *Metarhizium anisopliae* is an effective biocontrol of grasshoppers and locust swarms (Douthwaite 2001). This shows that there are differences not only between entomopathogenic species of fungi, but there are differences in

strains of a single species in strength against specific hosts coupled with other factors (Douthwaite 2001).

Moreover, many of these fungal biocontrols have proven to be host-adapted with limited non-target impacts. Still many obstacles remain in place that limit the use of entomopathogenic fungi as a management tool against HWA, such as limited knowledge on fungal biology and life cycle, fungal competition, as well as regulatory and commercialization aspects (Shipp et al. 2003). Previous studies by Reid et al. (2002), indicated the *Lecanicillium* was common from dead HWA throughout the eastern U.S. and might serve as an effective biocontrol. The genus *Lecanicillium* includes many generalist entomopathogenic fungi with a cosmopolitan distribution and are relatively common across the landscape (Sree and Joshi 2015). *Lecanicillium* also has a history of use as a control of greenhouse pests including whiteflies and aphids (Alavo 2015).

In a pilot study conducted in 2009 and 2010 in Tennessee, entomopathogenic *L. muscarium* was aerially deployed against HWA as a means of assessing its efficacy against HWA. This study utilized a commercialized form of *L. muscarium* named Mycotal® distributed by Koppert Biological Systems in the Netherlands. Mycotal® consists of spores of *L. muscarium* strain number ARSEF 5128 isolated from white fly (*Trialeurodes vaporariorum)* (Hempitera: Aleyrodidae) in the United Kingdom (Koppert 2015). One year post-inoculation data indicated a decline in growth of HWA populations following inoculation with the enhanced fungus, yet results were not significantly different from controls (Costa 2010). Regardless, the short term results of this study provided a glimpse into the potential application of aerially deployed fungal biocontrols against HWA and a foundation for follow-up studies. The long-term results from the pilot study were inconclusive due to many confounding factors, including the polar vortex of 2014, which reduced HWA populations to trace levels and in turn, limited the ability to accurately assess long-term efficacy against HWA. The ongoing pilot study site enables sampling opportunities to assess the long term persistence of *Lecanicillium* in the environment as significant amounts of exogenous inoculum was applied to this ecosystem.

Many fungal species occupy and colonize healthy and naturally senescing plant tissues and can competitively exclude or inhibit growth of other fungi (Carroll 1988). Fungi can utilize their host resource in two ways that preclude other fungi from accessing the resource: they can deplete the resource, or they can exude chemicals to prevent competitor access into the tissue

(Wicklow 1992). Competition is an important aspect to consider when introducing a fungal biocontrol because it may reduce the overall efficacy of the introduced fungus. Hemlock branchlets and adjacent leaf tissues serve as environmental reservoirs for many species of fungi occupying various ecological niches such as plant pathogens, saprotrophs and beneficial plant endophytes (Carroll and Carroll 1978, Marcelino et al. 2009, U'Ren et al. 2012). Because fungi have the ability to transition from a primary niche to a facultative niche depending on available food source, it is possible that entomopathogenic *Lecanicillium* species already persist in hemlock environments as saprotrophs or plant pathogens even in the absence of insect hosts (Marcelino et al. 2009). When HWA is introduced into the environment, *Lecanicillium* may have the ability to revert back to the entomopathogenic niche and infect HWA.

This study aims to elucidate the fungal community in hemlock ecosystems and the persistence of *Lecanicillium* as well as other potential entomopathogenic fungi both in areas of previous deployment such as the pilot study area in TN as well as untreated areas that may or may not harbor native entomopathogenic populations. The specific objectives will address the points of environmental reservoirs for *Lecanicillium* in the hemlock environment and if the target entomopathogen persists in the environment.

Materials and methods

Sampling locations and Experimental Design

Four sampling locations within a 150-mile radius of Morgantown, West Virginia were chosen for fungal community composition studies on the following criteria: basal area and stem density of hemlock, levels of HWA, and HWA management practices. Sites from west to east included Shade River State Forest, OH; West Virginia University's University Research Forest, WV; Ohiopyle State Park, PA, and Savage River State Forest, MD. With the exception of parts of Shade River State Forest, which is on the advancing edge of HWA's current geographic range, all other study locations had previous history of adelgid, with initial infestations reported in 2006 for WV, 2007 for MD, 2009 for PA, and 2014 for OH (Table 2-1). At three of the four locations (MD, PA, and WV), three plots were established. In OH, two additional plots were established on account of recent HWA infestations within the forest which allowed direct comparisons of infested and non-infested sites. By including sites not historically affected by HWA, direct comparisons of fungal community composition among long-infested, recently infested, and

uninfested sites could be made. Management methods also varied among geographic locations allowing for comparisons in fungal communities within and across sites with released predatory beetles, chemical stem injection, a mixture of chemical stem injection and predatory beetle releases or no management.

Permanent tenth-acre plots were established to allow for repeated sampling and was based largely on capturing variability in HWA incidence, management strategy and HWA residency time. Within each fixed radius plot, tree-level variables including species, diameter-at-breast-height (DBH), canopy class, and percent dieback (for hemlock only) were recorded. HWA density was rated on branchlets as well as overall for infested hemlock trees. An ordinal scale was used to rate HWA infestation levels and were as follows: uninfested, trace (zero individuals present, but evidence of previous HWA infestations), light (1-10 individual HWA masses / 10" branchlet), moderate (11-20 individual HWA / 10 inch branchlet), and heavy (>21 individual HWA / 10 inch branchlet), crown transparency and percent crown dieback ratings were based on 10% increments.

For hemlock sampling, trees were randomly selected from within each of three canopy classes (upper-story, mid-story, and suppressed) and branches destructively sampled using a pole pruner. Given the co-dominance of many of the hemlock across all sites, sampling in the higher parts of the canopy was not possible without felling the tree. Therefore sampling focused on the lower branches and up to 20 feet in height. Likewise, assessments of HWA density in the upper part of the crowns of over-story and mid-story trees could not be assessed. Two trees per canopy class were selected, flagged, and their locations recorded. From each selected canopy class representative, three categories of hemlock needles were sampled: asymptomatic (healthy), chlorotic (diseased), and necrotic (diseased and senesced). Six needles per category were carefully removed from branchlets to avoid damaging of the intact needle prior to surface disinfestation and subsequent fungal plating and placed inside pre-labeled microcentrifuge tubes for transport back to the lab. Three ten-inch randomly chosen branchlet sections from which representative needles were taken were also retained for fungal isolation. To examine possible linkages between infestation levels and fungal community structure, adelgid population density was assessed and noted for each of the sampled 10 inch branchlet as described earlier. In locations where extant HWA populations were found, infested branchlets were sampled and

brought back to the lab for adelgid sampling. A total of ten live and ten dead HWA were sampled per HWA-positive plot for fungal isolations.

Mycotal® Release Pilot Study Location, Campbell Co. Tennessee

In May 2009, a pilot study was initiated within a mature hemlock stand along Titus Creek immediately west of the North Cumberland Wildlife Management Area in Campbell County, TN. The purpose of the pilot study was to evaluate the efficacy of Mycotal®, a promising commercial formulation of *L. muscarium* approved for control of whitefly in greenhouse settings, against HWA (Costa 2011). A total of sixteen 1.25-acre hemlock plots were established from 2009-2010 on the basis of pre-treatment HWA density, twelve in 2009 and an additional four in 2010. All plots were aerially treated via helicopter at a volume of 10 liters/acre for each of three treatments. In 2009, plots received one of three treatments: 'no spray' control, Mycotal® (1×10^8 spores ml^{-1}) and Mycotal® at the same concentration enhanced with the microfactory formulation (5% w/v MycoMax®) (Table 2-2). Treatments were replicated three times. In 2010, 2009 plots were again treated but treatments were randomly assigned with some receiving the same treatment, no treatment or a new treatment in year two (Table 2-2). In addition four new plots were established in close proximity to 2009-treated plots and were treated with Mycotal® (1 plot), Mycotal® + Mycomax® (2 plots), or served as a control (1 plot) (Table 2-2). The purpose of the microfactory formulation, which consisted of whey protein, was to serve as an additional food source until the fungus came in contact with the insect host. The oil adjuvant Addit (0.25% v/v: Koppert Biological Systems) and the sticker Hyperactive (0.05% v/v : Helena Chemical) were added to both fungal treatments. The oil adjuvant Addit served to increase the effectiveness of the fungal biocontrol by increasing spore longevity. Previous formulation studies of *Metarhizium anisopliae* (Green Muscle®) indicated that fungal spore viability and efficacy is enhanced when covered in oil, especially in dry conditions (Prior and Greathead 1989).

Fungal community sampling was conducted in 2015 in sites previously established for the HWA aerial suppression pilot study. A total of 12 of the 16 previously established plots were successfully located and sampled. Sampling was as previously described for sites in MD, OH, PA, and WV with some exceptions. To allow for site comparisons 5-years post-inoculation, hemlock crown ratings were assessed and recorded for a subset of previously rated trees on 12 of

the original 16 plots. Measurements included HWA presence and abundance using an ordinal rating scale as previously described, crown transparency and percent crown dieback.

Fungal isolations

Field collected hemlock needles and branchlets as well as HWA were sampled to permit fungal community characterization and assess whether *Lecanicillium* was pervasive across sampled sites. Sampling occurred in fall-winter of 2014 and summer of 2015 to consider the possible influence of the seasons and abiotic factors on the fungal community. Three categories of hemlock needles previously described were surface disinfested in a 10% sodium hypochlorite solution for 20 seconds and plated on a Difco potato dextrose agar (PDA; BD and Co., Franklin Lakes, NJ, USA) amended with streptomycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) and tetracycline (Fisher Scientific, Pittsburgh, PA, USA) (+ST).

A total of thirty-six needles for each of the three needle categories were sampled per plot with a total of 324 needles from across three plots at each of three sampling locations in MD, PA, and WV and 540 needles across five plots in OH. In Tennessee 1,296 needles were sampled. Following surface disinfestation, needles were arranged three per 10 cm diameter petri plate. Branchlets were similarly surface disinfested as previously described and plated individually on PDA+ST. A total of 54 branchlets from across three plots at each of three sampling locations in MD, PA, and WV and 90 branchlets across the five plots in OH were sampled. In Tennessee 72 branchlets were sampled. Live and dead HWA were aseptically removed from the base of infested needles, surface disinfested (dipped) in a 95% ethanol and plated on PDA+ST. Two adelgid were plated per 10 cm diameter plate. A total of 119 adelgid from four sampling locations in MD, PA, OH and TN were collected. A total of 79 of the 119 HWA were alive at the time of plating and 40 were dead to allow comparisons between saprophytic and entomopathogenic fungal community members. The West Virginia site, despite previous confirmation of HWA, lacked detectable populations for sampling. A secondary round of sampling collections was conducted in the summer of 2014 to see if seasonal differences occurred throughout the year. 1,296 Needles were sampled in the summer collections, and 72 branchlets from the standard sampling locations in OH, PA, MD, WV. HWA were unable to be sampled in the summer months.

Soil samples were collected at each of the 26 plots. Soil samples were also included given recent work by Kasson (unpublished) that suggested *Lecanicillium* spp. was in high incidence in soils on account of high arthropod diversity. Initial soil sampling aimed to enhance *Lecanicillium* recovery with selective media, such as Ophiostoma Selective Agar (OSA). Given the high amount or organic matter in the soil samples collected, hemlock needles recovered from soil were separated from the remaining substrate and plated as previously described for branchlet-extracted needles but on OSA. For the remaining soil substrate, serial dilutions of soil were generated by adding 5 g of substrate to 50 ml of sterile distilled water. Following homogenization, serial dilutions were generated up through 1 x 10^{-7}. Because of the presumed high colony count from less diluted suspensions, only dilutions for 1 x 10^{-5} through1 x 10^{-7} were plated on OSA. Plates were kept at ambient temperatures for 7 – 10 days or until fungal growth appeared from hemlock tissues, soil dilutions and HWA.

For fungal characterization, fungi were initially grouped based on colony and spore morphology. Colony features such as presence or absence of aerial mycelium, presence and morphology of conidiomata and/or ascocarps/basidiocarps, presence and color of pigments, and general growth rates were used to distinguish fungal genera and species.

Storage and preservation of representative fungi

For long-term storage, representative isolates of morphotypes with an incidence of >3% of the total number of fungal isolates recovered or isolates of particular interest, were placed onto PDA slants for long-term storage and maintained at 4°C. From each site, representative isolates were retained for DNA and entomopathogenicity studies as well as inhibition assays. Singleton and other low incidence morphotypes were tallied but not retained. Representative slants are maintained in cold storage in the Kasson Plant Pathology Lab at West Virginia University and available upon request.

DNA extraction and molecular identification of isolates

Genomic DNA was extracted from fungal mycelial plugs harvested from Difco potato dextrose broth (PDB; BD and Co., Franklin Lakes, NJ, USA) following procedures described by Short and colleagues (2015). All PCR was performed on a MJ Research PTC-200 Peltier Thermal Cycler (GMI, Ramsey, MN) using primers (Integrated DNA Technologies, Coralville, IA, USA)

ITS 4 and ITS5 (White et al. 1990) to amplify the nuclear internal transcribed spacer regions ITS1-5.8S-ITS2 (ITS) and BioLine PCR Kits (Bioline USA Inc, Taunton, MA) in 25.5 µL reactions containing: 1 µL of each of two primers, 1 µL genomic DNA, 10 µL nuclease free water, and 12.5 Bioline PCR Mastermix. For gel electrophoresis, 4 µL of SYBR gold (Invitrogen, Grand Island, NY, USA) and 4 µL of loading dye (5Prime, Gaithersburg, MD) were added to PCR products which were then loaded into a gel comprising 0.5% Tris-Borate-EDTA buffer (Amresco, Solon, OH, USA) and 1.5% w/v agarose (Amresco, Solon, OH, USA). Electrophoresis was performed at 90 v for 45 min and DNA bands were visualized on a UV transilluminator (Syngene, Frederick, MD, USA). A 100 bp molecular ladder (Omega Bio-tek, Norcross, GA, USA) was included for size comparison. PCR products were purified using ExoSap-IT (Affymetrix, Santa Clara, CA). Representative PCR amplicons were Sanger sequenced with the same primers used for PCR (Eurofins, Huntsville, AL, USA).

Fungal inhibition assays

A preliminary inhibition assay was performed to see if dominant fungal community members recovered from hemlock and HWA inhibited *Lecanicillium* growth. Fungal taxa included *Colletotrichum fioriniae*, *Epicoccum nigrum*, *Pestalotiopsis microspora*, *Rhizosphaera macrospora.* To test inhibition, two fungal plugs, one from each of the two tested species, were co-cultured on a single 10-cm diameter PDA+ST petri plate and kept at ambient temperatures for 7 days. Tester plugs originated from 12-21 day old parent cultures that were cultivated from long-term storage slants for the sole purpose of this assay. Seven days post-inoculation, presence and extent of inhibition was recorded.

The *Lecanicillium spp.* isolated from the chlorotic needle, the Lepidopteran pupa (Figure 2-16) and Mycotal® were plated with a representative of the main saprophytic guild of *Penicillium*, *Aspergillus* and *Trichoderma*. None of these fungi inhibited the growth of *Lecanicillium*.

Results

Fungal diversity summary

A total of 3,132 needles, comprising chlorotic, necrotic, and asymptomatic categories, 378 branchlets and 119 HWA were sampled from 26 plots across all 5 sites. A total of 2,472 fungal colonies were recovered from needles, 351 from branchlets, and 120 from HWA from all five

sites in PA, OH, MD, WV and TN (Table 2-3)(Figure 2-2). From the fourteen plots in the four standard sites of PA, OH, MD, and WV from fall-winter 2014 a total of 1,836 needles, 306 branchlets and 90 adelgid were sampled. This round of sampling produced 1,303 fungal colonies recovered from needles, 244 fungal colonies from branchlets and 83 from HWA. From Tennessee, where Mycotal® was aerially deployed a total of 1,296 needles, 72 branchlets and 29 live adelgid were sampled. This round of sampling produced 1,169 fungal colonies from needles, 107 fungal colonies from branchlets and 37 from HWA (Figure 2-3).

Five fungal taxa were frequently isolated from surface-disinfected hemlock tissues and HWA in OH, MD, WV, PA and TN including *Colletotrichum fioriniae*, *Epicoccum nigrum*, *Pestalotiopsis microspora*, *Rhizosphaera macrospora* and an undescribed Leotiomycete (Figure 2-4 - 2-8). A high number of singleton taxa as reflected by a species richness value of 44 fungal genera across all sampling substrates, were present, albeit at low levels. As such these low incidence taxa were grouped into a combined category labeled as "other" in Figures 2-5 through 2-9. The ecological niches of the dominant fungi included previously-confirmed plant pathogens (Kou et al. 2015), plant endophytes (Carroll and Carroll 1978, U'Ren et al. 2012), saprotrophs and to a lesser degree, facultative entomopathogens (Marcelino et al. 2009, Pirttilä 2009). *Colletotrichum fioriniae* is a confirmed pathogen of apple, European blueberry, grape, olive, papaya, and strawberry (Damm et al. 2012) causing blight, leaf spots, cankers and dieback in these hosts. Recently, *Colletotrichum fioriniae* was implicated in causing seedling blight of poison ivy in Virginia (Kasson et al. 2014). *Colletotrichum* was isolated from all substrates with a uniform distribution. *Epicoccum nigrum* has been reported as both an endophyte and opportunistic plant pathogen in conifers (Kowalski 1993). During this study, *Epicoccum* was most commonly isolated from chlorotic (42%) and necrotic (45%) needles. *Pestalotiopsis microspora* is a weak secondary plant pathogen involved in mostly saprophytic activity of already stressed plant tissues and can be found in damaged areas and already diseased tissues (Sinclair 2005). Chlorotic (34%) and necrotic (49%) tissues yielded the highest incidence of *Pestalotiopsis* with some sites having no *Pestalotiopsis* recovered from green healthy tissues. *Rhizosphaera macrospora* is the causal agent of a needle cast disease which leads to premature death and casting of needles in conifers (Sinclair 2005). *Rhizospheara* was almost always isolated from only necrotic needles, but was also recovered in extremely low incidence from the other substrates. The Leotiomycete is suspected to be an endophyte of hemlock tissues as it is

present in all needle types, especially in healthy hemlock needles (U'Ren et al. 2012). This fungus could also be an opportunistic plant pathogen like *Epicoccum* in that it is the most common in chlorotic hemlock needles. *Colletotrichum* and the Leotiomycetes fungus were never recovered from HWA. All locations had similar representative numbers and percentages of key fungal species of interest.

Necrotic needles harbored the most fungi at 1,071 colonies, 871 were isolated from chlorotic needles, and 530 from green asymptomatic needles (Figure 2-9). There was a trend of fungal species recovered from the different needle types at each site. Average percentages of recovery for genera from needles were *Colletotrichum,* 7%; *Epicoccum,* 3%*; Pestalotiopsis,* 21%*; Rhizosphaera,* 4%; an undescribed Leotiomycete, 17% (Table 2-10). On average at all sites 49% of fungi belonged to the "Other" category which was comprised of fungal species in a saprophytic guild of common environmental contaminants. These environmental contaminants included: *Penicillium* spp., *Aspergillus* spp., *Trichoderma* spp., *Mucor* spp., and *Xylaria* spp. Average percentages of recovery for genera from all sites were *Colletotrichum*, 8%; *Epicoccum,* 3%*; Pestalotiopsis,* 24%*; Rhizosphaera,* 4%; and an undescribed Leotiomycete, 15% (Figure 2-11). On average, at all sites 47% of fungi belonged to the "Other" category. On average at all sites 43% of fungi belonged to the "Other" category isolated from branchlets. Average percentages of recovery for genera from branchlets across all sites were *Colletotrichum*, 13%; *Epicoccum,* 3%*; Pestalotiopsis,* 43%*; Rhizosphaera,* 3%; and an undescribed Leotiomycete, 1% (Figure 2-12). On average at all sites 48% of fungi belonged to the "Other" category isolated from HWA. Average percentages of recovery for genera from HWA from all sites were *Colletotrichum*, 0%; *Epicoccum,* 9%*; Pestalotiopsis,* 37%*; Rhizosphaera,* 6%; and an undescribed Leotiomycete, 0% (Figure 2-13).

A total of six out of 2,954 fungal colonies recovered across all plots were *Lecanicillium*. This included five isolates of L. *muscarium*, *Lecanicillium attenuatum* and one isolate of *Lecanicillium fungicola*. *L. fungicola* is a mushroom pathogen, with the ability to degrade a broad spectrum of proteins but is not described as an entomopathogen (St Leger et al. 1997, Zare and Gams 2001, Kim et al. 2008). In the fall-winter 2014 sample season, two colonies out of 2,472 needle fungal colonies recovered were *Lecanicillium* species resulting in a <1% incidence across all five sites. Two *Lecanicillium* spp. were successfully isolated from 582 fungal colonies

recovered from all substrates resulting in a 0.3% incidence. *Cordyceps confragosa* teleomorph of *L. muscarium* was isolated from hemlock needles *L. attenuatum* was isolated from a pupating Lepidopteran in hemlock needle bundles in Ohio. *Lecanicillium* was found in the soil of two sites (WV and OH) at extremely low incidence as well. One *C. confragosa* came from WV soil. Two isolates of *C. confragosa* came from soil dilutions from the OH site and one *L. fungicola* isolate from Ohio soil needles.

Total counts from soil substrates were not recorded because the application of selective media inhibited most fungi. Other cycloheximide tolerant entomopathogenic fungi were recovered from the soil environment. *Cordyceps brongniartii* is the teleomorph of *Beauveria brongniartii* a common generalist entomopathogen (Shimazu 1988). *Simplicillium* was recovered from the environment as well and is also an entomopathogen.

A secondary round of sampling was conducted in the summer of 2014 to see if seasonal differences occurred throughout the year. Needles (1,296) were sampled in the summer collections and 72 branchlets from the standard sampling locations in OH, PA, MD, WV. HWA were unable to be sampled in the summer months. This round of sampling produced 1,311 fungal colonies recovered from needles and 316 fungal colonies from branchlets (Figure 2-14). There was a shift in fungal species from the average fall-winter collections in that more needles produced the secondary pathogen *Pestalotiopsis.* This could be due to the possibility that evergreen trees drop their needles in fall, and the natural senescence of the needles in late summer created more reservoirs for the secondary pathogen (Terhonen et al. 2011). On average at all sites 27% of fungi belonged to the "Other" category Average percentages of recovery for genera from all sites and hemlock substrates were *Colletotrichum*, 12%; *Epicoccum,* 2%; *Pestalotiopsis,* 47%*; Rhizosphaera*, 2%; and an undescribed Leotiomycete, 10%.

No appreciable differences in fungal community structure were noted between upper canopy, mid-canopy and suppressed hemlock trees. Likewise, no significant differences were observed between levels of HWA on the branchlets and fungal community differences. There does not appear to be a significant difference between management types and the percentages of fungi recovered from sites. West Virginia (0.271322751) and Pennsylvania (0.275270037) are more diverse than OH (0.331098), MD (0.342391), and TN (0.335824) due to a lower diversity index. All sites complied together have a diversity index of (0.308104). No differences were

noticed from dead or alive adelgid. Fungal growth probably resulted from fungal spores on their woolly masses which were merely encountered from the environment, not from their infected bodies.

