

Assessing *Eucryptorrhynchus brandti* as a potential carrier for *Verticillium albo-atrum* from infected *Ailanthus altissima*.

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ABSTRACT

Significant mortality of the invasive tree of heaven (TOH), *Ailanthus altissima* (Mill.) Swingle, was first observed in Pennsylvania in 2002 to be caused by an apparently host-specific strain of *Verticillium albo-atrum* Reinke & Berthold, a soil-borne, vascular wilt fungus. A limited survey conducted in Virginia revealed two sites where TOH stands were infected with *V. albo-atrum*. A virulence test confirmed that fungal isolates from both states were found to be highly pathogenic on TOH, killing all inoculated seedlings in 9 weeks. After overwintering, 11% (n = 37) of TOH root sections tested positive for *V. albo-atrum*, although the origin of the colonies was not identified. The pathogenicity of this pathogen suggests that it could be used together with host-specific insects for the biological control of TOH. A host-specific herbivorous weevil from China, *Eucryptorrhynchus brandti* Harold (Coleoptera: Curculionidae) that has been extensively studied as another potential biological control agent for TOH is currently pending quarantine release. Quarantine experiments were conducted to test different forms of transmission with *E. brandti* and *V. albo-atrum* simultaneously. In one experiment, 75% (n = 32) of adult *E. brandti* transmitted *V. albo-atrum* to TOH seedlings after walking on an actively growing culture and feeding on infected plant material. In another study after feeding on infected TOH stems for 24, 48 and 72 h, respectively, 16.7% (n = 120), 15.0% and 12.5% of adult *E. brandti* ingested and passed viable *V. albo-atrum* propagules into feces. Surviving weevils (83%, n = 20) overwintering in infested potting mix carried viable *V. albo-atrum* propagules externally. In addition, all weevil progeny that emerged from infected TOH billets appeared to be as healthy as

weevils reared from non-infected billets and wild parents from China. Results from these laboratory studies indicate *E. brandti* has the ability to spread *V. albo-atrum* from tree to tree in a laboratory setting.

Dedication

I would like to dedicate this to my best friend, John Patrick Gannon II. His success and enthusiasm for science inspired me to continue my education in the field I love. Thank you for your patience, knowledge and encouragement during this journey.

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Chapter 1. Literature Review

1.1 *Ailanthus altissima* (Sapindales: Simaroubaceae)

Simaroubaceae are trees and shrubs that have conspicuous piths with quassinoid type triterpenoid compounds and secretory cells scattered in the leaves and bark (Judd et al. 2002). One important genus of this family is *Ailanthus* and the number of species within this genus is unclear. One report claimed that *Ailanthus* is comprised of about ten species from Asia to North Oceania and six species in China (eFloras 2008), while another report claimed five to six species are from India alone (CSIR 1985).

Ailanthus altissima (Mill.) Swingle, commonly known as tree of heaven (TOH), is native to China, Taiwan, Japan, and India (CSIR 1985) and is a widespread invasive species found throughout much of the continental United States. Tree of heaven was imported to the United States as a shade tree from Europe in the late 18th century and then again in the mid-19th century, when it was planted for medicinal purposes by Chinese laborers during the California gold rush (Tellman 1997). Almost all species of *Ailanthus* have been used in ancient times, particularly in India for the treatment of various ailments using traditional applications (Laskar 2010). The bark has properties that are astringent, anti-spasmodic, anthelmintic, and parasiticidal. It has been used to treat diarrhea, dysentery and parasitic worms (CSIR 1985). The root bark is recommended for heart ailments, epilepsy and asthma, and the leaves have astringent properties that have been made into lotions to treat scabies (Kirtikar and Basu 1933).

The genus *Ailanthus* is thought to be derived from the Moluccan name ‘Aylanto’ meaning “tree reaching for the sky” (Hu 1979). This tree is widely known for its ability to withstand high amounts of environmental pollutants and water stress. Nearly every part of this tree produces an unpleasant odor. TOH is a serious threat to native ecosystems because it is a strong competitor as it produces allelopathic chemicals that inhibit native tree growth. In addition, there are few herbivores that negatively impact its health and reproduction. Control of TOH is a large economic and agricultural concern because of its invasiveness and lack of long-term conventional control methods to combat it (Kok et al. 2008). It is for these reasons that biological control is being investigated as a potential control tactic for TOH.