There was no difference between fungi recovered from upper mid and suppress trees. There is no difference between canopy classes and fungal occurrence in incidence of species or count. There was not a difference between levels of HWA on the branchlets and fungal community differences. There does not appear to be a significant difference between management types and the percentages of fungi recovered from sites. No differences were noticed from dead or alive adelgid. Fungal communities associated with HWA were likely resulted from fungal spores on their woolly masses which were merely encountered from the environment, not from the HWA bodies.

There were differences between fungal isolation occurrences between HWA and hemlock tissues. The Leotiomycetes and *Collectotrichum* were only recoverable from plant tissues. HWA was found colonized by *Epicoccum*, *Pestalotiopsis*, and *Rhizosphaera* and species classified in the earlier described "Other" category.

Molecular identification

BLASTn searches of the NCBI GenBank database found 99 to 100% maximum identity matches with the fungal identity sequences deposited (Table 2-4). *Lecanicillium* spp. were confirmed recovered, which confirms that hemlock tissues can serve as a viable, albeit rare, reservoir for *L. muscarium*.

Fungal Inhibition assays

Results of the inhibition assay between *Lecanicillium* isolates and dominant fungal community members revealed that four of the five most commonly recovered fungi from hemlock and HWA (*Colletotrichum fioriniae*, *Epicoccum nigrum*, *Pestalotiopsis microspora*, *Rhizosphaera macrospora)* did not inhibit the growth of any of the tested *Lecanicillium* species, although overgrowth was observed in some of the pairings. The potentially novel Leotiomycete exhibited strong inhibitory effects against other fungal community members (data not shown) and against several species of *Lecanicillium* in inhibition assays. Over all across all pairings of *Lecanicillium* isolates with several Leotiomycetes, *Lecanicillium* were inhibited 35% of the time (Table 2-5)

(Figure 2-15). However, inhibition varied across strains of both species. Interestingly, the *Lecanicillium* strain in Mycotal® was not inhibited. Since the Leotiomycete fungus is present 17% of the time on average across all five sites, this could be a significant factor in the persistence of *Lecanicillium* in the environment.

Discussion

Sampling of hemlock tissues, soils, and HWA confirmed that *Lecanicillium* isolates*,* albeit rare, do exist in the environment in the absence of an insect host. The isolation from the chlorotic needle suggests that *Lecanicillium* may, in rare cases, survive as a facultative saprotroph and/or endophyte in addition to having an entomopathogenic lifestyle in hemlock needles. Although the primary goal of thesis study was to uncover insights into the biology of *Lecanicillium*, several observations on other common fungi in the hemlock ecosystem are noteworthy. First, *Colletotrichum fioriniae*, *Epicoccum nigrum*, *Pestalotiopsis microspora*, *Rhizosphaera macrospora* and the undescribed Leotiomycete all occupy a niche within the ecosystem. The absence of Colletotrichum and Leotiomycete from HWA but dominance within needle and branchlets samples suggest these fungi are dominant saprotrophs/ endophytes not capable of colonizing HWA. Interestingly previous studies by Marcelino and colleagues (2008) demonstrated that endophytic *Colletotrichum fioriniae* were opportunistic entomopathogens of another hemlock pest, elongate hemlock scale but the host range of this fungus does not appear to extend to HWA. There does not appear to be a significant difference between management types and the percentages of fungi recovered from sites indicating that previous management methods do not need to be considered if thinking about applying a fungal biopesticide.

Lecanicillium is inhibited, *in vitro*, by other fungal community members in the hemlock tissues. The presence of Leotiomycete fungi must be considered in the efficacy of using Mycotal® or a *Lecanicillium*-based fungal biocontol. Different *Lecanicillium* species/strains reacted differently, but the Mycotal® isolate was not inhibited by the Leotiomycetes. The Mycotal® strain stands as a best option against fungal competition. However, as an important aspect of this study, all pairings were performed on PDA media. There could be a difference in inhibition tendencies when plated on different media types which means these results are is inconclusive.

The Tennessee site had low incidence of HWA in the canopies during the summer 2015 collection. This could be due to the aerial application of Mycotal® however, it could also be a result of the 2014-2015 polar vortex winter or another unknown abiotic/biotic factor.

Lecanicillium should still be considered as a combat method against hemlock woolly adelgid as it is a more ecofriendly alternative to chemical insecticides. The application approach should be an augmentation of the natural population of *Lecanicillium* by an inundative release of millions of *Lecanicillium* spores. An aerial application of Mycotal® in the first year of HWA infestation could add to the already present *Lecanicillium* in the environmental reservoir of the soil and greatly reduce HWA populations until traditional chemical and biological methods can be implemented. *Lecanicillium* should not be used as a preventative measure since it does not persist in the environment, but instead as a reaction to HWA being present in the ecosystem.

Table 2-1: . Site details for standard sampling locations in Maryland, Ohio, Pennsylvania and West Virginia including latitudes and longitudes, subplots, year of HWA first detected, 2015 infestation levels and current management practices of HWA

Location	Plant Hardiness Zones Lat/Log	Subplots	HWA First Detected	Current 2014-2015 HWA infestation levels	Current/Previous Management
West Virginia University's Experimental Forest	6a	WV1	2006	Trace-light	Preventative CORE tablet
Morgantown, West Virginia	Lat: 39.6551 Long: -79.7280	WV2	2006	Trace	Preventative CORE tablet
Monongalia County		WV3	2006	Trace	Preventative CORE tablet
Shade River State Forest	6a	BH1	-	Not Present	None
Tupper Plains, Ohio		BH2	-	Not Present	None
Meigs County	Lat: 39.0841 Long: -81.8846	HTP1	2014	Uninfested - Light	Chemical Stem Injections
		HTP2	2014	Uninfested - Light	Chemical Stem Injections
		HTP3	2014	Uninfested - Light	Chemical Stem Injections
Ohiopyle State Park	6a	PA1	2009	Trace- Moderate	Chemical Stem Injections
Ohiopyle, Pennsylvania	Lat: 39.9207 Long: -79.4905	PA2	2011	Trace	Chemical Stem Injections
Fayette County		PA3	2013	Trace	1966 *L.n.* Releases in 2013
Savage River State Forest	6a	MD1	2007	Moderate - High	Preventative CORE tablet & *L.n.* Releases
Grantsville, Maryland	Lat: 39.5989 Long: -79.0762	MD2	2007	Trace	Chemical Stem Injections
Garrett County		MD3	2007	Moderate	1735 L.n. Releases in 2014

Table 2-2: . Site details for the aerial application of Mycotal® Pilot Study site in Tennessee

Location	Plant Hardiness Zones Lat/Log	Subplots	Included in Wickert Sampling	Current HWA infestation levels	2009 Treatment	2010 Treatment	Treatment Block
Royal Blue WMA	6a	1			Fungus and Whey	none	D
Pioneer, Tennessee		2	X OSA	Light	Control	Fungus and Whey	B
Cambell County	Lat: 36.3440 Long: -84.2822	3	X	Light	Fungus	none	
		4	X	Light	Fungus	none	
		5	X GYE	Moderate	Control	Control	B
		6	X	Light	Fungus and Whey	Fungus and Whey	C
		7	X	Light	Fungus	none	
		8	X	Light	Fungus	none	
		9	X	Light	Control	Control	B
		10	X	Moderate	Fungus and Whey	Control	C
		11	X	Light	Fungus and Whey	Fungus and Whey	D
		12		Light	Control	none	
		13	X	Light	none	Fungus0	B
		14			none	Fungus and Whey	A
		15	X	Light	none	Control	A
		16			none	Fungus and Whey	A

Table 2-3: Fungal colonies recovered from substrates of 5 sampling sites

	Needles				
	Chlorotic	Necrotic	Asymptomatic	Branchlets	HWA
Maryland	63	137	51	75	62
	Total Needles:	251			
Ohio	184	201	146	34	17
	Total Needles:	531			
Pennsylvania	74	115	44	72	4
	Total Needles:	233			
Tennessee	452	497	220	107	37
	Total Needles:	1169			
West Virginia	98	121	69	63	0
	Total Needles:	288			

Table 2-4: ITS confirmation of representative fungal isolates recovered from hemlock, HWA and soil

Site	Sampling Substrate	NCBI BLAST ID	% Identity	GenBank Accession #
MD1	**Chlorotic Needle**	***Colletotrichum fioriniae***	**100**	**JN121190**
OH HTP3	**Dead HWA**	***Pestalotiopsis* sp.**	**100**	**JX624316**
MD1	**Chlorotic Needle**	***Rhizosphaera macrospora***	**100**	**EU700368**
OH HTP1	**Necrotic Needle**	**Leotiomycete**	**99**	**JQ761460**
MD1	**Dead HWA**	***Epicoccum nigrum***	**99**	**KM519661**
OH HTP2	**Lepidopteran pupa**	***Lecanicillium attenuatum***	**99**	**JQ901939.1**
OH HTP3	**Chlorotic Needle**	***Cordyceps confragosa***	**99**	**AB111495.1**
WV 2	**Soil Needles**	***Cordyceps confragosa***	**99**	**KM678344.1**
OH HTP2	**Soil Dilution 10^7**	***Cordyceps confragosa***	**99**	**KM678344.1**
OH HTP2	**Soil Dilution 10^6**	***Cordyceps confragosa***	**99**	**KM678344.1**
OH HTP2	**Soil Needles**	***Lecanicillium fungicola***	**99**	**FJ810136.1**

Table 2-5: Leotiomycetes fungal inhibition on different species and strains of *Lecanicillium*. Distance of inhibition measured in centimeters. Isolates are from the Kasson Lab collection (KLW 80 & KLW 84) and the ARSEF collection at Cornell.

Leotiomycete isolates	***Lecanicillium* isolates**								
	KLW 84	KLW 80	5789	5165	5126	6046	5771	5772	Mycotol®
Leotio 1	0	0.1	0	0.2	0	0	0	0	0
Leotio 3	0	0	0	0	0	0	0	0	0
Leotio 4	0	0.5	0.9	0	0	0.2	0.2	0	0
Leotio 10	0	0.3	1.3	0.3	0	0	0	0	0
Leotio 17	0	1	1.4	0	0	0	0.3	0	0
Leotio 21	0	0.8	0.8	0.3	0	0	0	0	0
Leotio 31	0.3	0.2	0.4	0.2	0.3	0	0	0	0
Leotio R4N	0	0.4	0.8	0	0	0.4	0	0	0
Leotio TN2	0	0.5	1.5	0	0.3	0	0.3	0	0
Leotio TN3	0	0.9	0.4	0	0	0	0	0	0

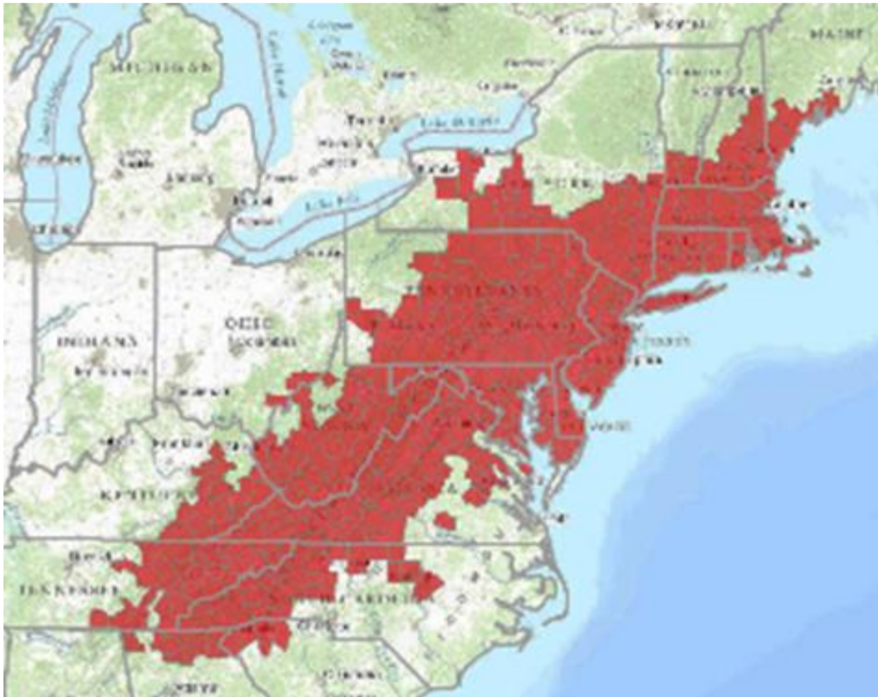

Figure 2-1: Current geographic range of the exotic HWA in the eastern U.S. Map provided by USDA Forest Service Northern Research Station Alien Forest Pest Explorer

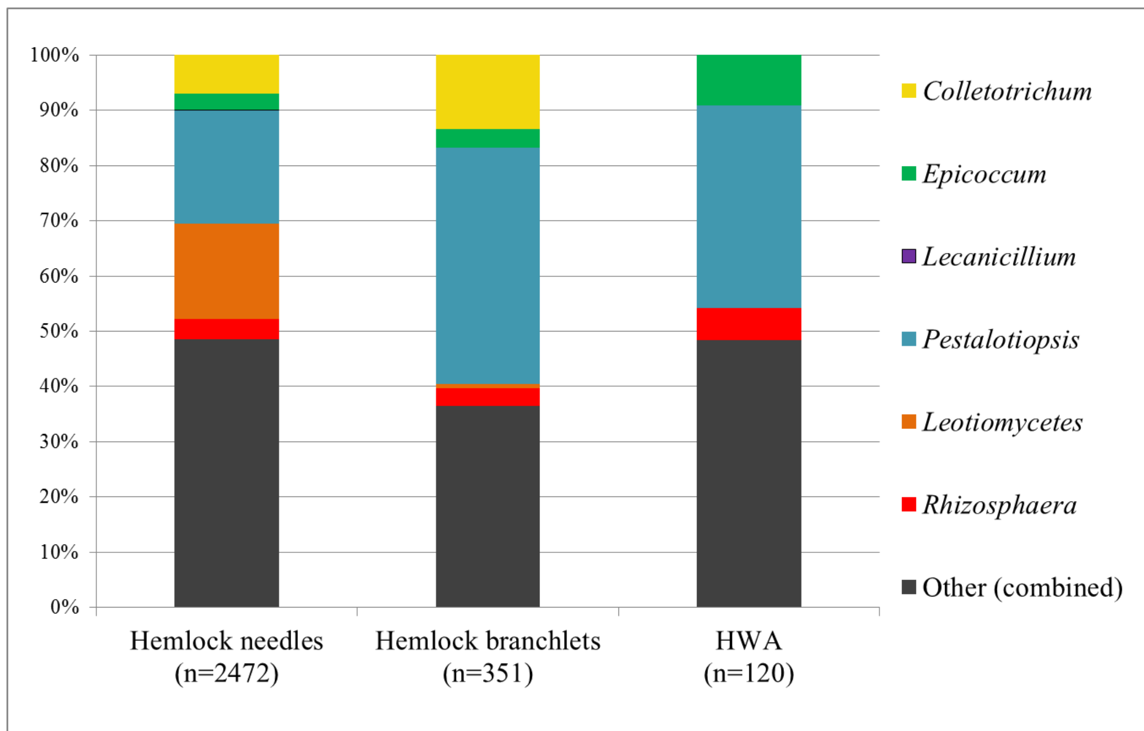

Figure 2-2: Fungal diversity from hemlock tissues and HWA across all 5 sites. Simpson's Diversity Index: 0.308104149

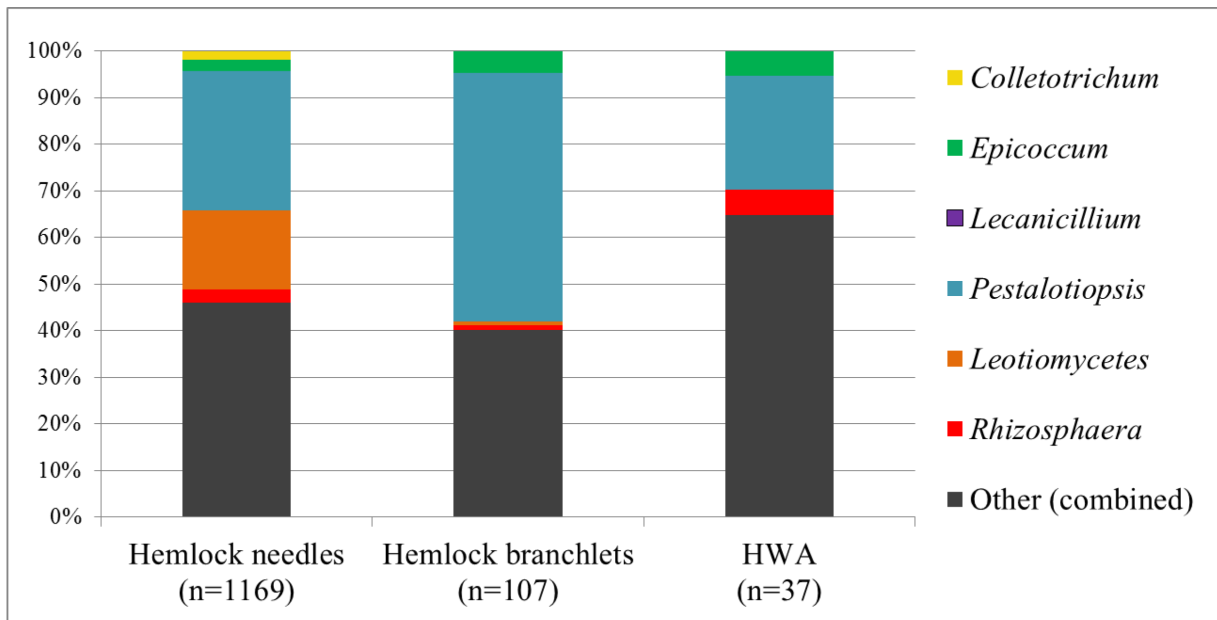

Figure 2-3: Fungal diversity from hemlock tissues and HWA, Royal Blue WMA, TN Simpson's Diversity Index: 0.335824448

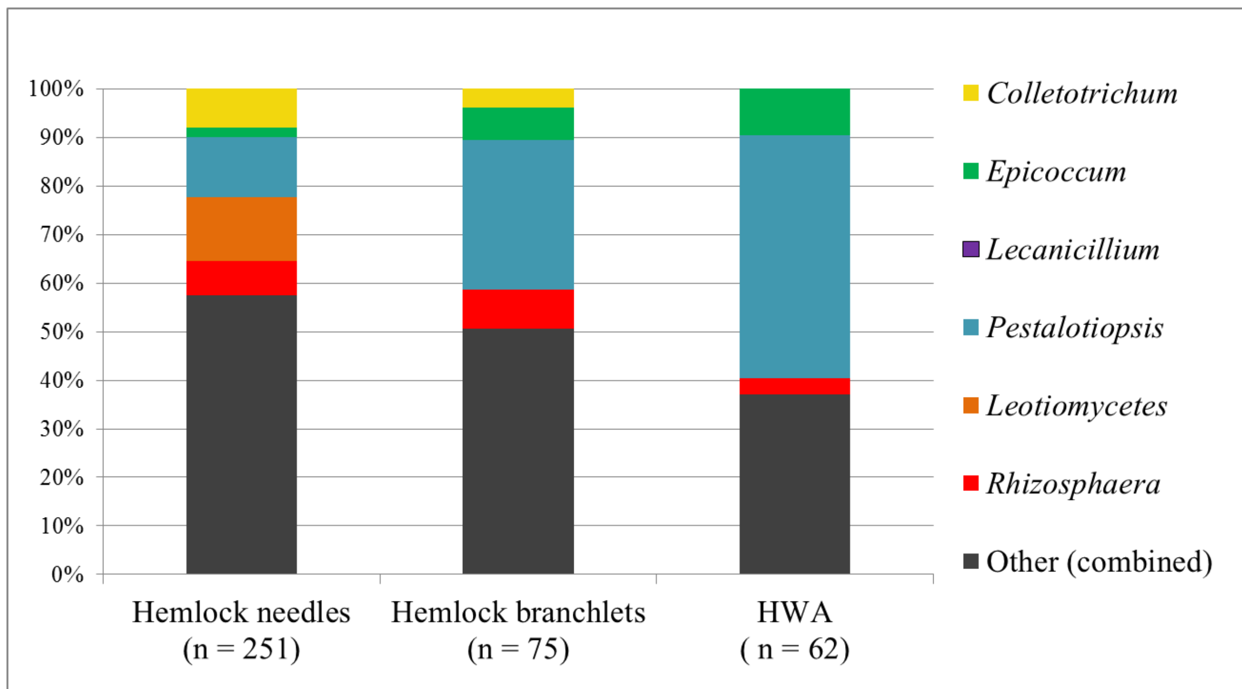

Figure 2-4: Fungal diversity from hemlock tissues and HWA, Savage River State Forest, MD Simpson's Diversity Index: 0.34239058

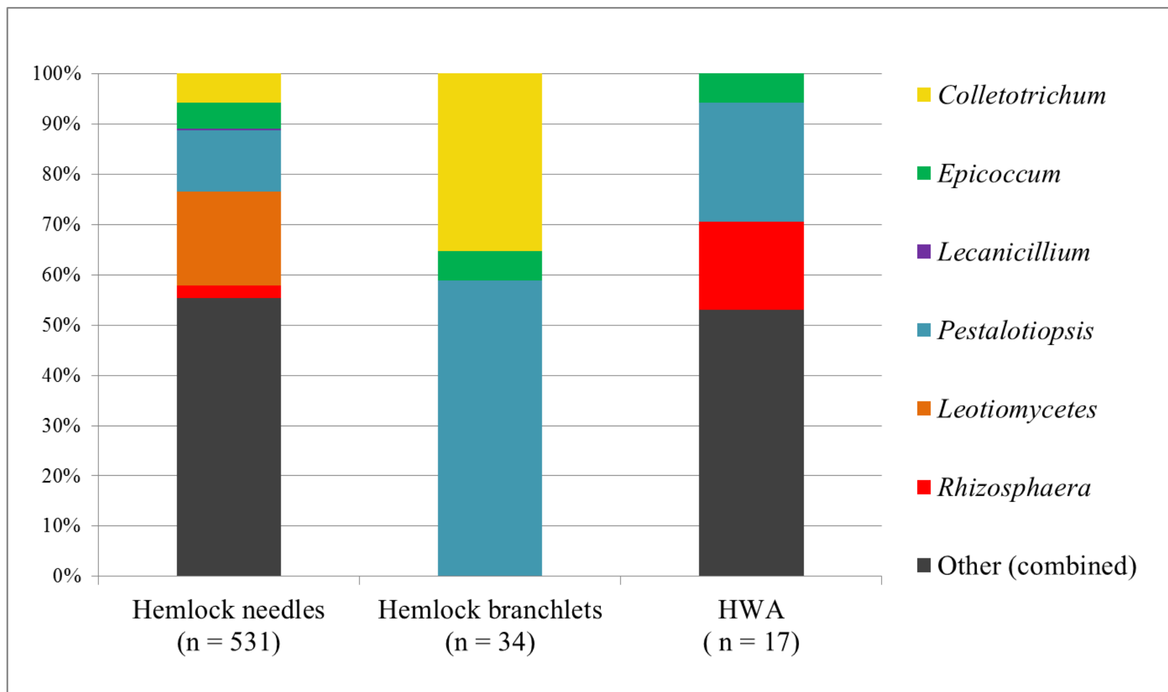

Figure 2-5: Fungal diversity from hemlock tissues and HWA, Shade River State Forest, OH
Simpson's Diversity Index: 0.331097586

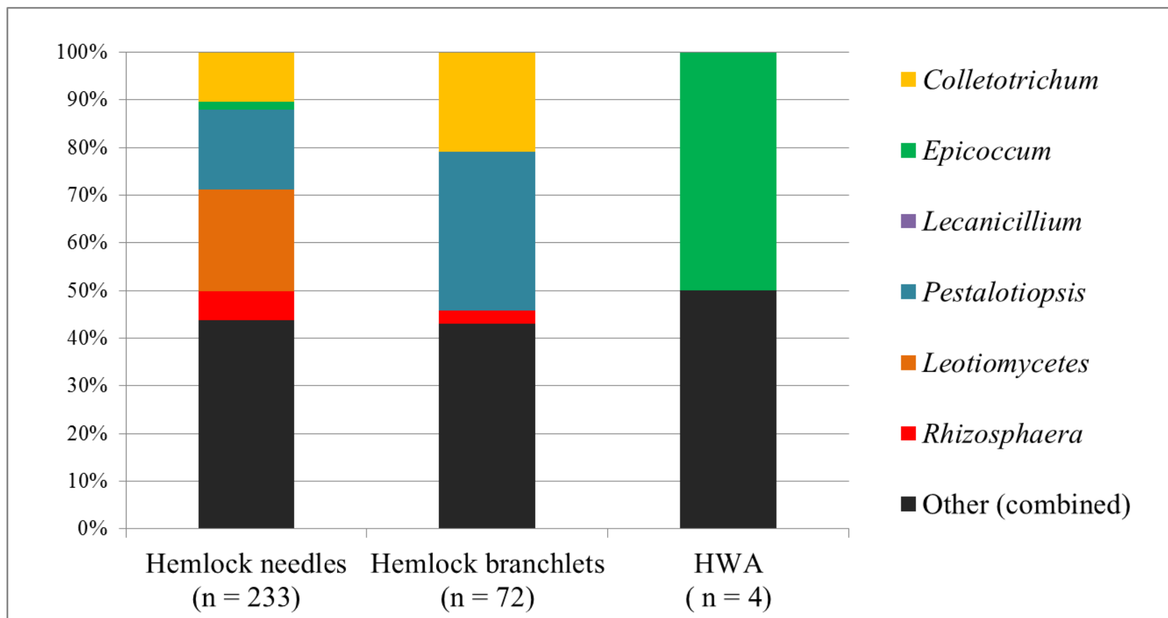

Figure 2-6: Fungal diversity from hemlock tissues and HWA, Ohiopyle State Park, PA.
Simpson's Diversity Index: 0.275270037

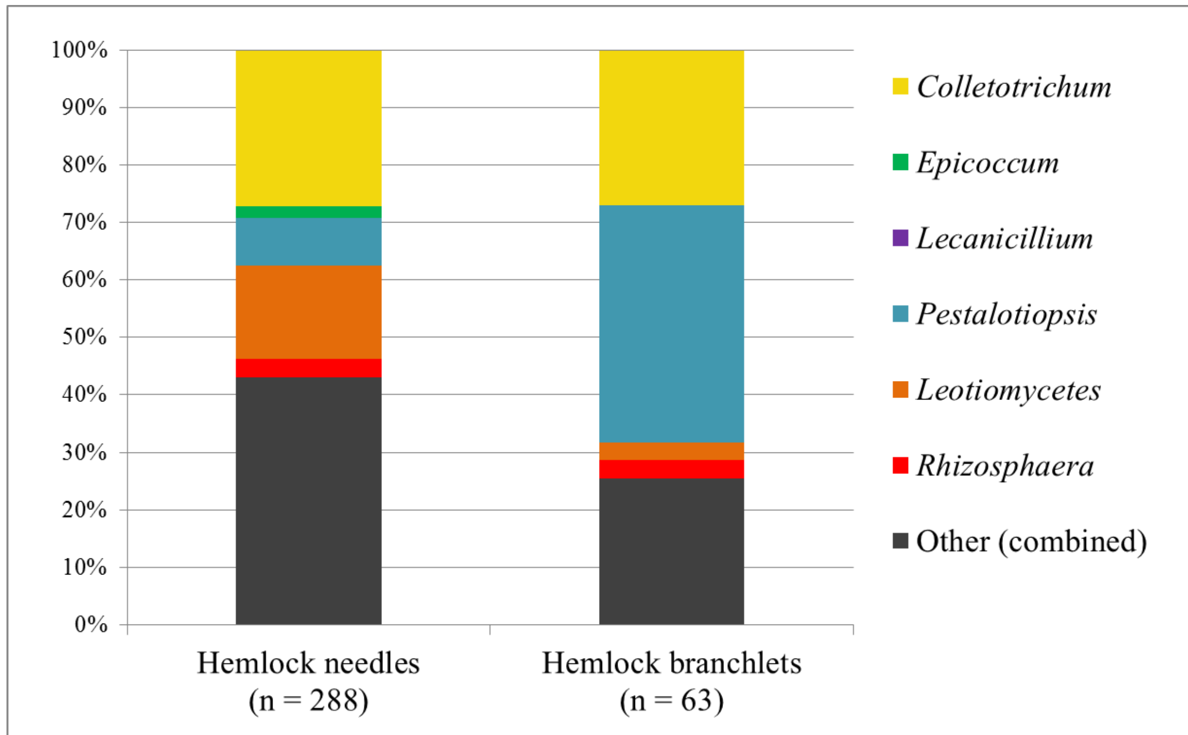

Figure 2-7: Fungal diversity from hemlock tissues, WVU University Forest, WV. Simpson's Diversity Index: 0.271322751

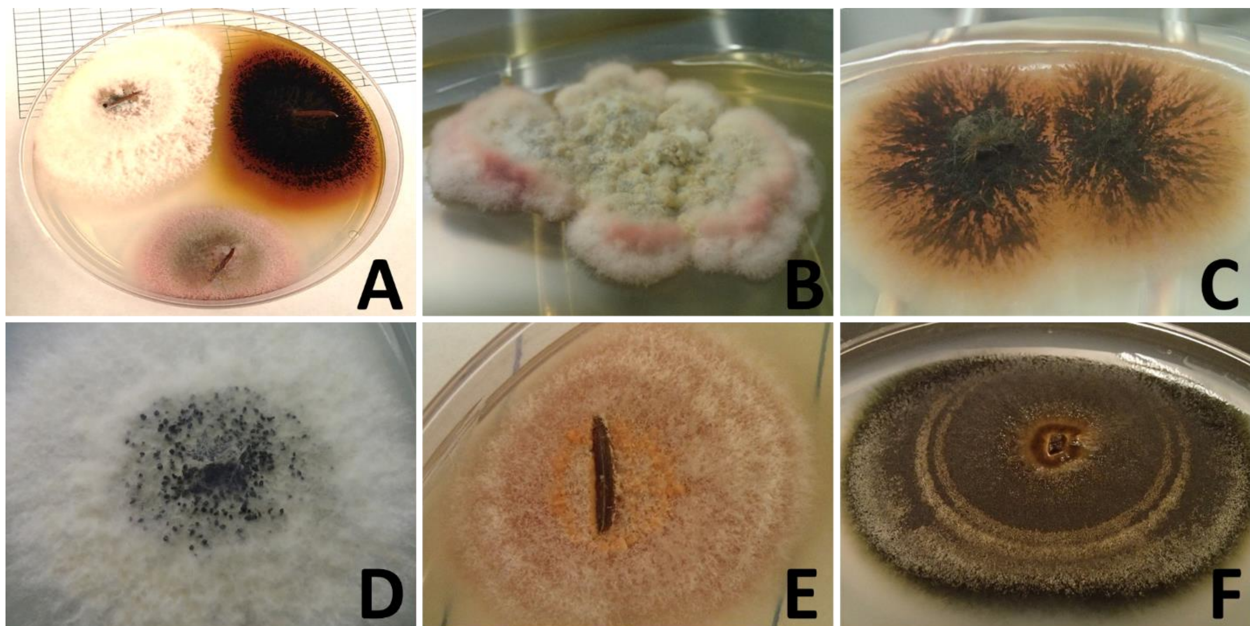

Figure 2-8: (A) Isolation of fungi from hemlock needles on Difco Potato Dextrose Agar and common fungi recovered from the substrates (B) Leotiomycete (C) *Epicoccum nigrum* (D) *Pestalotiopsis sp.* (E) *Colletotrichum fiorniae* (F) *Rhizosphaera macrospora*

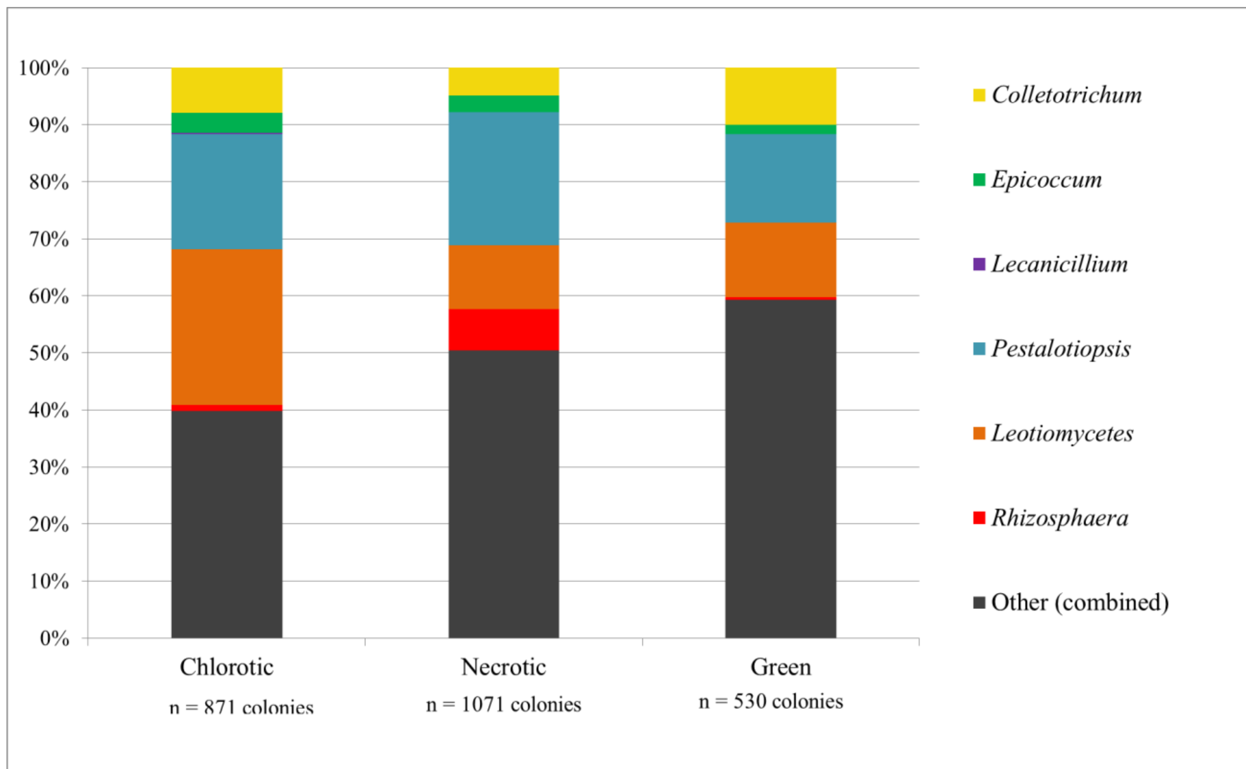

Figure 2-9: Fungal diversity from hemlock needles across all five study sites

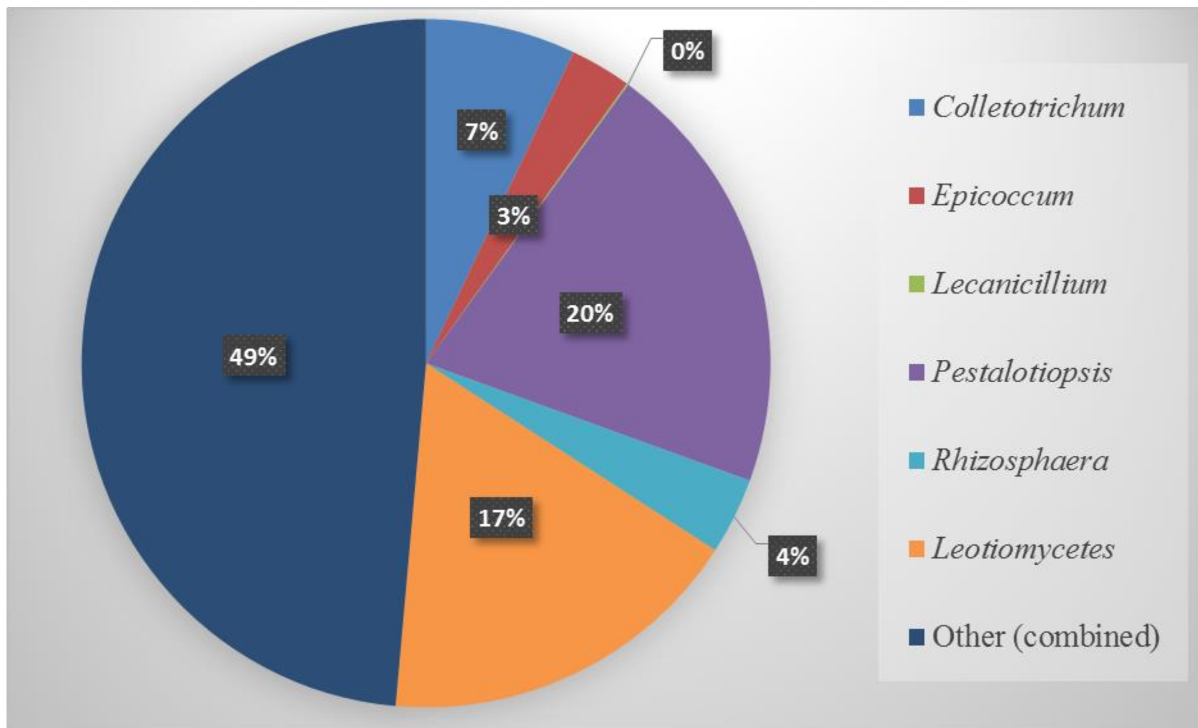

Figure 2-10: Fungal diversity across all sites isolated from hemlock needles (n=2472)

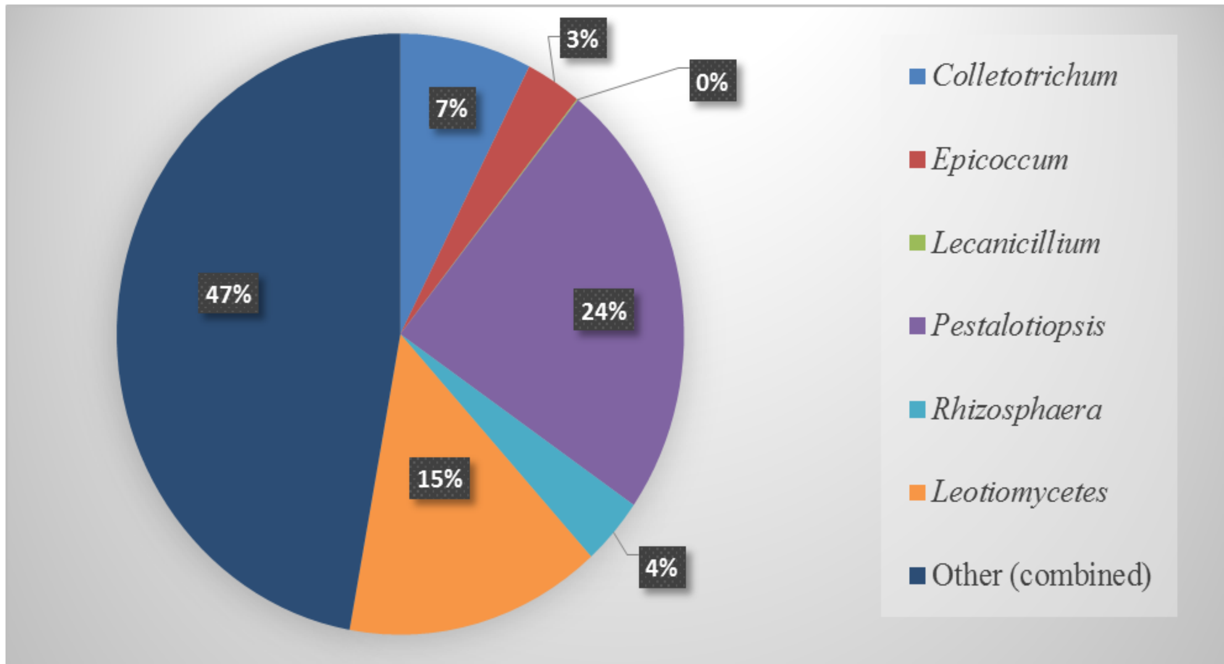

Figure 2-11: Fungal diversity recovered from HWA and hemlock needles, and branchlets

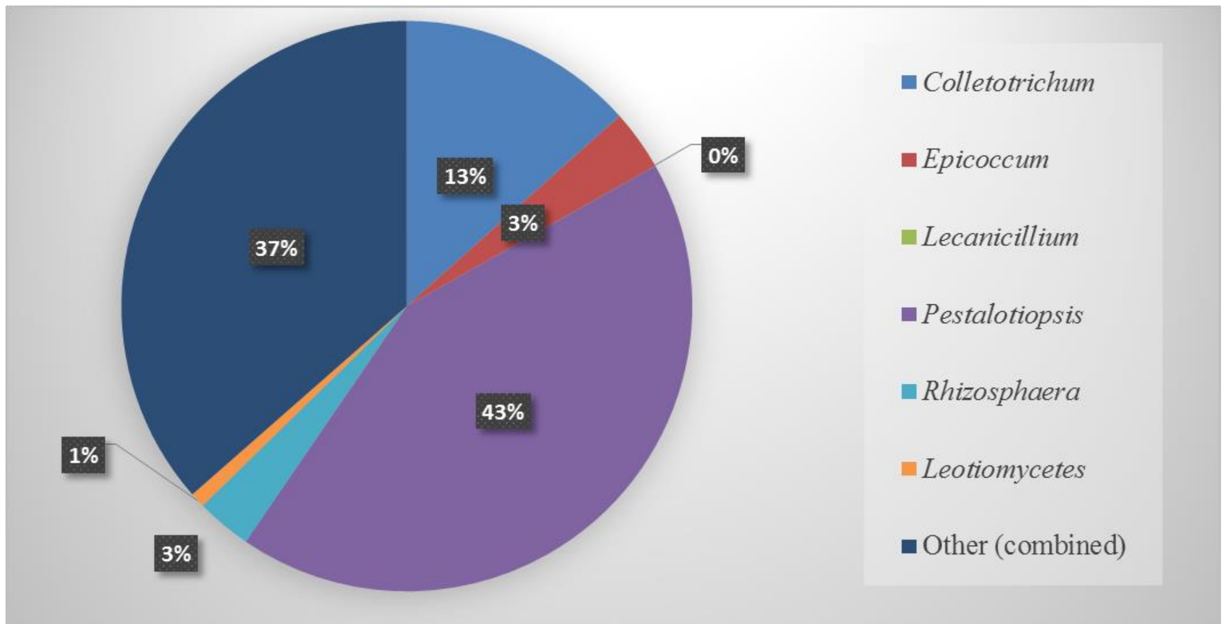

Figure 2-12: Fungal diversity across all sites isolated from hemlock branchlets (n=351)

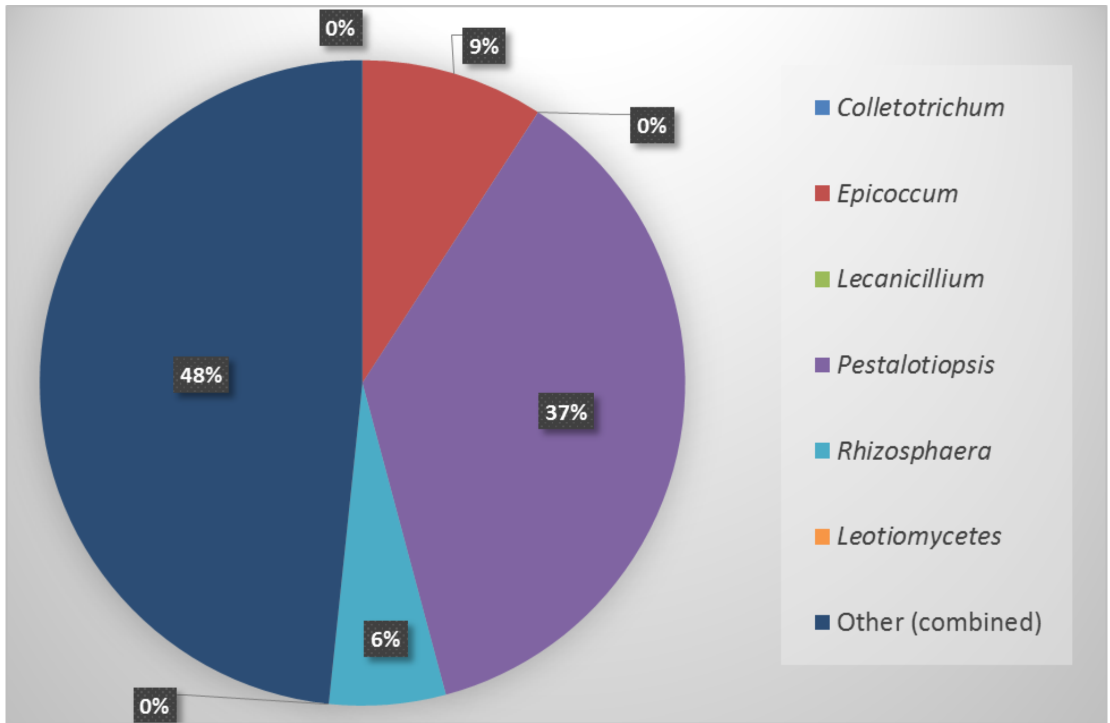

Figure 2-13: Fungal diversity across all sites isolated from HWA (n=120)

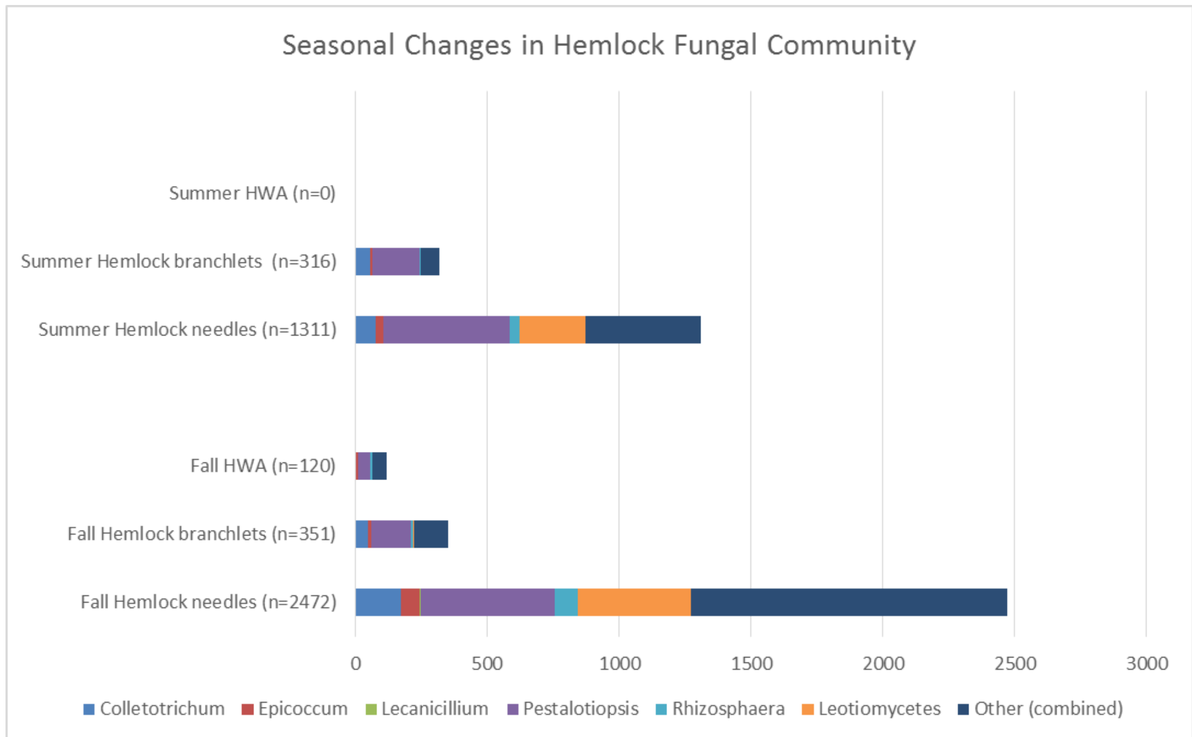

Figure 2-14: A second round of summer sampling occurred to evaluate the possibility of seasonal changes of fungi recovered from tissues in hemlock stands. There was an increase in saprophytic fungi recovered in the summer sampling, which is to be expected, due to naturally occurring conifer needle senescence.