Tree of heaven is a fast growing, dioecious tree reaching up to 27 m in height and 1 m in diameter over its 30–50 year life span (Miller 1990). The bark is usually light gray and leaves are large, odd-pinnately compound, pubescent, and can grow up to 1 m in length (Kowarik and Säumel 2007). Leaflets are ovate-lanceolate with glandular teeth at the rounded base (Hu 1979). The male flower has either rudimental or absent carpels with 10 stamina, each having a fertile anther and glandular green disc. A female flower can have ≥ 10 sterile stamina and a green glandular disc and pistil with five or six free carpels. Fruit later develops into spiral greenish yellow to reddish-brown samaras with one central seed (Hu 1979, Kowarik and Säumel 2007). Each seed is light and ideal for wind or water dispersal for distances of up to 100 m or more from the parent tree (Landenberger et al. 2007). A medium to large tree can produce as many as 300,000 viable seeds per year (Sheppard et al. 2006), with the most prolific period of seed production at 12–20 years of age (Asaro et al. 2009).

Seeds can germinate in highly compact or salty soils and retain their dormancy for about 1 year (Asaro et al. 2009). Once germinated, seedlings grow 1–2 m within the first growing season and continue to grow rapidly until age 20–25 when overall growth slows. Tree of heaven also spreads vigorously through asexual sprouting from roots and stumps (Hu 1979), often resulting in dense thicket growth. Root sprouts are very prolific and one established tree can produce enough sprouts to cover 1 acre of land (Asaro et al. 2009).

Tree of heaven produces weak, lightweight wood that is prone to breaking in heavy winds and snow or ice accumulation, making TOH dangerous in recreation areas and roadsides. It also produces several bitter chemicals or quassinoids, which have phytotoxic activity to more than 35 hardwood and 34 coniferous species (Mergen 1959). Among these are aianthone, amarolide, acetyl amarolide, 2-dihydroailanthone, ailanthinone, chaparrin, chaparrinone, quassin, neoquassin, shinjulactone, and shinjudilactone (Heisey 1996). Tree of heaven often can be seen growing along roadsides, newly harvested forest areas, agricultural land and other disrupted habitats. It is shade intolerant and prefers rich, moist soils, although it can also tolerate dry, poor soils (Ding et al. 2006). Currently, TOH is present in 41 of the lower 48 states and has been declared a noxious weed in Connecticut, Massachusetts, New Hampshire and Vermont (USDA-NRCS 2011). In Virginia, it is dominant along Interstate-81 and occupies hundreds of acres in the Shenandoah National Park (Marler 2000).

A recent survey of TOH in Virginia showed that canopy coverage of TOH ranged from 15-85% and 17 tree species were found to co-exist within the areas surveyed. Twenty herbivores were found associated with TOH, but they had little impact on healthy TOH. Ambrosia beetles,

(*Euwallacea validus* Eichoff and *Xyleborus atratus* Eichoff) were suspected to attack only dead or dying TOH and the *Ailanthus* webworm, *Atteva punctella* Cramer, was consistently found but these insects never caused more than 5% defoliation (Kok et al. 2008).

Mechanical control of TOH is expensive and labor intensive because the entire root system has to be removed. Physical removal will cause an increase in root sprouts and stand density. Therefore, additional herbicide treatment is needed to achieve complete eradication. Several general-use herbicides are available for TOH control in the form of foliar sprays, stump treatments, basal sprays, or injection. Registered herbicides currently include imazapyr, glyphosate, dicamba, triclopyr and metasulfuron methyl (Kok et al. 2008). However, all chemical treatments available are general-use herbicides that can kill more plant species than the target, leaving the treated area barren and ideal for re-intrusion of TOH. In one study, imazapyr injections on TOH caused 100% mortality, but also killed 17.5% of neighboring trees of eight different species (Lewis and McCarthy 2008). Long-term removal of TOH is taxing and requires constant follow-up monitoring with additional treatments for new growth. As a result, control is expensive and time consuming, as much as 8,750 USD/ha (Kok et al. 2008).