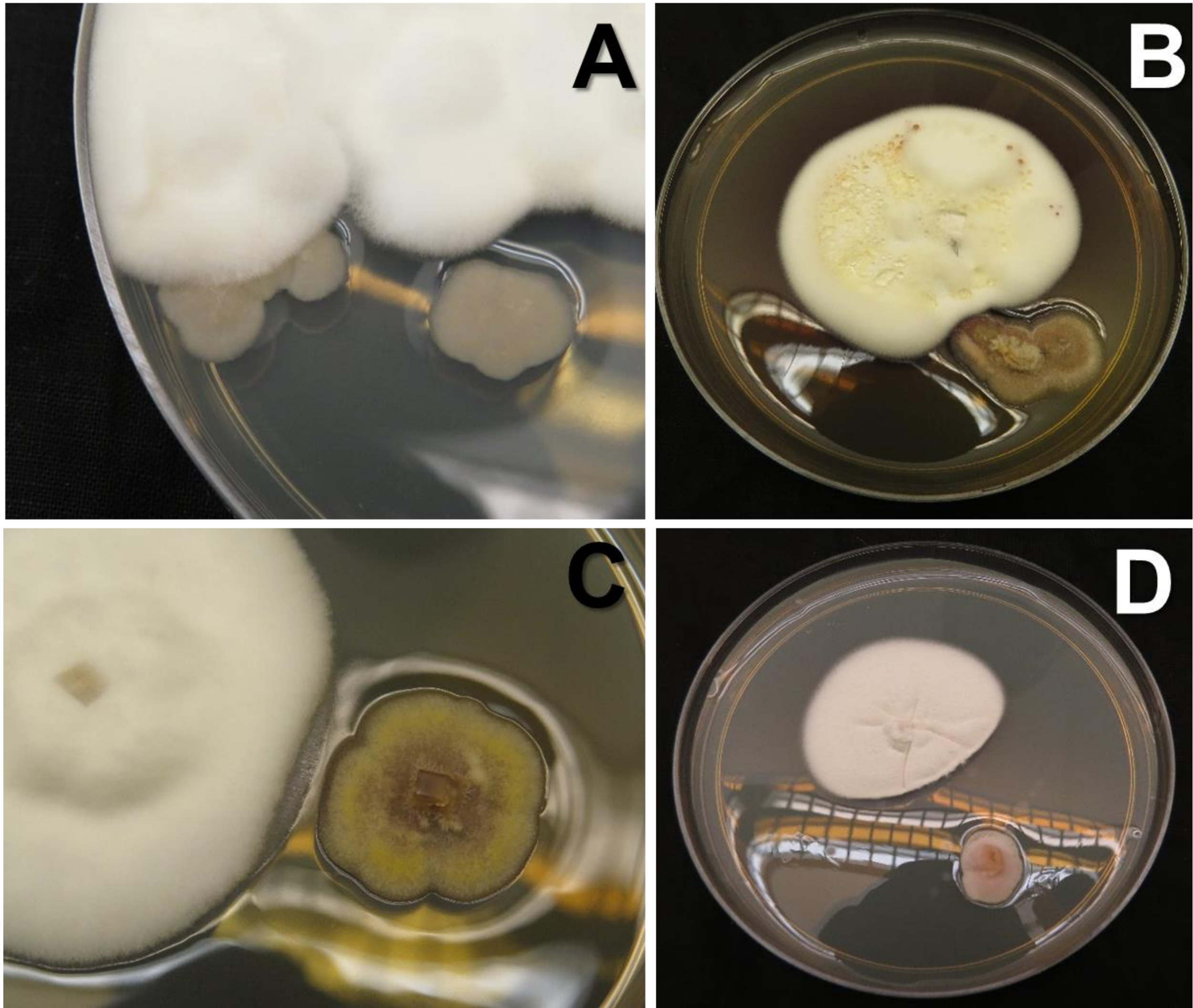

Figure 2-15: Inhibition by the common Leotiomycete fungus on different species of *Lecanicillium* plated on Difco Potato Dextrose Agar ranging from: (A) no inhibition, (B) moderate inhibition, (C) distinct lack of aerial mycelium on the *Lecanicillium* species on the left from the Leotiomycete specie on the right, (D) high inhibiton on the growth of the *Lecanicillium* species.

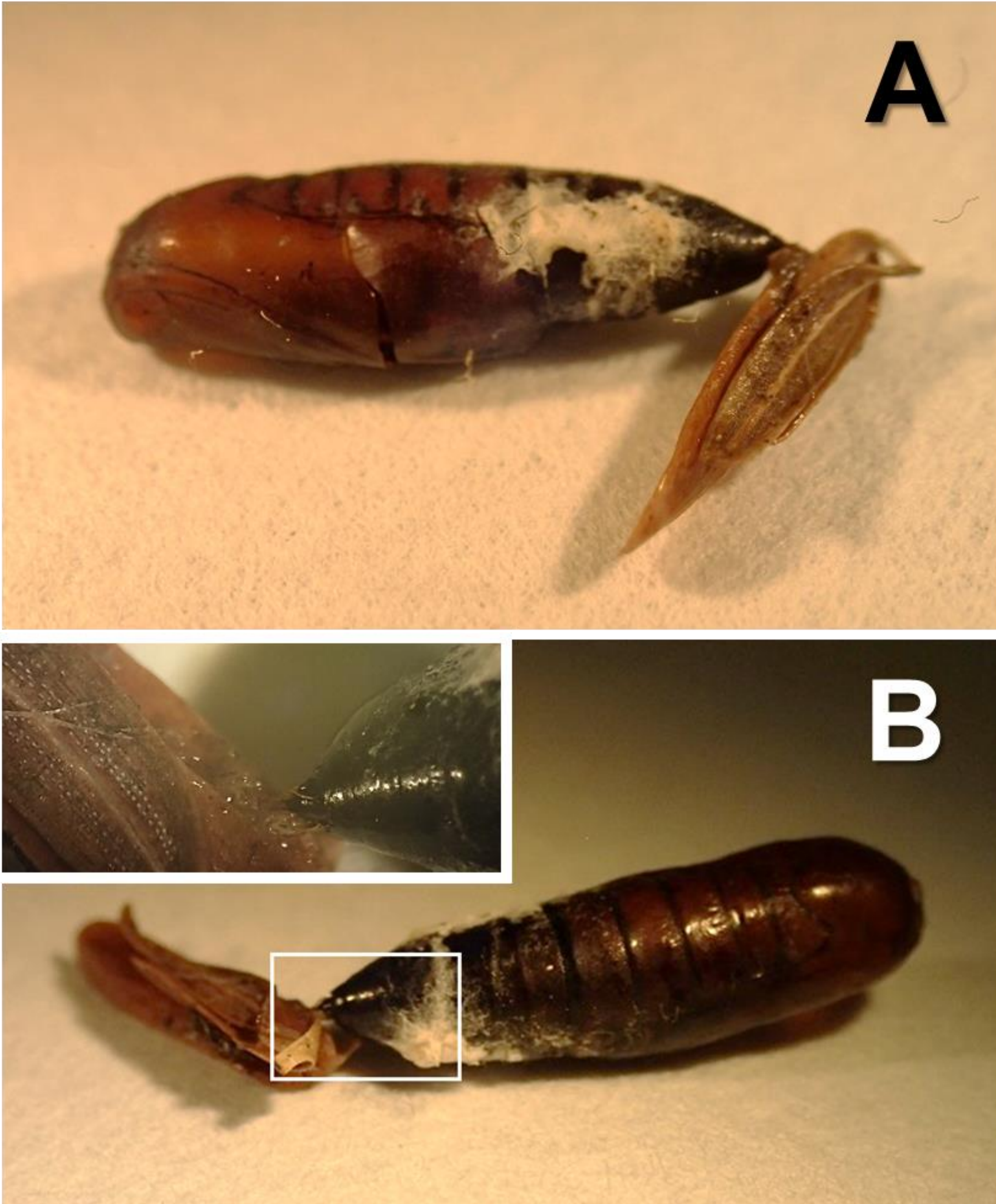

Figure 2-16: (A) Infected Lepidopteran pupa (B) showing hyphae of *Lecanicillium attenuatum.*

Acknowledgments

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Chapter 3

Lecanicillium phylogeny and comparative entomopathogenicity of *Lecanicillium* spp. and other fungi isolated from eastern hemlock stands Kristen L. Wickert

Abstract

Hemlock woolly adelgid is an invasive insect that is decimating eastern hemlock throughout most of hemlock's native range. Classical control methods of chemical insecticides and insect biocontrols are not enough to eradicate HWA. Methods of a fungal biocontrol are being investigated with a candidate being the entomopathogen *Lecanicillium muscarium*. There are many factors to consider when using a fungal biocontrol such as ecology, genetic distinction and mode of insect/pathogen contact. During this study multi-gene phylogenetic analyses were conducted to address the evolution of *Lecanicillium* (Clavicipitacaea, Ascomycota). Data presented in this study are for approximately 4,500 base pairs from portions of four genes and one mitochondrial gene*: β-tubulin*, elongation factor 1α (*EF-1α*), the largest and second largest subunits of RNA polymerase II (*RPB1* and *RPB2*), and NADH dehydrogenase subunit (*nad1*). Separate phylogenetic analyses, with data partitioned according to genes produced some complementary results and supported the monophyly of many *Lecanicillium* species. The phylogenetic trees informed selection of isolates to use in entomopathogenicity testing. The pathogenicity of selected isolates were tested on hemlock woolly adelgid adults and eggs separately. All isolates were found to be pathogenic to the insect but their virulence among species and isolates within species varied. The six isolates of *Lecanicillium* caused significantly higher mortality than the other fungal species. *Lecanicillium* isolates recovered from HWA were found to be more pathogenic than the Mycotal® isolate, which was isolated from white fly (*Trialeurodes vaporariorum)* (Hemiptera: Aleyrodidae). The most pathogenic isolates were 5165, 3531, 7375 and 5126 which all caused 100% mortality in adult trials. In egg mass trails these same isolates caused 73%, 64%, 94% and 87% mortality respectively. These results indicate that these isolates are promising candidates for the control of the HWA. *Lecanicillium* isolates 5795 and 7375 from the ARSEF collection caused significantly more mortality on HWA eggs than the other isolates. It is recommended that treatment of adelgid with a fungal biocontrol occur during May to select for progredien generation adults carrying sistens generation eggs. Due to higher mortality rates, the adults seem more vulnerable than the egg masses, therefore it is more efficient to utilize a different *Lecanicillium* strain than Mycotal® during this life stage.

Introduction

Hemlock woolly adelgid (HWA) has caused significant mortality of eastern hemlock throughout its introduced range in eastern North America. The past few decades have produced control methods that have mitigated the spread of HWA, but, ultimately, are unable to eradicate or this invasive insect. These methods are primarily traditional insect chemical controls or classical biocontrol releases of insect predators of HWA imported from outside the native range of eastern hemlock. Despite their effectiveness, insecticides are harmful to a diverse group of non-target insect species and can bioaccumulate in the environment (Cowles et al. 2006). The release of insect predators as biocontrols of HWA have proven useful in more ecologically sensitive areas where chemical controls are restricted yet these biocontrols have yielded mixed results (Onken and Reardon 2011). Nevertheless, when used together with chemical controls, these management methods are marginally successful at finer scale resolution but they are neither able to eradicate HWA from the larger landscape nor have they slowed hemlock mortality in heavily infested areas.

Fungal biocontrol might be an appropriate option to add to the ongoing integrated pest management against HWA but much remains unclear regarding efficacy and host specificity of candidate entomopathogens (Federici and Maddox 1996). This understanding is critical since many fungal biocontrols are native to the invaded ranges of the targeted insect pest and therefore have not co-evolved with these introduced pests (Kasson et al. 2014, 2015, Carrillo et al. 2014). Nevertheless, fungal biocontrols have proven to be a successful management method against other invasive species such as *Entomophaga maimaiga* against gypsy moth (*Lymantria dispar*) (Lepidoptera: Erebidae) and *Beauvaria bassiana* against aphids and other greenhouse pests (Hajek et al. 1996, Hong and Kim 2007).

Previous studies by Reid et al. 2010, showed that *Lecanicillium* was commonly associated with dead HWA throughout the eastern U.S. and might indicate its utility as an effective and naturally occurring biocontrol. The genus *Lecanicillium* is a generalist entomopathogenic fungus with a cosmopolitan distribution and is present across the landscape and reported from numerous hosts (Meyling and Eilenberg 2006, Sree and Joshi 2015, Sun et al. 2008). This entomopathogen also has a history as a control for greenhouse pests (Alavo 2015).

Lecanicillium is a member of the Clavicipitaceae within the Hypocreales. The genus was erected to accommodate entomogenous and fungicolous *Verticillium*-like anamorphs in the Claviciptaceae. Specifically, members of the Clavicipitaceae include pathogens of arthropods (e.g., *Cordyceps*, *Hypocrella*, and *Torrubiella*), parasites of truffles (e.g., *Elaphocordyceps*), and pathogens, endophytes and epiphytes of the Poaceae (e.g., *Claviceps*, *Balansia*, and *Epichloe*) (Sung et al. 2007). Recent multi-gene phylogenetic analyses of the of Clavicipitaceae revealed species of the genus *Cordyceps* (teliomorph of many entomophagous *Lecanicillium spp.*) are found in all three clavicipitaceous clades, highlighting the need for heightened resolution prior to utilizing these fungi as biocontrols (Sung et al. 2007). Although there is morphological overlap with some closely related Claviciptaceae, species within *Lecanicillium* generally form slender aculeate phialides, mostly with procumbent or postrate aerial hyhae, singly or in terminal and intercalary whorls. Conidia are generally elongate adhering in heads or fascicles at the tips of phialides, often at right angles to the phialide, a morphological feature exclusive to *Lecanicillium* (Zare and Gams 2001).

Recent multi-locus phylogenetic analysis conducted by Sung et al. (2007) and Koevelis et al. (2008) demonstrated several mitochondrial and nuclear genes including NADH dehydrogenase subunit 1 (*nad1*) gene (mitochondrial gene), DNA-dependent RNA polymerase II second largest subunit (*RPB2*) gene, translation elongation factor 1 alpha (*EF1-α*) gene, and DNA-dependent RNA polymerase II largest subunit (*RPB1*) gene had utility for resolving members of the Clavicipitaceae.

Koevelis et al. (2008) characterized sixty-five strains of *Lecanicillium* from different geographical regions and hosts. The combined use of mitochondrial gene sequences with ITS sequences, supported close relationships among *L. muscarium*, *L. psalliotae*, *L. lecanii, L. longisporum and L. nodulosum* as well as the the monophyly of the latter three species. In addition these studies helped place uncharacterized *Verticillium lecanii* and *Verticillium* sp. firmly into *Lecanicillium sensu stricto*. For example, the combined mt data resolved the uncertainty of Mycotal®, a commercially available formulation of *L. muscarium* (Koppert Biological Systems - The Netherlands), which had been previously identified among a group of isolates within a mixed *L. muscarium/L. longisporum* clade in the ITS dataset, by placing it clearly into *L. muscarium*. Results from these same studies failed to uncover any geographic

association of strains clustered in one species or another, but they clearly showed association with hosts in *L. lecanii* (scale insects) and *L. longisporum* (aphids).

In a pilot study, conducted in 2009/2010 in Tennessee, Mycotal® was aerially deployed to assess its efficacy against HWA. Although aerial application resulted in a decrease in growth of HWA populations, overall results were inconclusive (Costa 2010). Moreover, follow-up evaluations of these same hemlock trees revealed little if any sustained control and/or persistence of the fungus in the environment, emphasizing the need for reevaluation of previous methodologies as well as alternative strategies to permit meaningful observations along with successful outcomes in future attempt using fungal biocontrols (Costa 2010). Entomopathogenic infections likely require direct contact of conidia with specific life stages of HWA under specific environmental conditions, some of which may have been sub-optimal during aerial deployment.

The specific biology of HWA and the ecology of the environment will most likely also need to be considered in the implementation of a fungal biocontol for HWA. As an example, *Entomaphaga maimaiga* is only virulent on larval stages of gypsy moth (Andreadis and Weseloh 1990). Confirming whether such limitations exist on HWA is vital in understanding *L. muscarium*'s potential as a biocontrol. Furthermore, numerous studies indicate a lag-effect in populations' growth and subsequent efficacy (Tobin and Hajek 2012). Regardless, the short term results of this study provided a glimpse into the potential application of aerially applied fungal biocontrols against HWA.

Beginning in 2012, the previous field study using Mycotal® against HWA was re-evaluated and, as a result, modified to include phylogenetic resolution and comparative entomopathogenicity testing. Both of these additions to the study included native, naturally-occurring *Lecanicillium* sp. and closely related species, in addition to Mycotal®, with emphasis on potential HWA-adapted strains. This revision included strains recovered by the author during concurrent studies examining the potential environmental reservoirs for *Lecanicillium*. The purpose of the proposed project was two-fold. First, phylogenetic studies would resolve relationships among Mycotal®, closely related *Lecanicillium* species recovered from HWA, and numerous strains from other geographic locations and insect hosts. In doing so, phylogenetic diversity could be used in a targeted manner to aid in the selection of candidate isolates for efficacy studies representing the breadth of diversity within *Lecanicillium*. As an example, Green

Muscle® is a successfully commercialized fungal biocontol that utilizes an extremely virulent strain of *Metarhizium anisopliae* and has been used successfully against swarms of grasshoppers and locusts in Africa (Douthwaite 2001). The use of an aggressive strain supports the previous point that there are stark differences not only between different entomopathogenic species of fungi, but between strains of a single species (Douthwaite 2001). In this study, once selected, isolates were used experimentally against wild caught HWA to determine their efficacy against HWA and better understand the interaction between life stage of HWA and the fungal biocontol candidate.

Materials and methods

Fungal Isolates and Culture Maintenance

Fungal isolates were obtained from the USDA Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF) housed at Cornell University in Ithaca, NY and the Kasson Lab Culture Collection (KLCC) at West Virginia University in Morgantown, WV. Isolates acquired from the ARSEF included a diverse set of 58 *Lecanicillium muscarium* isolates and closely related species (*L. longisporum, L. psalliotae*, *L.* sp., and *Verticillium lecanii*) from two classes of arthropods, seven orders of insects, 12 insect families, and 17 species including 35 isolates from HWA (Table 3-1). Of the 46 included isolates originating from within the United States, 30 were from HWA and spanned 5 states. The additional 16 domestic isolates from other arthropods originated from seven states. Twelve non-domestic isolates were recovered from Canada, the Peoples Republic of China, Russia, and the United Kingdom including the reference isolate, Mycotal® ARSEF 5128, isolated from *Trialeurodes vaporariorum* (whitefly) (Hempitera: Aleyrodidae) in the U.K. (Figure 3-1) (Koppert 2015). All five Chinese isolates were recovered from HWA and represent the only *Lecanicillium* spp. from HWA not recovered from within the U.S. Although efforts were primarily focused on charactering and testing pathogenicity of HWA associated *Lecanicillium*, efforts were made to include isolates from other closely related Homopteran insects as well as geographically diverse isolates spanning six other insect orders. At least eleven of the isolates included in this study had been previously characterized with regard to phylogenetic placement (Koevelis et al. 2008), as well as a varying numbers of these same isolates used in entomopathogenicity testing against HWA (Reid et al. 2002), HWA predators used in classical biocontrol (Parker et al 2004), and other insects (Parker et al. 2003, Pas et al. 1996) (Table 3-1).

Kasson Lab Culture Collection isolates included six isolates of *Lecanicillium* spp. recovered from hemlock tissues and soil in hemlock stands as part of a parallel study aimed at identifying environmental reservoirs of *Lecanicillium* and other entomopathogens in hemlock stands with and without HWA (Wickert and Kasson, unpublished data). In addition, two *Lecanicillium* isolates recovered from rinses of *Laricobius nigrinus* predatory beetles in a recapture program of previously released HWA predators after one year in hemlock forests infested with hemlock woolly adelgid were also included. Four additional isolates associated with fungivorous millipedes were included in the study because they represented species not available or limited in availability through ARSEF (*L. psalliotae*, *L. saksenae*, *L. fungicola*) or whose identification could not be resolved with previous ITS rDNA sequencing (*Verticillium* sp.). Finally, five isolates served as the outgroup and included four isolates of *Ponchonia bulbillosa*/*Metacordycepts bulbillosa*, all previously recovered from hemlock needles in the soil described from the author's parallel study (Table 3-2).

For long-term storage, subcultures of all isolates were maintained on PDA slants and/or colonized Whatman GF/A 60-mm glass microfiber filter paper (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) and placed in individual coin envelopes. To revive cultures from long-term storage, colonized slant plugs or filter paper pieces were excised and placed onto Difco potato dextrose agar (PDA; BD and Co., Franklin Lakes, NJ, USA) plates amended with streptomycin sulfate and tetracycline.

DNA extraction, amplification and sequencing of rDNA

For DNA extraction, isolates were transferred to Difco potato dextrose broth (PDB; BD and Co., Franklin Lakes, NJ, USA) and incubated for seven days. Mycelia were harvested, dried between filter papers, and transferred to 1.5 mL Eppendorf tubes. Genomic DNA was extracted as described (Short et al. 2015). Following DNA extraction, a portion of the internal transcribed spacer (ITS) region was PCR amplified and sequenced to validate putative identifications based on morphology. GenBank BLASTn searches were used to confirm fungal identification following sequencing. Morphological features were subsequently confirmed to acknowledge congruence between morphological features and molecular identification. This step is critical in that long-term storage can result in both contamination and/or phenotypic changes (e.g. reduction/loss of sporulation) that render subcultures unrecognizable from parent strains (Marx and Daniel 1976).

Portions of the following five genes were PCR amplified and sequenced based on their proven utility for resolving phylogenetically distinct members of the Claviciptaceae and *Lecanicillium sensu strictu* (Koevelis et al. 2008, Sung et al. 2007): NADH dehydrogenase subunit 1 (*nad1*, 569bp alignment) gene (mitochondrial gene), DNA-dependent RNA polymerase second largest subunit (*RPB2*, 1154 bp alignment) gene, translation elongation factor 1 alpha (*EF1-α*, 951 bp alignment) gene, DNA-dependent RNA polymerase II largest subunit (*RPB1*, 786 bp alignment) gene, and *β-tubulin* gene (*BTUB*, 823 bp alignment).

All PCRs were performed using primers (Integrated DNA Technologies, Coralville, IA, USA) and BioLine PCR kits (Bioline USA Inc., Taunton, MA) in 25.5-µl reaction mixtures containing 1 µl genomic DNA, 10 µl nuclease-free water, and 12.5 BioLine PCR master mix. Each primer was used at 1 µl at 10 pmol. Thermal cycling profiles were based from previous phylogeny studies for each gene (Castlebury et al. 2004, Kouvelis et al. 2008, Sung et al. 2007).

Gel electrophoresis was performed for each reaction to confirm positive amplifications. Prior to electrophoresis 4 µl of SYBR gold (Invitrogen, Grand Island, NY, USA) and 4 µl of loading dye (5Prime, Gaithersburg, MD, USA) were added to PCR products. This mixture was then loaded onto a 1.5%, wt/vol, agarose gel (Amresco, Solon, OH, USA) made with 0.5% Tris-borate-EDTA buffer (Amresco, Solon, OH, USA). Electrophoresis was performed at 115 V for 45 minutes, and bands were visualized on a UV transilluminator (Syngene, Frederick, MD, USA). For size comparison, 100-bp and 1-kbp molecular ladders (Omega Bio-tek, Norcross, GA, USA) were included in gels. Representative PCR amplicons were Sanger sequenced with the same primers used for PCR (Eurofins, Huntsville, AL, USA). Sanger sequences were edited and consensus files created using Codon code aligner (CodonCode Corporation, Centerville, MA, USA).

Phylogenetic analyses

Multi-gene phylogenetic analyses were conducted to address the evolution of *Lecanicillium* (Clavicipitacaea, Ascomycota). A concatenated 64-taxon five-locus alignment was generated using CLUSTAL-W (http://www.genome.jp/tools/clustalw) followed by manual improvement. Separate partitions were created for each gene to permit analyses for both individual genes and the combined dataset. Mega 6.0's Modeltest was used to find the best parameters to permit phylogenetic analyses. Maximum likelihood (ML) analyses were conducted using MEGA 6.0

(Tamura et al. 2013). ML bootstrap analyses of the individual and combined dataset were generated for individual and combined phylogenies.

Fungal isolates used in entomopathogenicity testing

Isolates selected for the entomopathogenicity testing included *Lecanicillium* species from both the ARSEF and Kasson Lab Culture Collection. In addition to *Lecanicillium* isolates, several hemlock fungal community members previously recovered from hemlock tissues and HWA as part of a peripheral study were utilized for entomopathogenicity testing. These included *Colletotrichum fioriniae*, *Epicoccum nigrum*, *Pestalotiopsis microspora* and *Rhizosphaera macrospora,* and *Simplicillium lamellicola* based off literature that these genera have incidences of facultative entomopathogenic abilities (Marcelino et al. 2009). Ten *Lecanicillium* strains were included in the entomopathogenicity testing. Isolate selection was based primarily on phylogenetic diversity (i.e. genealogical exclusivity) followed by geographic origin and host. Isolates used in adult/crawler and egg stages varied. In both studies, Mycotal® served as a positive control in that it is a vetted entomopathogen of HWA (Table 3-3).

HWA field collections

Live HWA were collected from the field on two separate occasions to cover different periods in the adelgid lifecycle. In June of 2015 a collection occurred to select progredien generation adults carrying sisten generation eggs which hatched into crawlers. Progredien generation eggs masses were collected in late February, 2016. Treatments for adelgid are separated by adult/crawler and egg experiments.

HWA adult inoculations

HWA infested hemlock branches were collected from the field mid-June 2015 and transported to the lab. The source of infested branches was Ohiopyle State Park, Ohiopyle, PA, USA. In the lab, a total of 320 live adult adelgid were aseptically removed from their woolly masses and surface disinfected with 95% ethanol. Following disinfestation, four adult HWA were then plated onto 10 cm diameter petri dishes lined with sterile filter paper (VWR, Radnor, PA, USA) at five replicates per treatment. Inoculum consisted of conidial suspensions of previously mentioned ARSEF and Kasson Lab cultures (Table 3-3). Inoculum was prepared by adding 5 to 10 ml of sterile distilled water to 2-week-old cultures for each of the fifteen isolates tested on PDA and

scraping the surface with a sterile cell spreader. The resulting spore suspensions were collected, vortexed, and passed through sterile milk filters (KenAg, Ashland, OH, USA) to separate mycelial fragments out of the conidial suspension. Conidial concentrations were determined using a hemocytometer and adjusted to 8.5×10^5 conidia ml^{-1}. Viability of conidia was evaluated by examining growth after four days of plating suspensions on each of the fungal treatments onto PDA plates. Each of the 15 treatments received 1 ml of conidial suspension divided among the four replicates. For fungal treatments, filter paper helped ensure inoculum remained in close contact with HWA to permit infection whereas in controls it served to ensure HWA did not succumb on account of desiccation. Plates were parafilmed to maintain moisture and sterility and kept at room temperature. Adult adelgid plates were monitored and allowed to incubate for seven days or until signs of infection occurred. To assess entomopathogenicity, fungal growth from treated HWA adults was quantified, cultured from symptomatic HWA cadavers, and subjected to morphological and molecular protocols as previously described to confirm identity.

HWA egg mass inoculations

The source of the egg masses was on West Virginia University's Evansdale Campus, Morgantown, WV, USA. Egg masses were collected and plated near the end of February 2016 due to the specific life stage of progredien eggs being present. The adult sisten generation were dead after laying eggs and therefore could not be tested concurrently with progredien eggs. Adelgid egg masses were removed from their waxy coverings and hemlock branchlets using a sterilized dissecting needle. HWA were surface disinfested in 95% ethanol and plated onto 10-cm diameter petri plates with sterile filter paper. Three egg masses were plated onto single 10-cm diameter petri plates and replicated three times per treatment for a total of nine egg masses per treatment. Conidial suspensions were created as previously described and inoculum concentration ranged from 5.6×10^5 and 8.2×10^5. An average goal of 6.5×10^5 conidial suspension was created for 17 treatments (Table 3-3). Filter paper was provided to maintain moisture levels while creating a surface on which the eggs would not be submerged. Each of the treatments including the H_2O (negative) control received 1 ml of conidial suspension divided among the three adelgid egg masses per replicate plate. Two of the controls received no moisture as they were to remain untreated. Conidial suspension was aliquoted equally to the petri dish over adelgid egg masses.

Additionally, to assess whether eggs might desiccate following removal of their woolly coverings, entire 3-4 inch long branchlet sections with an average of 2-5 egg masses were placed on filter paper inside sterile petri dishes. Entire branchlets with attached adelgid egg masses were dipped in 95% ethanol as a surface disinfectant. These branchlets were placed on filter paper and the same conidial solution used on the single egg mass experiment was applied in a dip inoculation method. Entire branchlets were submerged in the conidial suspension and placed on the filter paper. One milliliter of conidial suspension was added to the filter paper to provide moisture during the one week incubation period. Three branchlets were dipped and plated for each of the 17 treatments.

Plates were parafilmed to maintain moisture and kept at room temperature. Egg mass plates were monitored and allowed to incubate for seven days or until signs of infection occurred. Eggs were counted on inoculated plates after the allotted time period passed and images were taken. Symptoms of infection, such as desiccation, presence of aerial hyphae, and lack of hymolymph, of the eggs was checked by observing the presence of red hemolymph during recording of infection.

Microtome cross sectioning of HWA eggs

Fungus-treated and negative control HWA egg masses were assessed for the presence of fungal colonization using a microtome. Eggs were prepared as previously described by Li et al. (2015) and Kasson et al. (in review). Eggs were not orientated in any certain direction, rather masses were embedded together to permit simultaneous longitudinal and transverse visualization of HWA eggs. Five-µm transverse sections were cut with a Microm HM 325 rotary microtome (Walldorf, Germany) at the West Virginia University School of Medicine. Selected slides confirmed by immediate viewing were dried at 60°C for 24 h, double-stained with Harris-hematoxylin and eosin-phloxine by hand, and examined and photographed using a Nikon Eclipse E600 compound microscope (Nikon Instruments, Melville, NY, USA) equipped with a Nikon Digital Sight DS-Ri1 high-resolution microscope camera and Nikon NIS-Elements BR 3.2 imaging software.

Anthroquinone assessments

Previous work by Jones (2012, 2014) indicated that anthroquinones present in HWA eggs might have inhibitory effects on fungal infection and colonization. In order to test anthroquinone inhibitory fungal capabilities during entomopathogenicity testing, eggs were plated on GYE + A plates that were inoculated with conidial suspensions from the egg mass entomopathogenicity treatments. Two egg masses were 100% ethanol surface disinfested and placed on the inoculated GYE + A plate per each treatment. Observations of halos around egg masses were recorded one to two days after inoculation.

Results

DNA sequencing results

All amplicons were sequenced and identified by BLASTn searches of the NCBI GenBank database and found to have 99 to 100% maximum identity matches with the fungal sequences deposited (Table 3-2).

Phylogenetic analyses

Sequence data for 64 taxa were obtained from portions of four nuclear protein-coding genes (*RPB2*, *RPB1*, *EF-1α*, *BTUB*) as well as a portion of the mitochondrial gene *nad1*. Phylogenetic inference using maximum likelihood on both individually partitioned genes as well as concatenated gene alignment resolved most *Lecanicillium* spp. in a strongly supported monophyletic group, within which two of five conserved lineages contained isolates or were isolated exclusively from HWA. In the absence of formal names, a numeric system was applied to each of the novel multilocus sequence types (MLSTs). Conserved linages include: MLST #1 which contained a majority of North American *Lecanicillium* isolates included in the study; MLST #2, which contained two isolates, one from Aphididae and a second from Coleoptera; MLST #3, a novel lineage (5-6 isolates) of *Lecanicillium* sp. exclusive to HWA from China; MLST #4 (*L. longisporum* clade, 2-3 isolates); and, MLST #5, which contains two isolates, one from Coccidae and one from the fungivourous millipede, *Brachycybe lecontii*. Individual genes supported additional genealogically exclusive lineages including two for *nad1*, MLST #6 and MLST #7, which contained a lineage recovered exclusively from HWA and a second lineage which contained two isolates, one from hemlock tissues and a second from Coleoptera. Additional genealogically exclusive lineages were identified from the *RPB1* (Figure 3-2) and

BTUB (Figure 3-3) phylogenies, MLST #8 and MLST #9, respectively. MLST #8 contained a lineage recovered exclusively from HWA whereas MLST #9 contained isolates from both HWA and from Geometridae (Lepidoptera).

ML bootstrap analyses of the individual partitions as well as the concatenated alignment indicated very strong support (>90% ML BS) for each of the five clades with few exceptions. Additionally, both combined and individual partitions showed strong support >75% ML BS for the monophyly of *Lecanicillium* with the exception of *EF1-α* (Figure 3-4), which showed weaker support (ML BS = 50%). Combined and *EF1-α* ML BS support for MLST #4 was 61% and 62%, respectively. *EF1-α*, and *RPB2* (Figure 3-5) ML BS support for MLST #5 was <50% and 84%, respectively. ML bootstrap analyses of the individual partitions for MLST #6-#9 were 63%, 78%, 61%, and 62%, respectively.

Three other well supported lineages which comprised a second clade sister to and outside *Lecanicillium* included a lineage containing four isolates of *Ponchonia bulbillosa*/*Metacordycepts bulbillosa,* a lineage of *Lecanicillium psalliotae* containing isolates from both Aphididae and *Brachycybe lecontii,* and a lineage containing two isolates, one from Thripidae and a second identified as *V. insectorum* from *Brachycybe lecontii*. A fourth lineage revealed incongruence between individual gene genealogies and contained two isolates of *Lecanicillium fungicola*. Phylogenies based on *nad1* (Figure 3-6) and *BTUB* as well as the concatenated (Figure 3-7) (Figure 3-8) dataset support *L. fungicola* as a member of *Lecanicillium* whereas *EF1-α* and *RPB2* resolve its placement among the second clade sister to and outside *Lecanicillium*.

ML bootstrap analyses of the individual partitions as well as the concatenated alignment of a second clade sister to and outside *Lecanicillium* indicated some lineages had strong support (>90% ML BS) whereas others could not be resolved in this study. Fortunately, this applied almost exclusively to outgroup taxa but also included lineages with known members of *L. psalliotae* and *L. fungicola.* Combined and *EF1-α* ML BS support for *Ponchonia bulbillosa*/*Metacordycepts bulbillosa* was <50% and 88%, respectively, compared to 91-100% for the remaining four genes. *EF1-α* and *RPB2* ML BS support for *V. insectorum* lineage was 77% and 62%, respectively, while ML BS support for *nad1* and *RPB1* was 99%. The combined and *BTUB* datasets only included one isolate from the *V. insectorum* lineage therefore ML BS

values were not available. Despite very strong ML BS support (99% for all genes), the phylogenetic placement of *Lecanicillium fungicola* could not be fully resolved. For combined, *nad1*, and *BTUB*, *L. fungicola* resolved with other *Lecanicillium* forming a monophyletic group whereas *EF1-α* and *RPB2* data resolved its placement among outgroup taxa.

ML bootstrap analyses of the individual partitions revealed *nad1*and *RPB1* (Table 3-4) possessed the highest proportion of informative characters and supported the largest number of nodes (3 and 4 at P>65% ML BS, respectively). By contrast, the *EF1-α* partition was the least informative with all nodes receiving P<50% ML BS. *EF1-α* number of amplicons was also the smallest of all the gene regions contained a total of 255 bp compared to >500 bp for all other products. ML bootstrapping of the combined five-locus data set provided support for five nodes with P>50% ML-BS.

Entomopathogenicity testing

After constructing the phylogenetic trees and analyzing phylogenetic diversity and structure, isolates ARSEF 9925, 5165, 6035, 3531, 5126, 7375 were selected for entomopathogenicity experiments. Three isolates were selected primarily because they had been isolated from HWA, however they differed in geographic origin. Isolate 9925 is from New Hampshire, 5165 from Massachusetts and 6035 from The People's Republic of China. Three additional isolates from hosts other than adelgid were include in the pathogenicity testing. These isolates are 3531 (*L. muscarium*) from gypsy moth (*Lymantra dispar*) (Lepidoptera: Erebidae) from WV, 5126 (*L. longisporum)* from chrysanthemum aphid (*Macrosiphoniella sanborni*) (Hemiptera: Aphididae) from the United Kingdom and 7375 (*L. muscarium*) from a *Ceroplastes* scale species (Hemiptera: Coccidae) from Massachusetts.

Strains selected for entomopathogenicity testing spanned four phylogenetically confirmed MLSTs including MLST #1 (9925, 5165, and 3531), MLST #3 (6035), MLST #4 (5126), and MLST #5 (7375).

HWA adult and crawler entomopathogenicity

The results of this study confirmed pathogenicity of most fungal treatments including non-entomopathogenic fungi commonly recovered from hemlock plant tissues despite significant differences in infection rates among tested isolates. This significance was proven with a Tukey's

pairwise comparison. Inoculations using *Lecanicillium* isolates recovered from HWA (5165), gypsy moth (3531), scale (7375), and hemlock needles (KLW 84) in North America and aphid (5126) in the UK all resulted in 100% mortality on HWA adults. Inoculations with MLST #3 (6035) from HWA as well as two inoculations of Mycotal® (MLST #1) also resulted in high infection rates ranging from 90-95% of the adult adelgid. *Lecanicillium* isolate KLW 80 recovered by the author as part of a peripheral study of environmental reservoirs of *Lecanicillium* resulted in 80% infection rate.

Needle, branchlet, and HWA associated fungi without previously confirmed reports of entomopathogenicity appeared to infect some adult HWA. Percent infections ranged from 25% (K6 – *Rhizosphaera macrospora,* K31 – *Colletotrichum fioriniae*), 35% (K28 – *Pestalotiopsis sp.*), and 70% (K14 - *Epicoccum nigrum*). Percent infections for negative controls were 10% (Figure 3-9). All crawlers that emerged from woolly masses of plated HWA adults onto *Lecanicillium* treated plates were infected and succumbed. In comparison, no crawlers exposed to non-entomopathogenic fungi were infected despite apparent colonization of immobile adults on the same plates (Figure 3-10).

Egg mass entomopathogenicity

The results of the egg mass entomopathogenicity study not only confirmed pathogenicity of all *Lecanicillium* treatments used in this study but also further confirmed that entomopathogenic isolates spanned the phylogenetic diversity of *Lecanicillium*. Significant differences in infection rates were noted among tested *Lecanicillium* isolates. This significance was proven with a Tukey's pairwise comparison. Isolate 5795 (HWA) had the highest mortality (96%) followed by isolates 7375 (MLST #5), 94%, 5126 (MLST #4) 87%, 6035 (MLST #3) 85%, KLW 84 76%, KLW 80 74%, 5165 73%, 3531 64% and 9925 causing 41% mortality, respectively, and Mycotal® at 33% (Figure 3-11).

Needle, branchlet, and HWA associated fungi without previously confirmed reports of entomopathogenicity appeared to infect HWA eggs, albeit at low levels. *Epicoccum nigrum* (K14) had the highest mortality (18%) followed by *Pestalotiopsis* sp. (K28) with 9%, and *Rhizosphaera macrospora* (K6) and *Colletotrichum fioriniae* (K31), both with 0% (Figure 3-13, 3-14).

Percent infections for water inoculated (negative) controls were 8% whereas egg masses left untreated as a second (negative) control showed no signs of infection. The common natural environmental contaminants for this study were *Alternaria* sp. and *Aspergillus* spp. Branchlets showed similar rates of infection compared to the egg mass plating's at one week post inoculation (Figure 3-14).

Anthroquinone fungal inhibition assessments

Antroquinones already present in the eggs did not produce any inhibitory effect around the egg masses, indicating that they do not have an inhibitory effect on fungal growth and infection. However this could be due to the large inoculum source of the 10^6 conidial suspension, which would be what representative of the inoculation load used in field applications.

Microtome cross sectioning of HWA eggs

Microtome visualization of hyphae invasion of HWA tissues was present in unstained and stained images versus the control egg masses (Figure 3-15, 3-16).

Discussion

The *Lecanicillium* concatenated tree shows that the genus *Lecanicillium* appears to be monophyletic with strongly supported bootstrap values over 50. This monopyly is indicative of a clonally reproductive organism. The entomopathogenicity testing of isolates from the phylogenetic tree answer a few questions about the efficacy of a fungal biocontrol. Mycotal® utilizes a virulent strain for an inundative augmentative approach to bolster naturally low population of *Lecanicillium* present in hemlock stands. However, its low infection rate on egg masses (33%) could indicate that other *Lecanicillium* isolates used in this study, especially North American strains, might be a better candidate for widespread application against HWA in the eastern United States. It is hypothesized that Mycotal® is not able to compete with other fungi as well as other stronger isolates, as was seen in the entomopathogenic testing. The Mycotal® repetitions had *Alternaria* contamination arising from fungal propagules found in the egg masses from the natural environement and Mycotal® fungal hyphae/conidiophores appeared in low incidence when *Alternaria* was present; however, other *Lecanicillium* isolates had *Alternaria* contamination but were still able to outcompete and infect the host at high percentages. There may be possible host adaptation supported by the virulence of the strains of *Lecanicillium*

recovered from HWA. All adelgid recovered isolates caused 90 to 100% infection in adelgid adults. Location of the *Lecanicillium* isolates did not seem to show a difference in virulence. Comparing the two life stages infected in this study, it is recommended that treatment of adelgid with a fungal biocontrol occur during the month of May to select for progredien generation adults carrying sisten generation eggs. Due to higher mortality rates, the adults seem more vulnerable than the egg masses. The month of May also has more appropriate weather and humidity conditions for a fungal biocontrol than winter months. Adults experienced higher mortality than eggs masses did, and by killing progredien adults, sisten eggs will be removed from the system.

Table 3-1: Fungal isolates from the ARSEF Collection and recovery hosts.