1.2 *Eucryptorrhynchus brandti* (Coleoptera: Curculionidae)

There are 46 phytophagous arthropod species associated with TOH in China (Ding et al. 2006). While most are defoliators that do not impact the growth of TOH, a few attack trunks and branches. Two Curculionidae species, *Eucryptorrhynchus brandti* Harold and *Eucryptorrhynchus chinensis* Oliver, are major pests of TOH in China and are thought to feed exclusively on this tree (Ge 2000).

Adult *E. brandti* feed on leaves, stems and petioles; larvae feed under the bark, destroying cambial tissue (Ding et al. 2006). Larvae overwinter under the bark and adults overwinter in soil near the host plant (Ge 2000). Females oviposit eggs under the bark where the larvae feed until they become adults and emerge through small exit holes on the bark (Kok et al. 2008). In addition to causing cambial damage, adult exit holes may leave the tree susceptible to pathogen infection. In China, adult *E. brandti* on the bark surface and emergence holes were found from the tree base to 4 m above the soil line (S. Salom, personal communication, Department of Entomology, Virginia Tech). The amount of damage these weevils can cause to TOH is still unknown, but studies are ongoing to assess whether they are primary or secondary killers of TOH in China (T. McAvoy, personal communication, Department of Entomology, Virginia Tech).

In some areas of China, 80–100% of TOH were attacked by *E. brandti* and *E. chinensis*, and 12–37% of those trees died (Ding et al. 2006, Ge 2000). Although the insects could have caused enough damage to kill entire trees, additional secondary factors could have been involved such as stress and disease. If laboratory tests reveal *E. brandti* to be host-specific to TOH, it could be reared and released as part of a low-risk biological control effort (Kok et al. 2008).

Studies of the general biology, development, and an efficient rearing method at the Virginia Tech Beneficial Insect Quarantine Laboratory were initiated in 2004 with importation of *Eucryptorrhynchus* spp. from China to the United States (Herrick et al. 2011). Attempts at rearing *E. chinensis* have not been successful and as a result this species was not evaluated.

Eucryptorrhynchus brandti has been successfully reared with high fitness through several generations making it the focal species for use as a biological control agent.

Eucryptorrhynchus brandti sex can be differentiated by observing the metathoracic sternite and first abdominal segment, which are convex in females and concave or flat in males. Females are larger than males with heavier weights and longer sagittal and transversal measurements (Herrick et al. 2011). Preliminary quarantine egg production in the lab is very low, averaging 3.4 eggs per female. More work is needed to determine fecundity. Development studies at 25°C revealed that the egg stage averaged 5 d, the larval stage with six stadia averaged 110 d, and the pupal stage averaged 16 d. *Eucryptorrhynchus brandti* adult choice and no-choice feeding tests on North American TOH and 30 test species resulted in significantly more feeding on TOH foliage when compared with all test species (Kok et al. 2008). When larvae were inoculated into plant stems, larval development only occurred in seedlings of *Leitneria floridiana* Chapm. (20%, n = 10) and TOH controls (70%). Further testing showed that adults were unable to oviposit on seedlings of *L. floridiana* (0%) when compared with TOH controls (70%) (Herrick 2011). Although more development testing of *E. brandti* on larger specimens of *L. floridiana* is required, *E. brandti* appears to be highly host-specific for TOH.

1.3 *Verticillium albo-atrum*

Verticillium wilt is a vascular wilt and root disease that can be caused by seven different species of *Verticillium*. Of these seven species, *Verticillium albo-atrum* Reinke & Berthold and *Verticillium dahliae* Kleb. are the two most important pathogens affecting agronomic plants (Pegg and Brady 2002), and both can cause disease in TOH. *Verticillium albo-atrum* has been a documented pathogen of TOH in Virginia, Pennsylvania, and New York since the early 1950's

(Weiss and Muriel 1950-1953) and is recently responsible for acute TOH decline (Schall and Davis 2009a).