Order	Family	Species	Location	Fungal species	ARSEF No.	Internal ID	Concatenation
Homoptera	Aphididae	*Rhopalosiphum nymphaeae*	FL, USA	*L. longisporum*	321*	1	x
Homoptera	Adelgidae	*Adelges tsugae*	NH, USA	*L. sp.*	9924	2	x
Homoptera	Aphididae	*Macrosiphum euphorbiae*	ME, USA	*L. muscarium*	204*	4	x
Coleoptera	Buprestidae	*Agrilus planipennis*	MI, USA	*L. muscarium*	8163	5	
Diptera	Culicidae	*Ochlerotatus triseriatus*	KY, USA	*L. muscarium*	810*	7	x
Homoptera	Eriococcidae	*Cryptococcus fagisuga*	UK	*L. muscarium*	3740	8	x
Lepidoptera	Lymantriidae	*Lymantria dispar*	OR, USA	*L. muscarium*	2065*	10	x
Lepidoptera	Lymantriidae	*Lymantria dispar*	NY, USA	*L. muscarium*	3600*	12	x
Homoptera	Aphididae	*?*	VT, USA	*V. lecanii*	5166	13	x
Hemiptera	Scutelleridae	*Eurogaster sp.*	Russian Fed.	*V. lecanii*	6010	14	x
Homoptera	Adelgidae	*Adelges tsugae*	NJ, USA	*V. lecanii*	5795	15	x
Homoptera	Adelgidae	*Adelges tsugae*	CT, USA	*V. lecanii*	5759	16	x
Hymenoptera	Formicidae	*Formica sp.*	VT, USA	*V. lecanii*	*5168*	17	
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5165	18	x
Homoptera	Adelgidae	*Adelges tsugae*	Pr China	*V. lecanii*	6035	19	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5777	20	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5798*	21	x
Homoptera	Aphididae	*Myzus cerasi*	NY, USA	*L. muscarium*	8714	22	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*L. muscarium*	5828	23	x
Homoptera	Adelgidae	*Adelges tsugae*	NH, USA	*L. sp.*	9926	24	x
Lepidoptera	Lymantriidae	*Lymantria dispar*	WV, USA	*L. muscarium*	3531	25	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*L. sp.*	9176	26	x
Homoptera	Adelgidae	*Adelges tsugae*	NH, USA	*L. sp.*	9925	27	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*L. sp.*	9175	28	x
Homoptera	Adelgidae	*Adelges tsugae*	VA, USA	*V. lecanii*	5821	29	
Araneida	Araneae	*?*	VT, USA	*V. lecanii*	5167	30	x
Homoptera	Adelgidae	*Adelges tsugae*	Pr China	*V. lecanii*	6045	31	x
Homoptera	Adelgidae	*Adelges tsugae*	Pr China	*V. lecanii*	6047	32	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5783	33	x
Homoptera	Adelgidae	*Adelges tsugae*	Pr China	*V. lecanii*	6046	34	
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5793	35	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5789	36	x
Homoptera	Eriococcidae	*Cryptococcus fagisuga*	UK	*V. lecanii*	3741	37	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5781	39	x
Homoptera	Adelgidae	*Adelges tsugae*	VA, USA	*V. lecanii*	5824	40	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5820	41	x
Homoptera	Adelgidae	*Adelges tsugae*	VA, USA	*V. lecanii*	5771	42	x
Homoptera	Adelgidae	*Adelges tsugae*	Pr China	*V. lecanii*	6050	43	x
Homoptera	Coccidae	*Ceroplastes sp.*	MS, USA	*L. muscarium*	7375	44	x
Homoptera	Adelgidae	*Adelges tsugae*	VA, USA	*V. lecanii*	5772	45	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5778	46	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5779	47	
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5780	48	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5782	49	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5785	50	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5787	51	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5791	52	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5794	53	x
Homoptera	Adelgidae	*Adelges tsugae*	VA, USA	*V. lecanii*	5822	54	x
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5829	55	x
Hemiptera	Aphididae	*Diuraphis noxia*	ALB, CAN	*L. muscarium*	3000*	56	x
Thysanoptera	Thripidae	*Taeniothrips inconsequens*	PA, USA	*Lecanicillium sp.*	3255	57	
Homoptera	Adelgidae	*Adelges tsugae*	MA, USA	*V. lecanii*	5786	58	
Hemiptera	Aphididae	*Macrosiphoniella sanborni*	UK	*L. longisporum*	5126	59	x
Hemiptera	Aphididae	*Macrosiphoniella sanborni*	UK	*L. muscarium*	314*	60	
Hemiptera	Aphididae	*Sitobion avenae*	ID, USA	*L. psalliotae*	2332*	61	x
Hemiptera	Aphididae	*Macrosiphoniella sanborni*	UK	*L. longisporum*	5126*	62	
Hemiptera	Aleyrodidae	*Trialeurodes vaporariorum*	UK	Mycotal	none	63	
Hemiptera	Aleyrodidae	*Trialeurodes vaporariorum*	UK	Mycotal	none	64	
Hemiptera	Aleyrodidae	*Trialeurodes vaporariorum*	UK	*L. muscarium*	5128	11N	x
Homoptera	Aphididae	*?*	HI, USA	*L. muscarium*	7034	3,9	x
Thysanoptera	Thripidae	*Taeniothrips inconsequens*	NH, USA	*L. muscarium*	10178	6N	x

*- denotes previous use in phylogenetic or pathogenicity testing and therefore a priority isolate

Table 3-2: Kasson Culture Collection isolates incorporated in the phylogeny of *Lecanicillium* during this study.

ID	Blasted	State	Recovery Substrate	NCBI Number	*RPBI*	*RPB2*	*nad1*	*EF1-α*	*BTUB*	*Concatenation*
KLW T7	*Metaponchonia bulbillosa*	WV	Soil Needles	DQ132810	x	x	x	x	x	x
KLW T8	*Cordyceps confragosa*	WV	Soil Needles	KM678344	x	x	x	x		
KLW T9	*Ponchonia bulbillosa*	PA	Soil Needles	AB378552	x	x	x	x	x	x
KLW T10	*Ponchonia bulbillosa*	PA	Soil Needles	JQ272440	x	x	x	x	x	x
KLW T11	*Metaponchonia bulbillosa*	OH	Soil Needles	DQ132810	x	x	x	x	x	x
KLW T13	*Cordyceps confragosa*	OH	Soil Dilution 10^7	KM678344	x	x	x	x	x	x
KLW T16	*Lecanicillium fungicola*	OH	Soil Needles	FJ810136		x	x		x	
KLW T17	*Cordyceps brongniarti*	OH	Soil Dilution 10^6	AB258368				x		
KLW T18	*Cordyceps confragosa*	OH	Soil Dilution 10^6	KM678344	x	x	x	x	x	x
KLW T19	Mycotal (*Cordyceps confragosa*)	UK	White fly	KM678344	x	x	x		x	
KLW T20 / KLW 80	*Cordyceps confragosa*	OH	Chlorotic Needle	AB111495	x	x	x	x	x	x
KLW T21 / KLW 84	*Lecanicillium attenuatum*	OH	Hemlock Looper / Chlorotic Needle	AB111495	x	x	x	x	x	x
MTK B12	*Lecanicillium saksenae*	VA	*Brachycybe lecontii*	KF472156	x	x	x	x	x	x
MTK B15	*Lecanicillium psalliotae*	VA	*Brachycybe lecontii*	AB360367	x	x	x	x	x	x
MTK B52	*Verticillium sp.*	WV	*Brachycybe lecontii*	GU183119		x		x		
MTK 482	*Verticillium insectorum*	TN	*Brachycybe lecontii*	AB214655	x	x	x	x	x	x
MTK 488	*Verticillium fungicola*	TN	*Brachycybe lecontii*	KJ80871		x	x		x	
MTK 676	*Cordyceps confragosa*	SC	*Brachycybe lecontii*	KM678344	x	x	x	x	x	
KLW D1	*Cordyceps confragosa*	MD	*Laricobius nigrinus*	KM678344	x	x	x	x	x	x
KLW D3	*Cordyceps confragosa*	MD	*Laricobius nigrinus*	KM678344	x	x	x			

Table 3-3: Fungal isolates used in entomopathogenicity testing against HWA adults and egg masses applied through a conidial suspension

Treatment	**Isolated From**	**Internal ID number**	**Tested on**
Simplicillium lamellicola	Chlorotic Needle OH	77	Adults
Cordyceps confragosa	Hemlock needles	80	Adults and Egg
L. attanuatum	Lepidopteran	84	Adults and Eggs
L. muscarium	HWA Mass.	5165 MTK 18	Adults and Eggs
L. muscarium	HWA China	6035 MTK 19	Adults and Eggs
L. muscarium	Lymantra dispar WV	3531 MTK 25	Adults and Eggs
L. longisporum	Macrosiphoniella sanborni UK Aphid	5126 MTK 59	Adults and Eggs
L. muscarium	Ceroplastes sp. Coccidae Mississippi	7375 MTK 44	Adults and Eggs
L. muscarium	HWA NH	9925 MTK 27	Eggs
Verticillium lecanii	HWA NJ	5795 MTK 15	Eggs
Rhizosphaera macrospora	Hemlock needles	K6	Adults and Eggs
Epicoccum nigrum	HWA Ohio	K14	Adults and Eggs
Colletotrichum fioriniae	Hemlock needles	K31	Adults and Eggs
Pestalotiopsis microspora	HWA Ohio	K28	Adults and Eggs
Mycotal	Commercialized Product	N/A	Adults and Eggs
Untreated Control			Eggs
Ethanol Dipped Untreated Control			Eggs
H20 Control			Adults and Eggs

Table 3-4: Maximum likelihood analysis and percent of informative characters for the five single gene phylogenies and concatenation of these five genes.

	Taxa	Number of Characters	Total Number of Characters	Informative Characters
RPB2	76	130	1218	11%
RPB1	71	181	796	23%
nad1	74	488	584	84%
EF1-α	74	80	2551	3%
BTUB	72	193	858	22%
Concatenation	64	1112	3775	29%

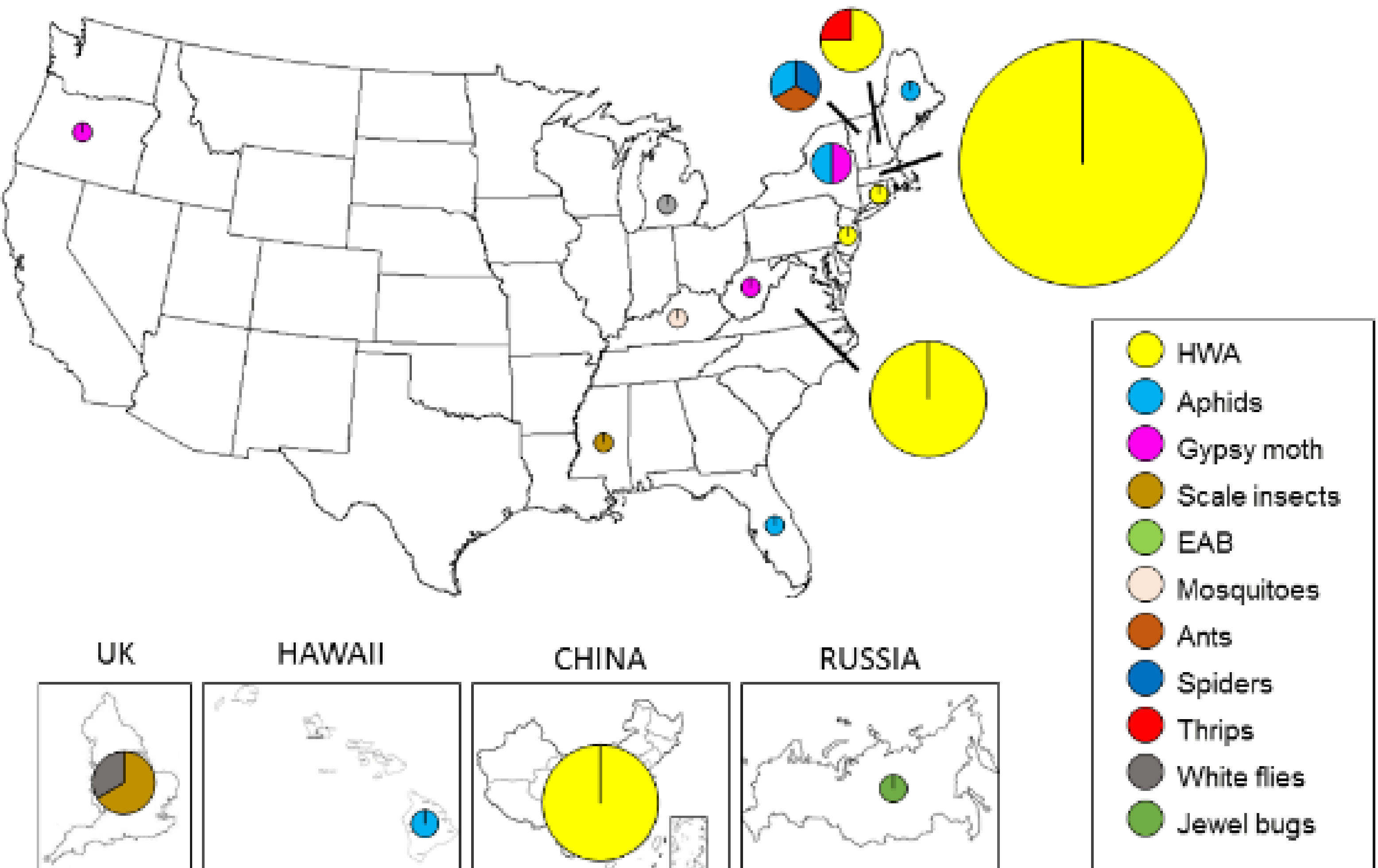

Figure 3-1: Geographical distribution of 62 *Lecanicillium* isolates from two classes of Arthropods , seven orders of insects, 12 insect families, and 16 species including 36 isolates from *Adelges tsugae* from 5 U.S. states and the Peoples Republic of China as well as the reference isolate Mycotal® isolated from *Trialeurodes vaporariorum* (whitefly) in the U.K.

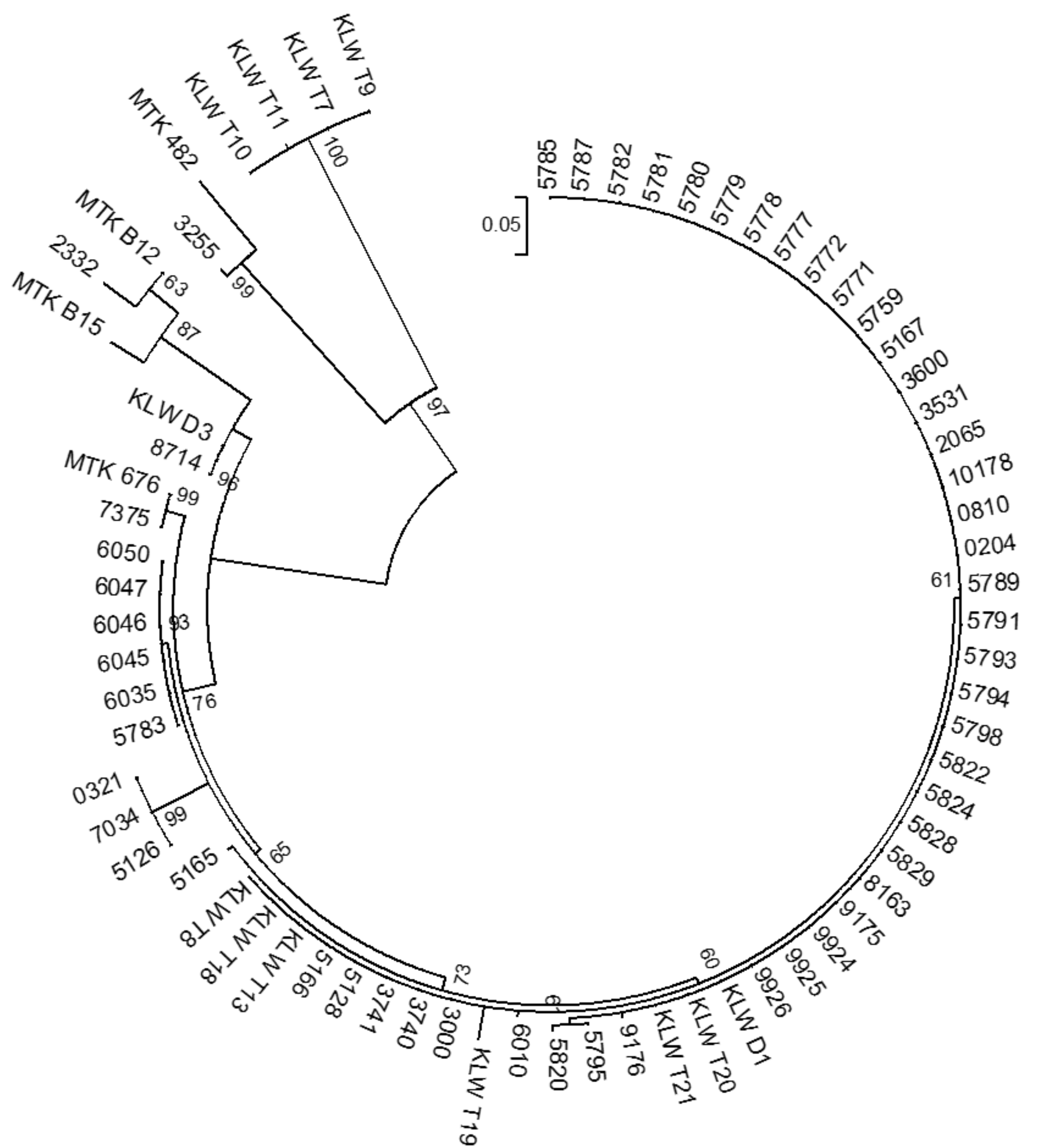

Figure 3-2: Phylogenetic tree created in Mega 6 for the *RPB1* gene utilizing Maximum Likelihood and a bootstrap value of 1000.

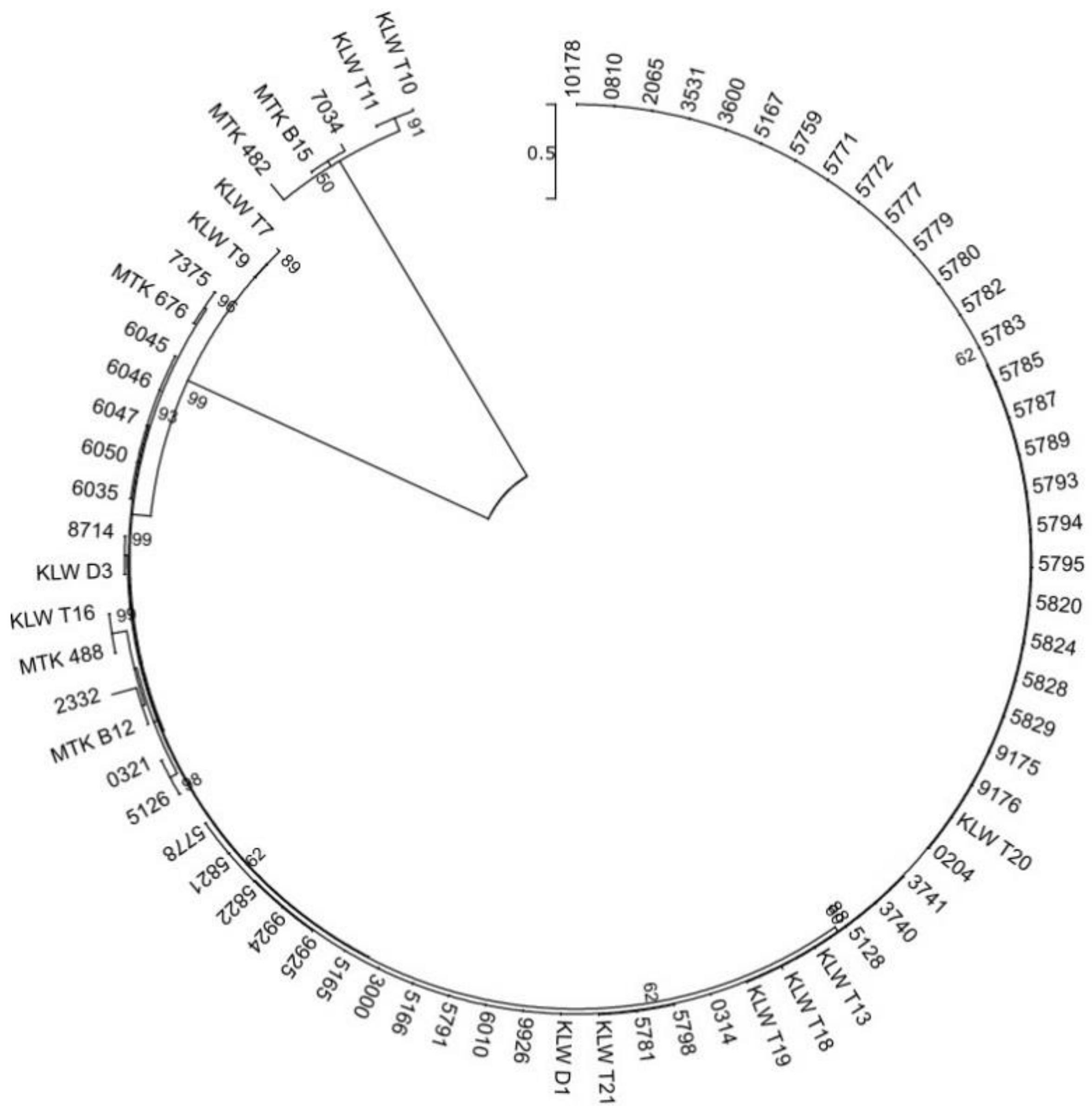

Figure 3-3: Phylogenetic tree created in Mega 6 for the *βtubulin* gene utilizing Maximum Likelihood and a bootstrap value of 1000.

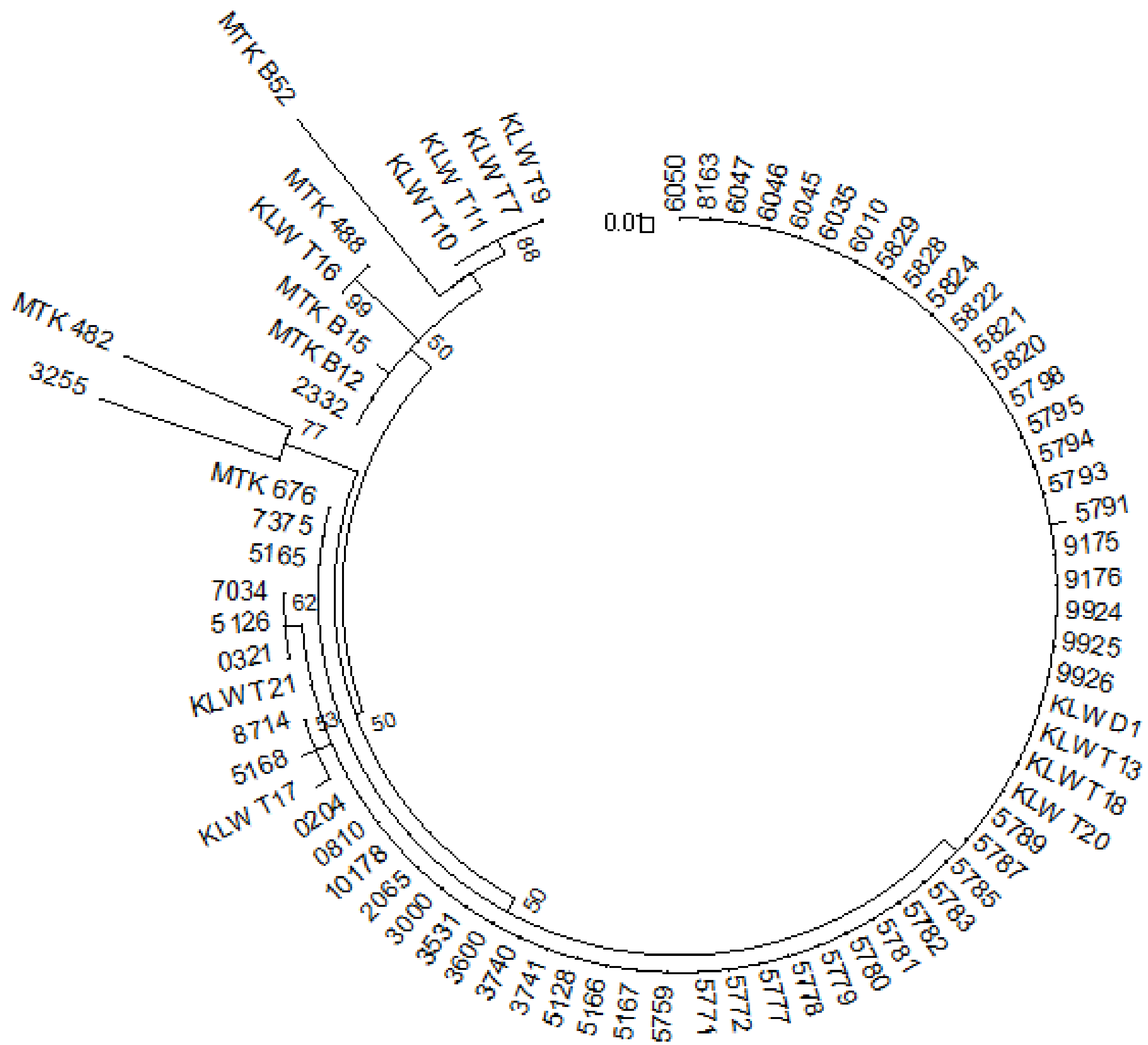

Figure 3-4: Phylogenetic tree created in Mega 6 for the *EF1-α* gene utilizing Maximum Likelihood and a bootstrap value of 1000.