Verticillium wilt symptoms include wilting of leaves, branch death sometimes in irregular patterns, and eventually death of the entire tree. A characteristic symptom is brown or greenish streaking in the xylem of branches, roots, and stems. However, correct identification of the disease can only be verified by culturing the fungus (Manion 1990).

Verticillium albo-atrum belongs to the Kingdom Fungi, Phylum Ascomycota, Subphylum Pezizomycotina, Class Sordariomycetes, and Order Phyllachorales (Fradin and Thomma 2006).

Verticillium albo-atrum is distinguished by the presence of specialized hyphae, which melanize to become dark, thick-walled resting structures. The hyphae and conidia are mostly haploid with conidiophore bases. The phialides are arranged in a verticillate whorls that contain a mass of oval shaped conidia (Pegg and Brady 2002).

Verticillium albo-atrum conidia and hyaline hyphae can survive in the soil for 3–4 weeks while melanized mycelium can remain dormant for 9 months to 4 years depending on soil conditions (Pegg and Brady 2002). Germination occurs when propagules become initiated by host root exudates (Pegg and Brady 2002, Tjamos 1993). *Verticillium albo-atrum* hyphae can directly invade actively growing host plant root tips or enter through older roots or xylem through mechanical injury (Tjamos 1993). Hyphae also have been reported to directly penetrate intact root hairs in tomatoes (Bewley 1922, Selman and Buckley 1959). Once the host root is penetrated, the pathogen is restricted to the xylem of the plant and spreads by transpiration flow

into vessel end plates. Here conidia accumulate and eventually become trapped until hyphae germinate and spread to surrounding vessels where the cycle continues. *Verticillium albo-atrum* releases enzymes such as endopectin lyase, which allow the fungus to lyse host cell walls and expose food reserves (Tjamos 1993). In addition, conidial and mycelial development forces the host to produce gums or tyloses, which clog the xylem, resulting in wilt symptoms (Pegg and Brady 2002).

Verticillium albo-atrum conidia have been found in xylem tissue in hops (*Humulus lupulus* L.) at a concentration of 6.87×10^3 conidia per ml^{-1} of xylem fluid (Sewell and Wilson 1964). Once infection occurs, conidia move rapidly through the vascular tissue. Garber (1973) found 50–100 *V. dahliae* conidia in a single cotton vessel and Presley (1966) noted conidia could colonize a 115-cm tall cotton plant in 24 h. Conidial development appears to be dominant over hyphal growth when the plant is alive and water is present in the vascular tissue. However after plant death, conidial growth is replaced by hyphal growth until the remaining host tissue is colonized (Heinz et al. 1998, Sewell and Wilson 1964).

Several wilting stands of TOH have been observed in south-central Pennsylvania (Schall and Davis 2009a). Isolation, and use of Koch's postulates, concluded that *Verticillium albo-atrum* PSU 140 (GenBank accession # FJ424082) and *Verticillium dahliae* PSU 154 (GenBank accession # FJ424083) were the causal agents of the wilt (Schall and Davis 2009a). From 2000 to 2008, nearly 10,000 TOH host trees died as a result of the two species. Inoculation studies have shown that TOH is susceptible to both *V. albo-atrum* and *V. dahliae* in both greenhouse and field canopy studies. However, *V. albo-atrum* PSU 140 is much more aggressive and pathogenic,

making it more suitable as a potential biocontrol agent. Tree of heaven stem inoculations with *V. albo-atrum* PSU 140 resulted in 100% mortality of TOH greenhouse seedlings in 9 weeks and 100% mortality of TOH canopy trees in 3 months (Schall and Davis 2009a). In host-range susceptibility tests, canopy stem inoculations of eight tree species growing intermingled with healthy TOH were found to be non-symptomatic. The only species found to be susceptible to stem inoculations was understory striped maple (*Acer pennsylvanicum* L.), which exhibited 100% mortality. When measuring susceptibility by naturally infected forest plots, 17 tree species growing intermingled within various stages of dead and dying TOH were found non-symptomatic except striped maple, where only 1% of saplings exhibited Verticillium wilt. This discrepancy may be due to the high dosage of conidial inoculation, which bypassed the root defense system and is not indicative of a natural setting. These host-range susceptibility tests provide important preliminary risk analysis for *V. albo-atrum* PSU 140 as a potential biological control agent for TOH, but need to be expanded.

1.4 Insects and Fungal Associations

Most true curculionids do not have specialized structures for holding fungi, but some are known to transmit fungal spores via legs or snout (Kok and Abad 1994). Fungal associations with wood-boring weevils are closely connected with phloem and inner bark feeding. Insertion of a fungal-infested snout into this highly nutritious part of the tree can be a pathway for fungus inoculation (Lieutier 2004). The most anterior part of the snout or rostrum includes the chewing mouthparts, labial palps, mandible, maxilla and maxillary palps, while the rostrum itself is a slender modification of the head which can sometimes be as long as the body (Triplehorn and Johnson 2005). In addition to feeding, weevils are often contaminated internally through ingestion of

infected plant material and externally with fungal spores after emerging from pupal galleries (Lieutier 2004).

The relationships between insects and pathogens have been extensively studied in human, animal, and plant systems. Insects, especially bark and ambrosia beetles, are important carriers of both beneficial and harmful fungi. Oak wilt, caused by the fungus *Ceratocystis fagacearum* (Bretz) J. Hunt, is responsible for widespread oak mortality and can be transmitted by sap feeding beetles from the Nitidulidae family or by oak bark beetles (Rexrode and Brown 1983). Dutch elm disease caused by *Ophiostoma ulmi* (Buisman) Nannf. and *Ophiostoma novo-ulmi* Brasier, is responsible for the national decline of American elm trees (*Ulmus americana* L.) The pathogen is spread by the smaller European elm bark beetle, *Scolytus multistriatus* Marsham (Manion 1990). These insect-pathogen relationships are well known because of the severity of damage caused in North American forests, but there are many other less well-known relationships.

Weevil-fungus associations are currently a problem in North America, especially with root disease and stain fungi (Lieutier 2004). *Leptographium wageneri* (W.B. Kendr.) M.J. Wingf., the causal agent of black-stain root disease, can kill a variety of western conifer species. The fungus can be transported by *Hylastes gracilis* LeConte and *Hylastes longicollis* Swaine at densities of 1×10^1 to 1×10^4 spores per individual (Schweigkofler et al. 2005). The European pine weevil, *Hylobius abietis* L., can carry spores externally and infect new trees with blue stain fungi [*Leptographium procerum* (W.B. Kendr.) M.J. Wingf.]. Spores were found in abundance on pronotal setae positioned in cuticular depressions on the anterior dorsal and lateral sides of the

pronotum (Piou 1993). This study found 3–47% of emerging pine weevils were associated with the fungus and 18% of seedlings fed upon by the weevils died.

Weevil dispersal patterns are widely unknown, but flight migration by the pine weevil, *H. abietis*, in Scandinavia has been studied. When temperatures reach 8–9°C in late May, weevils break quiescence from overwintering and begin to fly (Eidmann 1968, Solbreck and Gyldberg 1979). During this time, females begin to develop eggs in the oviducts, and after feeding, are reproductively mature (Örlander et al. 2000). At this time, all weevils are photopositive and generally fly downwind with wind velocities at 3–4 m s⁻¹, reaching elevations of 30–50 m (Solbreck and Gyldberg 1979). Individual adult weevils undertake most of their flight activity during the first 10 d of migration, but there is great variation in flight duration. Some adults fly for only 0.5 h, others do not fly at all, while some fly for longer periods of time. In one experimental group, frequency distribution estimations suggested 50% of the population flew only 1.5 km, but others flew greater than 80 km (Solbreck 1980). Weevils detect host volatiles to locate desired areas, such as freshly felled coniferous trees suitable for progeny (Nordenhem and Eidmann 1991, Nordlander et al. 1986). After the migratory phase, flight muscle degeneration follows, weevils become photonegative (Nordenhem 1989), and females enter a reproductive phase.