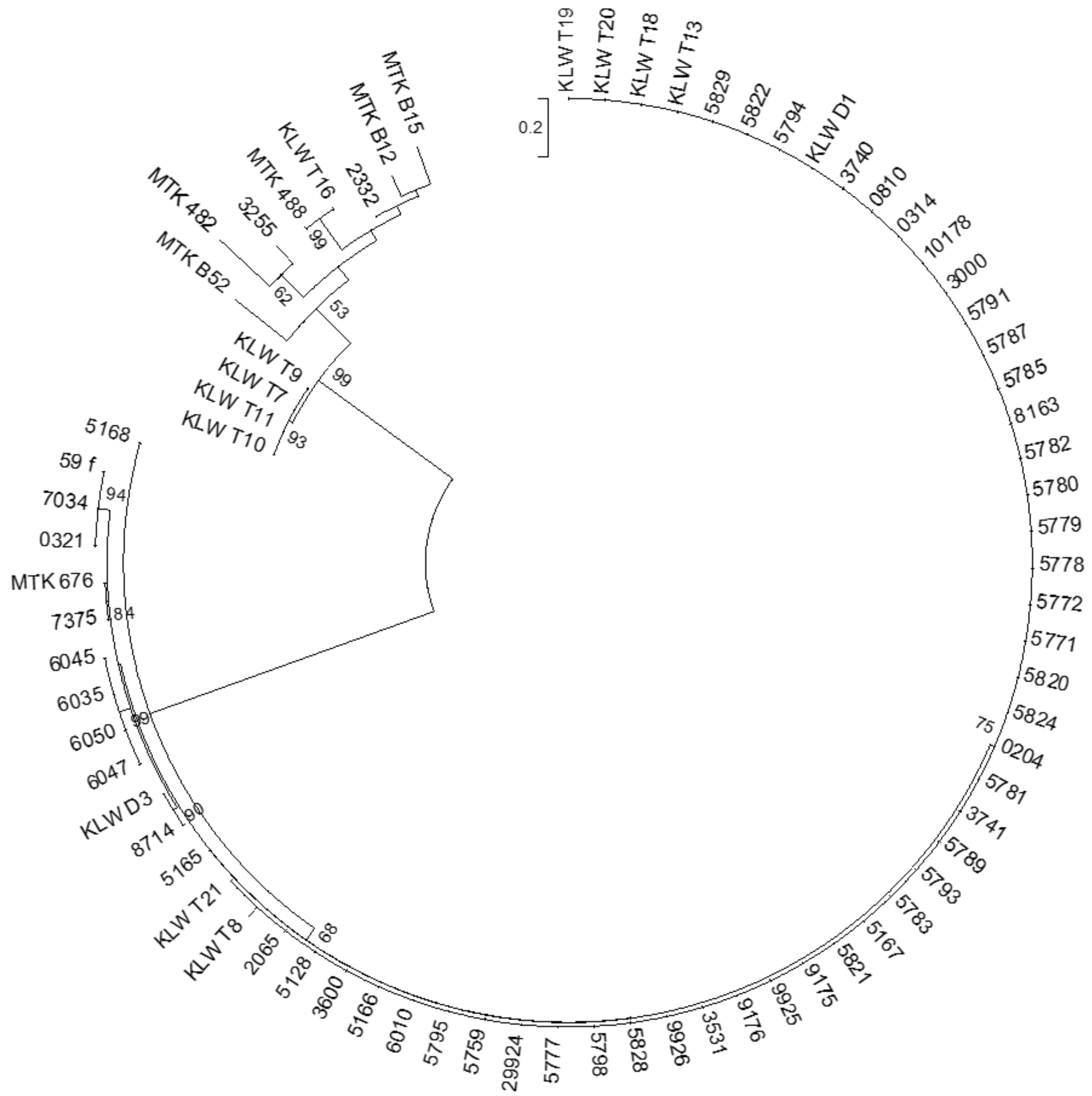

Figure 3-5: Phylogenetic tree created in Mega 6 for the *RPB2* gene utilizing Maximum Likelihood and a bootstrap value of 1000.

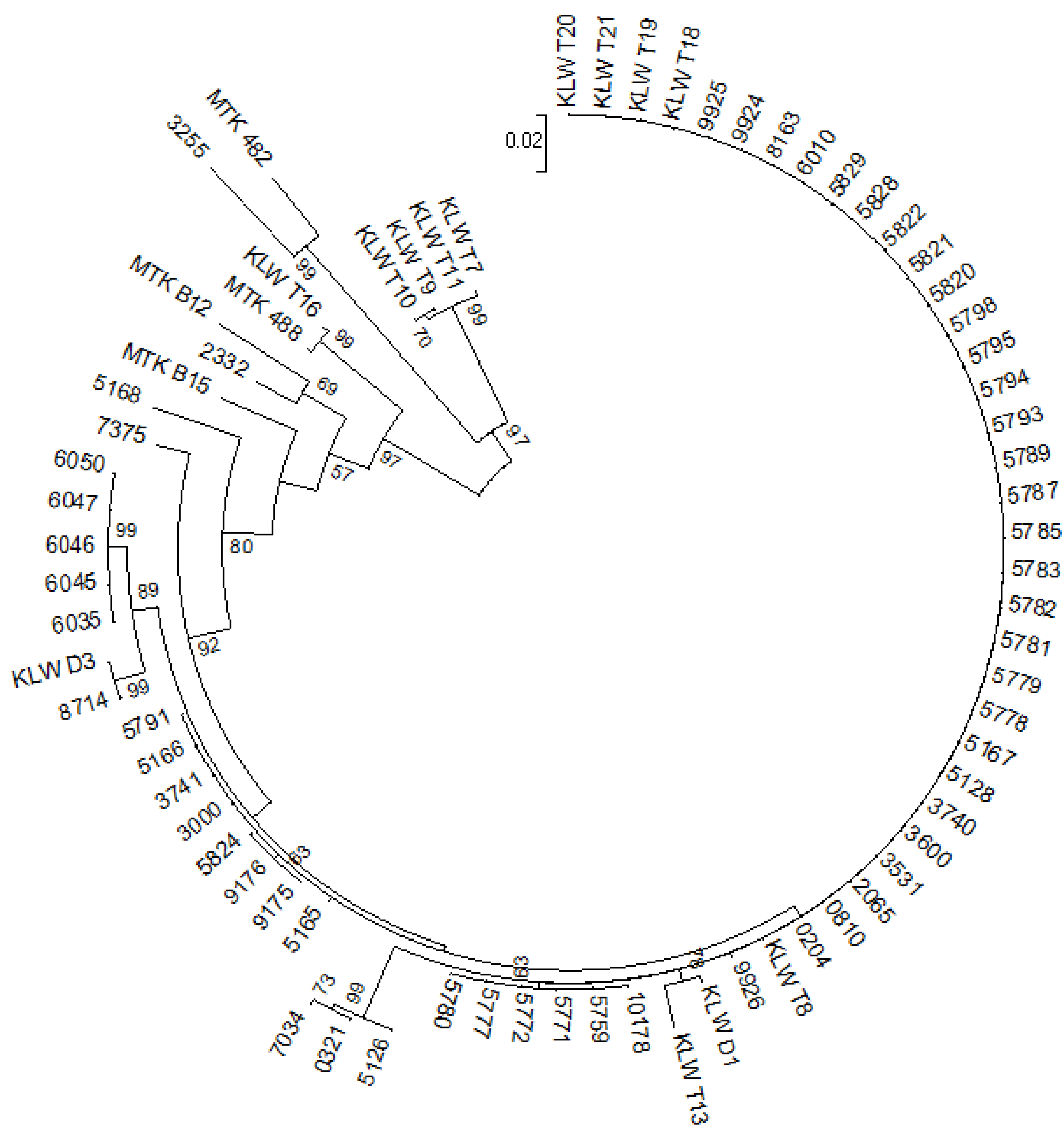

Figure 3-6: Phylogenetic tree created in Mega 6 for the *NAD1* gene utilizing Maximum Likelihood and a bootstrap value of 1000.

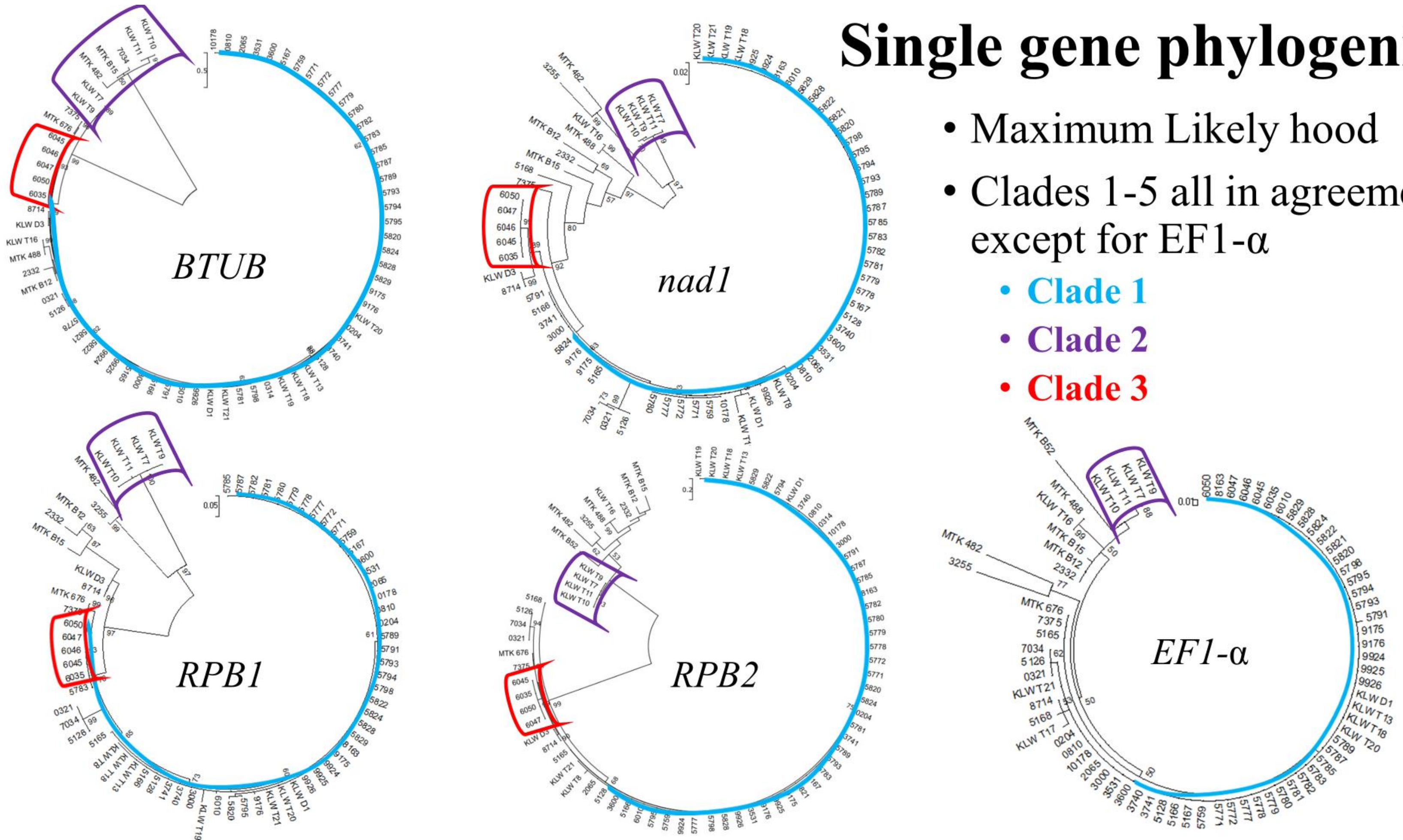

Figure 3-7: Comparison of all five single gene phylogenies with distinct clades highlighted that are in congruence with each other.

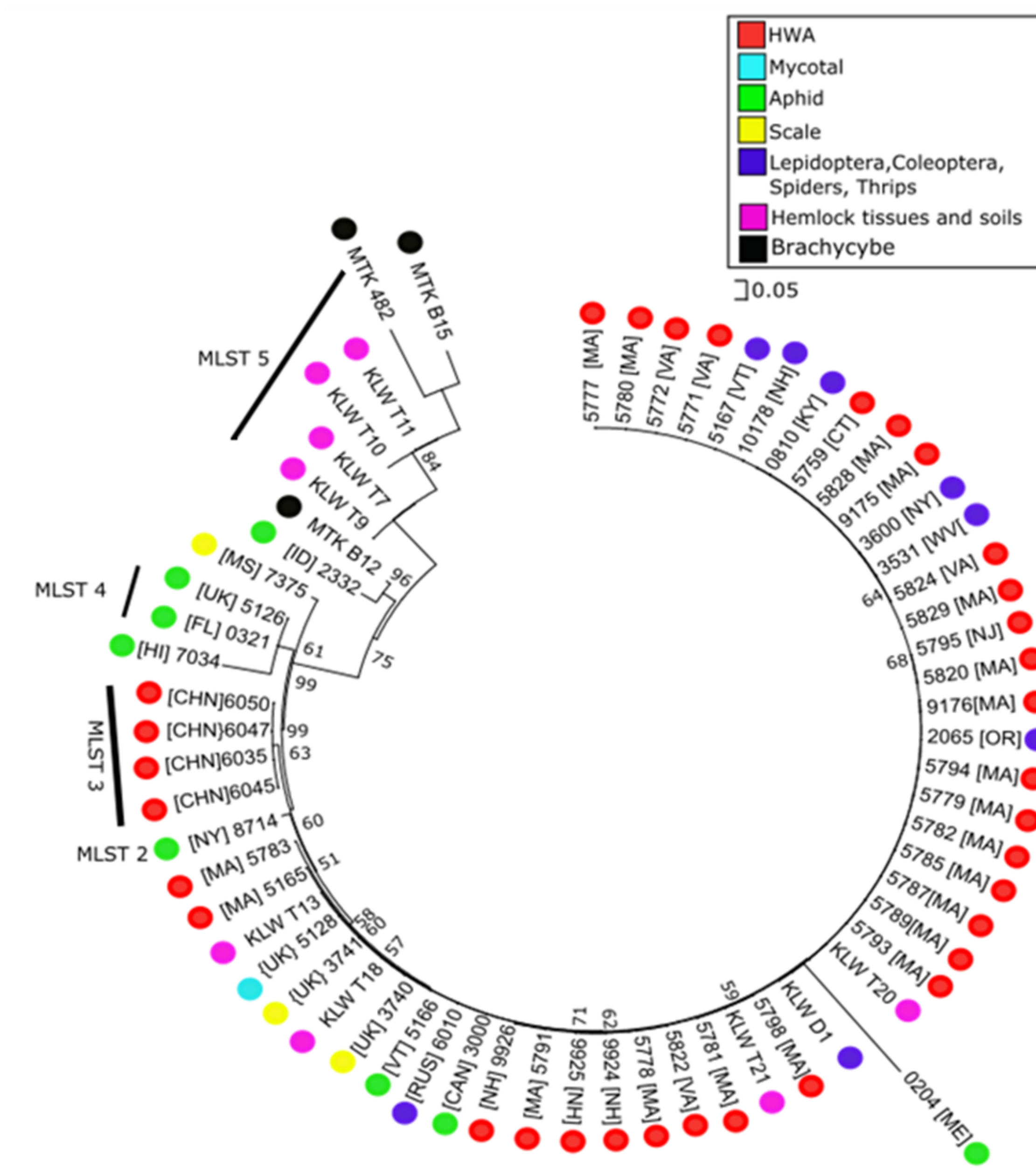

Figure 3-8: Concatenation of all five genes for 64 taxa. Designation of state or nation in brackets appears next to ARSEF isolate ID or Kasson Lab collection ID. Host of isolation is represented by a colored circle corresponding to the above key. Five main multilocus sequence types (MLST) were observed in the above concatenation. MLST 1 represents the monophyly of *L. muscarium.* MLST2 represents a single isolate from an aphid in New York. MLST3 groups isolates from HWA all in China showing some geographic separation. MLST4 supports some host affinity in a pairing of two isolates from aphids. MLST5 groups together as it includes *Pochonia* spp. to serve as outliers.

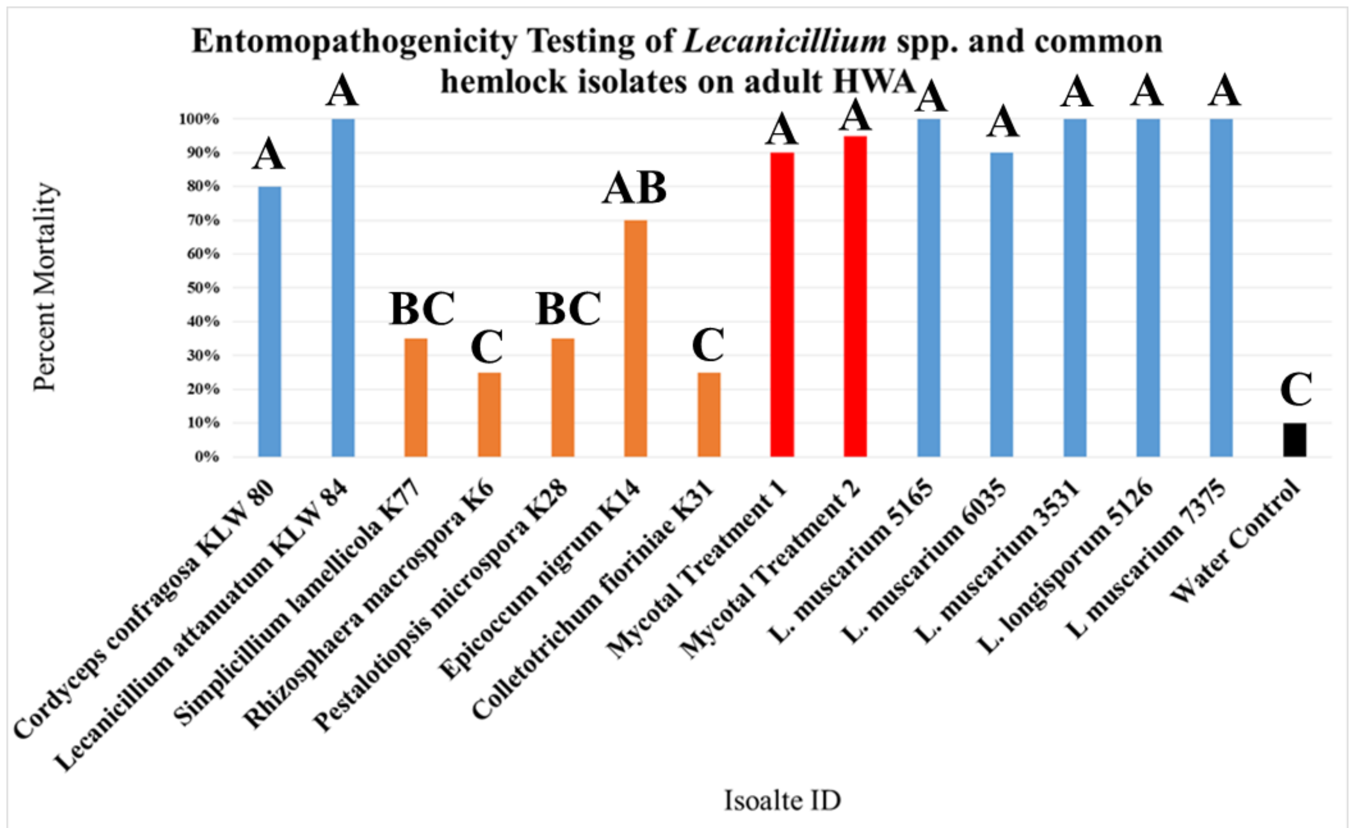

Figure 3-9: Entomopathogenicity testing of isolates on progredien generation adults. Blue represents *Lecanicillium* species, orange bars represent species of interest recovered from hemlock stands in a parallel study, red represents Mycotal® isolates and black represents the (negative) control. ANOVA results in an F-value of 21.63 with a significant P-Value of 0.000, showing there are significant differences between treatments. *Lecanicillium* isolates are from both the ARSEF collection and the Kasson lab.

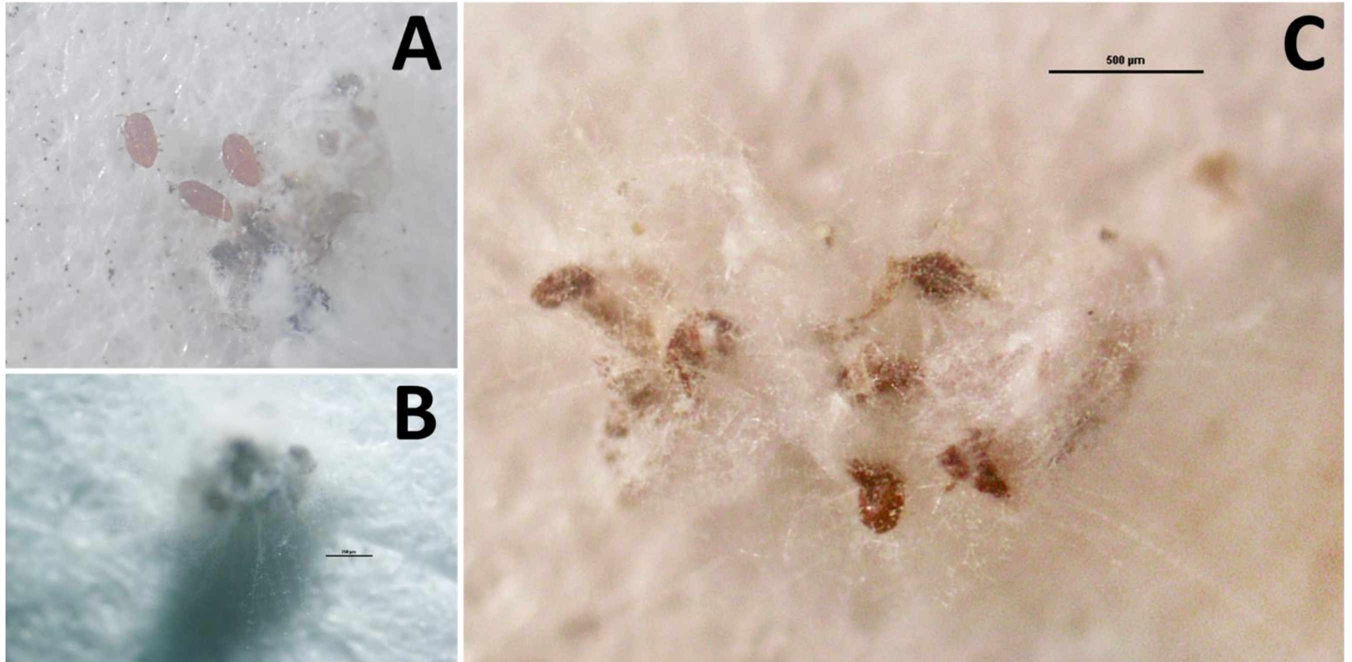

Figure 3-10: Entomopathogencity of adults and crawlers (A) HWA crawlers uninfected by *Rhizospheara* conidial suspension (B) *Lecanicillium* conidiaphores emerging from the wooly mass of an infected HWA adult (C) HWA crawlers emerging from an egg mass to only be infected by *Lecanicillium* from the previous hemlock sampling.

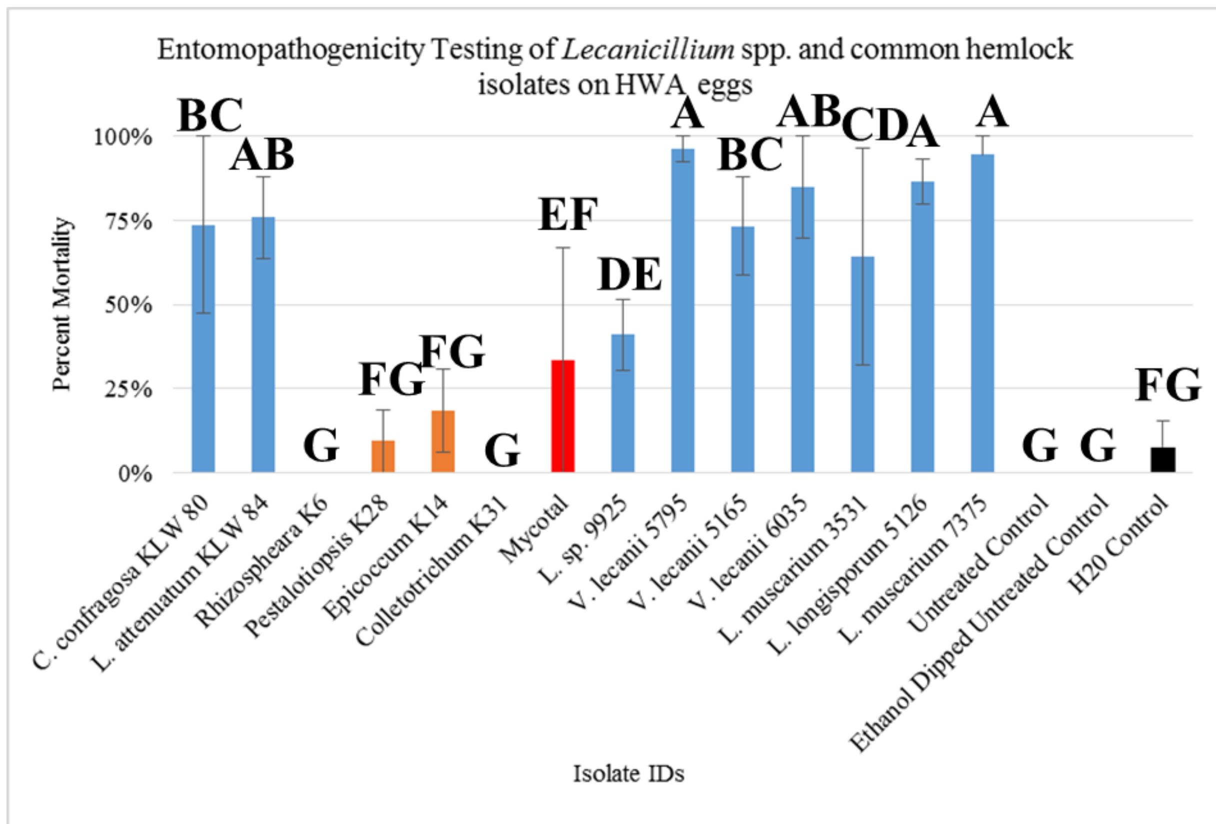

Figure 3-11: Entomopathogenicity testing of isolates on progredien generation eggs masses. Blue represents *Lecanicillium* species, orange bars represent species of interest recovered from hemlock stands in a parallel study, red represents a Mycotal® isolate and black represents the (negative) controls. ANOVA results in an F-value of 76.09 with a significant P-Value of 0.000, showing there are significant differences between treatments. *Lecanicillium* isolates are from both the ARSEF collection and the Kasson lab.

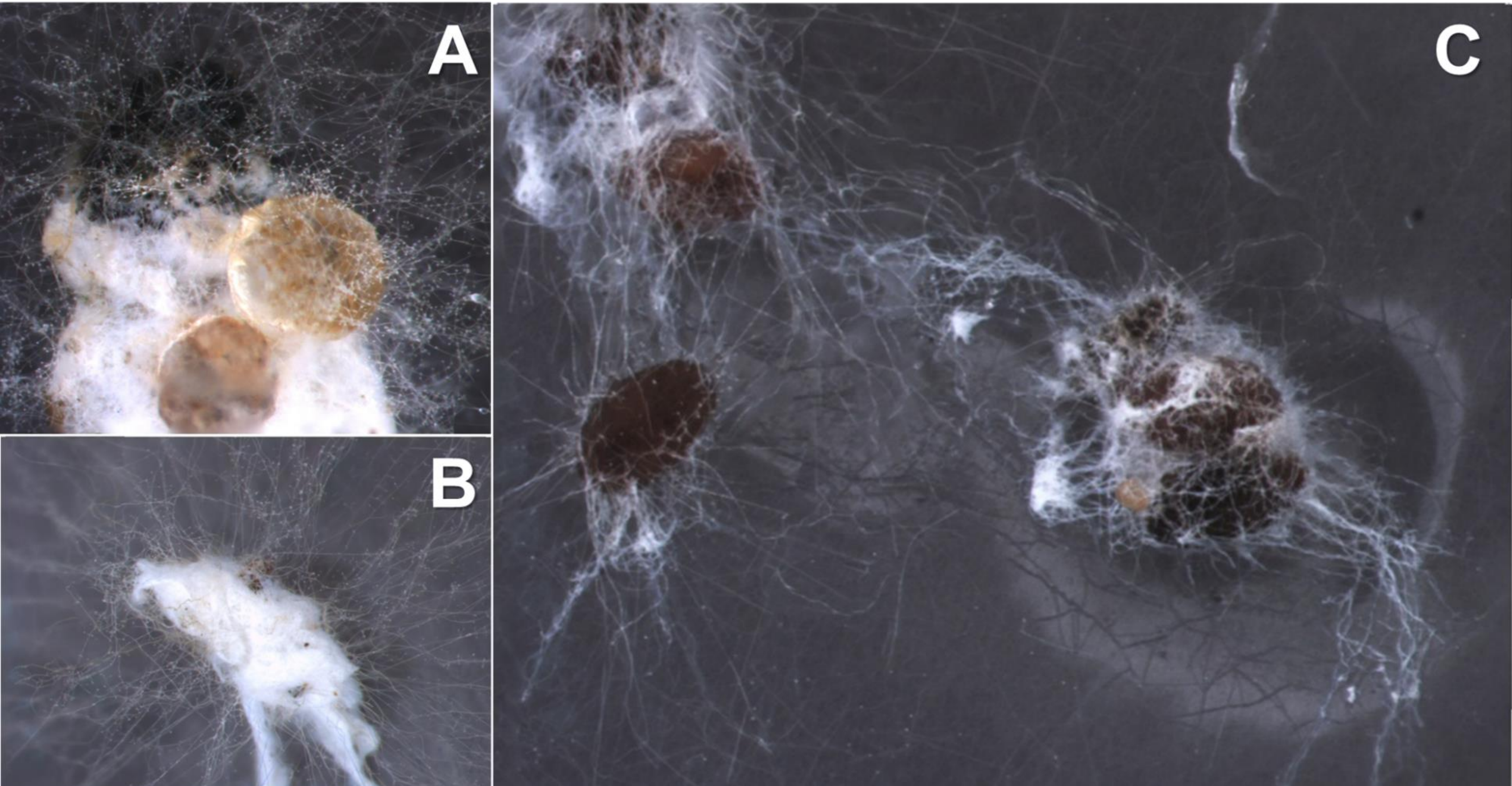

Figure 3-12: *Lecanicillium* isolated from a chlorotic hemlock needle infecting a hemlock woolly adelgid egg mass. Notice the distinct whorled conidiophores of the genus *Lecanicillium*. (C) Mycotal® infecting individual eggs removed from the woolly mass

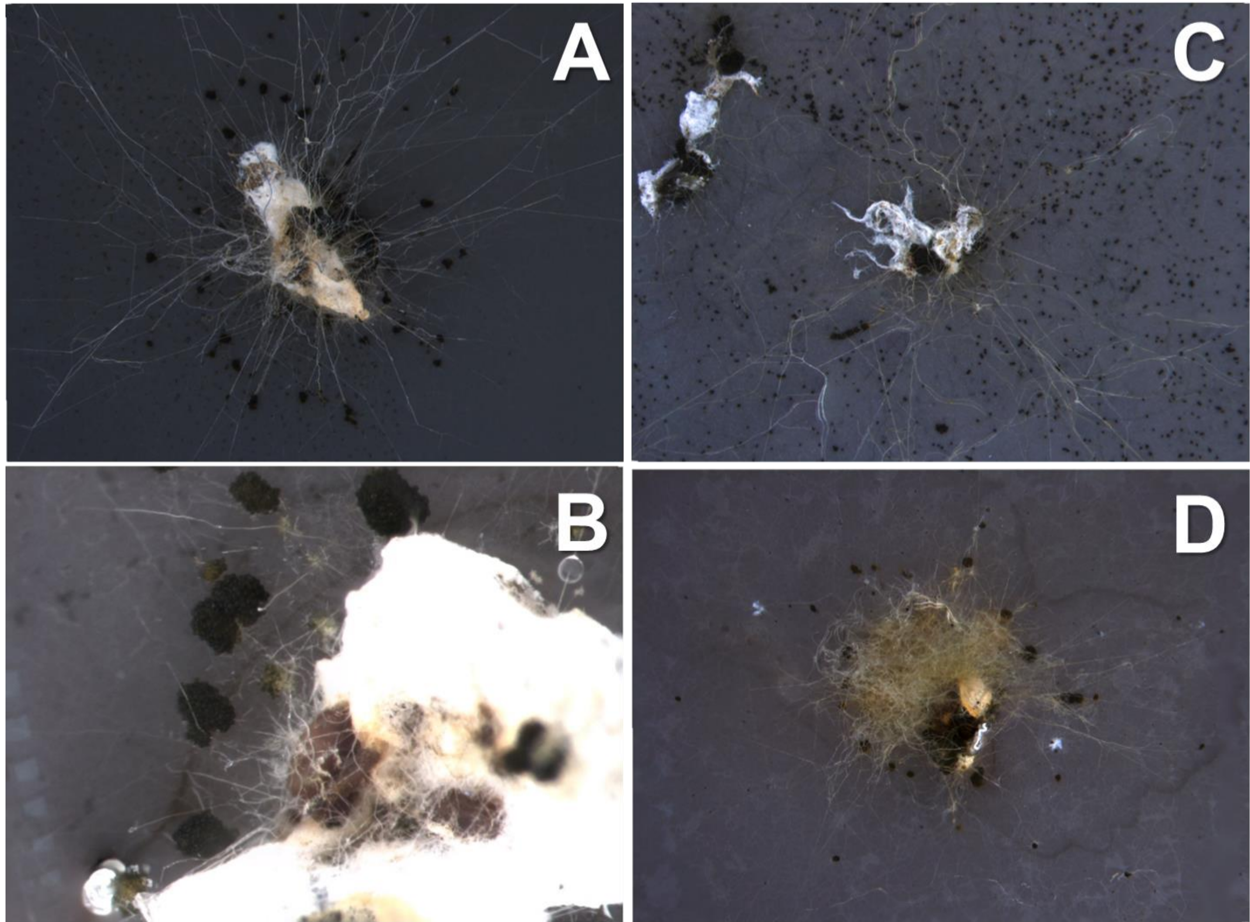

Figure 3-13: (A) *Rhizosphaera* infecting the wooly masses but not the eggs in the masses. (B) *Epicoccum* infecting an HWA egg exposed from the egg mass (C) *Rhizosphaera* sporulation over the entire filter paper due to the fungus utilizing remnants of the wooly masses but not utilizing the eggs. (D) *Pestalotiopsis* infecting the eggs.

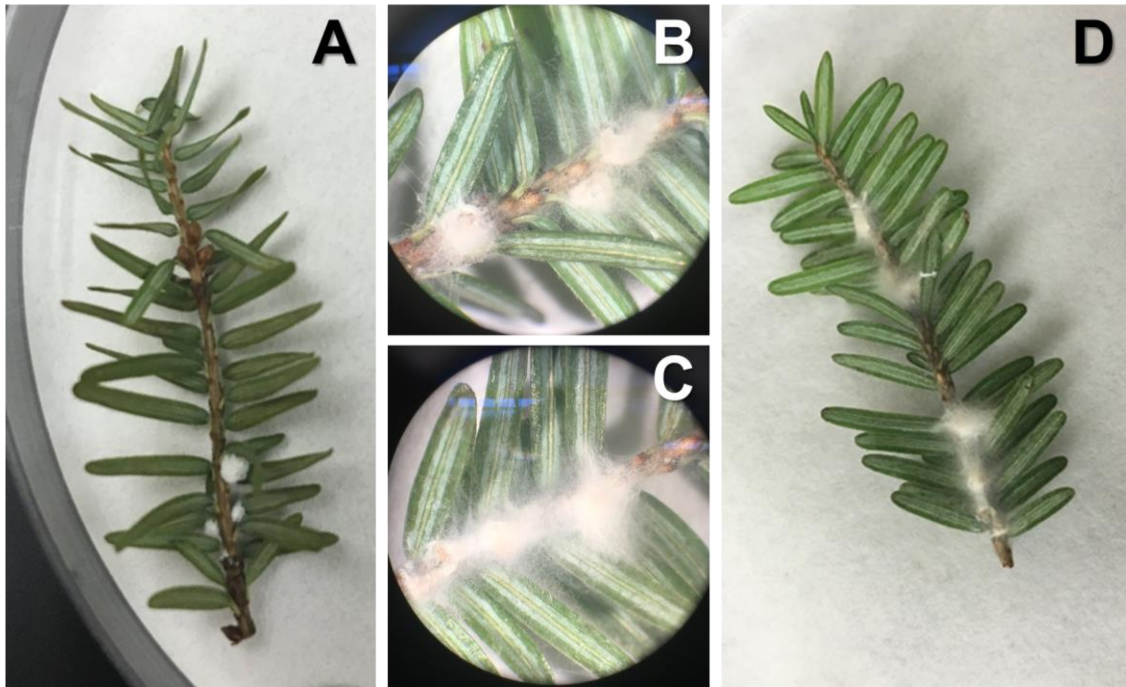

Figure 3-14: Microcosm hemlock branchlets during entomopathogenicity testing. (A) Untreated branchlet with three woolly egg masses showing no signs of fungal infection. (B) HWA egg masses infected with a virulent *Verticillium lecani*i isolate (5795). (C) HWA egg masses infected with isolate *Lecanicillium muscarium* (3531) showing a stronger infection. (D) All eggs masses in the 3531 treatment were infected at the end of the experiment.

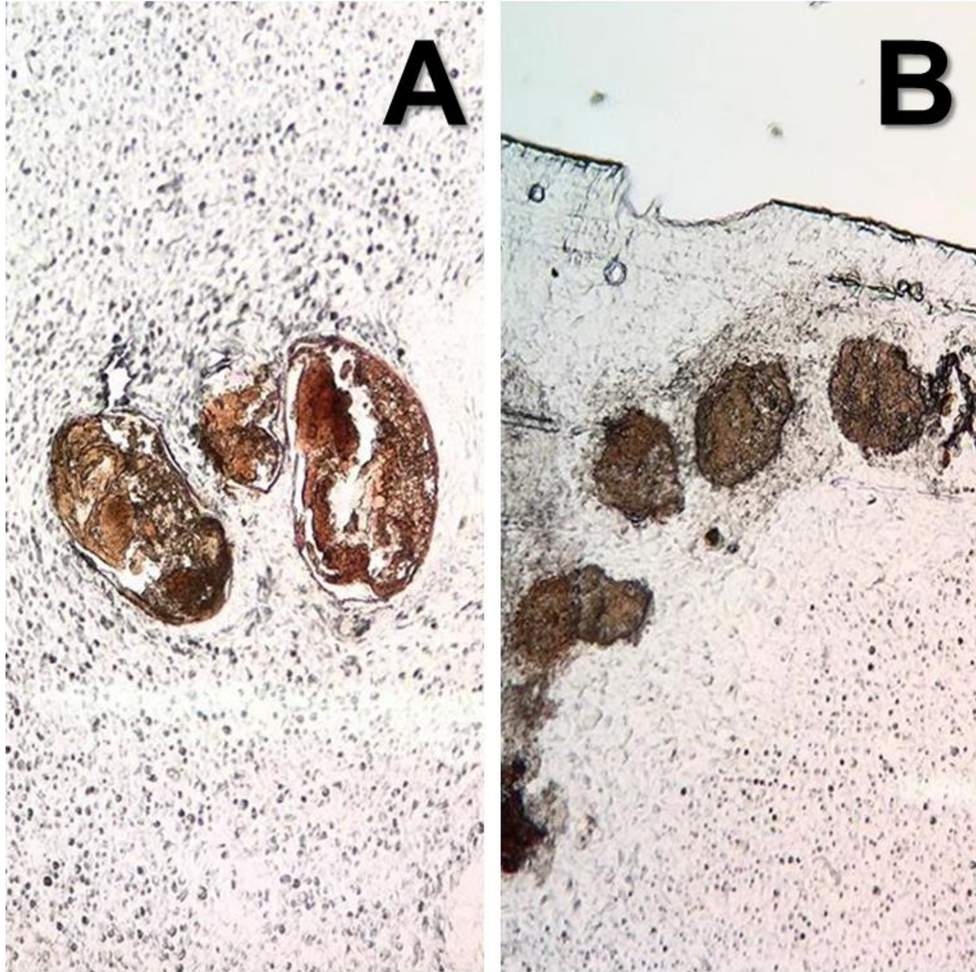

Figure 3-15: Unstained microtome images of HWA eggs. (A) HWA Eggs treated as controls showing full rounded healthy edges (B) Infected HWA eggs showing withered shells and cavities full of fungal tissue.

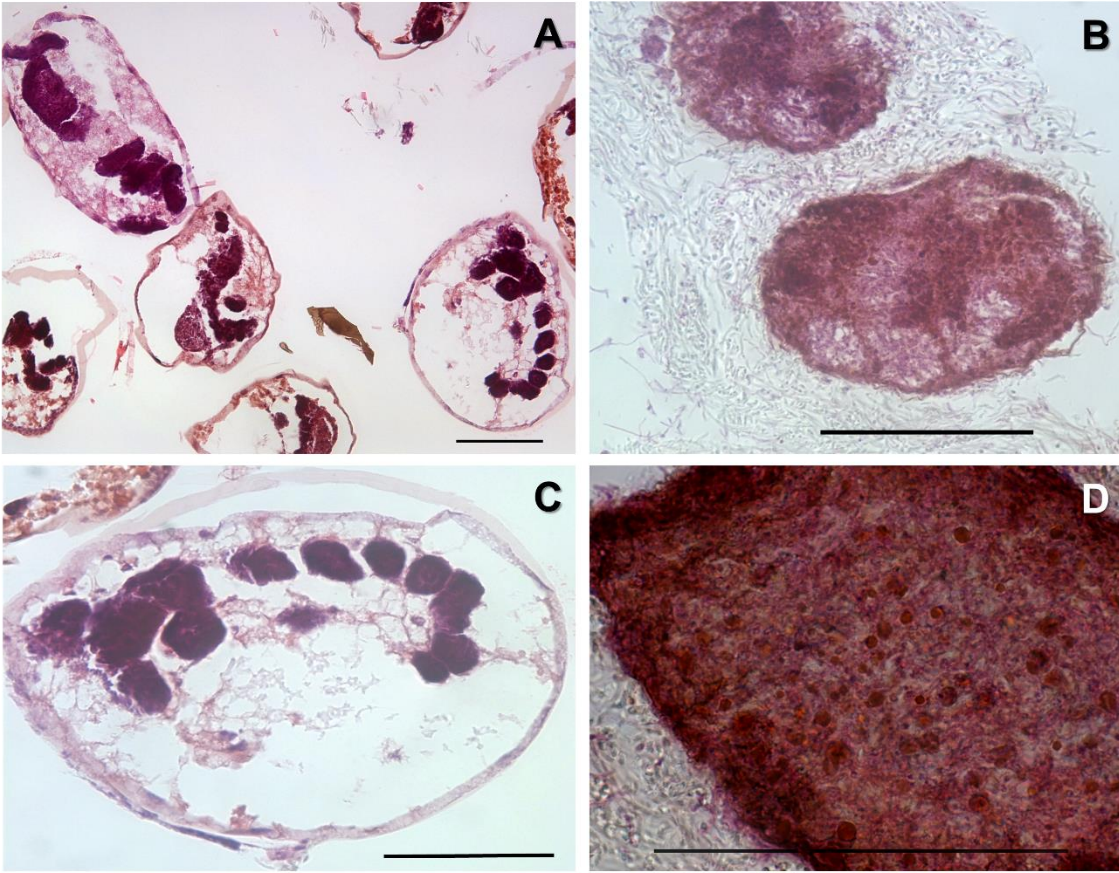

Figure 3-16: HWA egg microtome images after being stained for visualization. (A) Eggs in the control treatment with clear uninfected cavities. (B) HWA eggs infected with ARSEF 5795 *Verticillium lecanii* showing their cavity full of fungal protoplast (C) Close up of control egg (D) Close up of infected egg from the 5795 treatment. All photos utilize a 100 µm scale bar.

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CHAPTER 4

CONCLUSIONS

A good classical biocontrol method has the ability to persist in the environment where it would be long lasting, is inexpensive, and has a selective host range. Even though *Lecanicillium* does not seem to meet all of these criteria, I still believe that *Lecanicillium* should still be considered as a combat method against hemlock woolly adelgid as it is a more ecofriendly alternative to chemical insecticides. The application approach should be an augmentation of the natural population of *Lecanicillium* by an inundative release of millions of *Lecanicillium* spores. An aerial application of Mycotal® in the first year of HWA infestation could add to the already present *Lecanicillium* in the environmental reservoir of the soil and greatly reduce HWA populations until traditional chemical and biological methods can be implemented. *Lecanicillium* should not be used as a preventative measure since it does not persist in the environment, but instead as a reaction to HWA being present in the ecosystem. Timing, dosage, HWA life stage and percent coverage are all important factors in considering Mycotal® and other *Lecanicillium* as biocontrols for HWA.

Sampling during this study may have not been truly representative of what is in the environment in normal climatic situations. *Lecanicillium* could have been recovered in such low incidence during 2015 due to the Polar Vortex of 2014. Such cold temperatures for a prolonged period of time killed many adelgids and many other insects. This could have greatly reduced the food source for the entomophagous fungi. It is also uncertain if these cold temperatures impacted the fungus in the environment directly. A repeat of sampling in winter of 2016 and summer of 2017 could add to the certainty that *Lecanicillium* is found in low numbers in the environment. Future directions that this study need to address the host range of *Lecanicillium*.