If an herbivorous insect carrying a pathogen can breach barriers of a host plant such as intact bark, it may be able to inoculate the plant and cause a new infection. Since *V. albo-atrum* occurs naturally on TOH, releasing a possible long-range insect carrier may spread the pathogen to stands that are unreachable for short-range transmission. Such relationships may be occurring

naturally, based on evidence of the pine weevil's infection capabilities of blue stain fungus. Although no studies have been conducted on the dispersal and carrying capabilities of *E. brandti*, this weevil could act similarly as the pine weevil in both flight and transmission. Due to the aggressiveness and high pathogenicity already seen with *V. albo-atrum* PSU 140 and the wide distribution of *E. brandti* on TOH in China, the manipulation of an insect-pathogen combination might lead to a novel solution for the control of TOH.

Research Objectives

The purpose of this work is to examine the relationship between *V. albo-atrum* and the rate of infection on TOH by using *E. brandti* as a carrier. The underlying hypothesis is *E. brandti* is capable of carrying *V. albo-atrum*. All forms of passive transmission are meant to mimic a natural source of infestation that may be present in the field due to *E. brandti* pending quarantine release. Specific objectives are listed below.

Specific Objectives:

1. Survey for *V. albo-atrum* in Virginia, evaluate virulence among *V. albo-atrum* isolates, and examine TOH root sections for colonization by *V. albo-atrum*.
2. Assess transmission of *V. albo-atrum* from feeding and tarsal contact of *E. brandti* to TOH.
3. Assess carrying ability and propagule quantity from *E. brandti* feces in *V. albo-atrum* feeding tests.
4. Assess emerging adult *E. brandti* weight, carrying ability and transmission of the fungus from *V. albo-atrum*-infected billets.
5. Measure transmission of *V. albo-atrum* by *E. brandti* overwintering within infested potting mix.

Chapter 2. A preliminary assessment of *Verticillium albo-atrum* in Virginia

Abstract

Significant mortality of the invasive tree of heaven (TOH), *Ailanthus altissima* (Mill.) Swingle, in Pennsylvania was first observed in 2002 and determined to be caused by an apparently host-specific strain of *Verticillium albo-atrum* Reinke & Berthold, a soil-borne, vascular wilt fungus. A limited survey conducted in Virginia revealed two sites where TOH stands were infected with *V. albo-atrum*. A virulence test was conducted to determine the pathogenicity of each state's isolate, and both were found to be highly pathogenic on TOH, killing all inoculated seedlings in 9 weeks. This indicates they may be the same strain, although more work is needed. After overwintering, 11% (n = 37) of root sections tested positive for *V. albo-atrum*, although the origin of the colonies was not identified. This suggests new infections may be achieved in the field through root-to-root contact or by root grafting.

2.1. Introduction

Tree of heaven is native to China, Taiwan, Japan, and India (CSIR 1985) and is a widespread invasive species found throughout much of the continental United States. Tree of heaven was imported to the United States as a shade tree from Europe in the late 18th century and again in the mid-19th century when it was planted for medicinal purposes by Chinese laborers during the California gold rush (Tellman 1997). Tree of heaven is a serious threat to native ecosystems because it is a strong competitor as it produces allelopathic chemicals that inhibit native tree growth. In addition, there are few herbivores that negatively impact its health and reproduction. Control of TOH is a large economic and agricultural concern because of its invasiveness and

lack of long-term conventional control methods to combat it (Kok et al. 2008). These reasons make TOH a suitable species for biological control.

Tree of heaven is a fast growing, dioecious tree capable of producing as many as 300,000 viable seeds per year (Sheppard et al. 2006). Each seed is light and ideal for wind or water dispersal for great distances from the parent tree (Landenberger et al. 2007). Tree of heaven also spreads vigorously through asexual sprouting from roots and stumps (Hu 1979), often resulting in a dense thicket growth. The tree produces weak, lightweight wood unsuitable for most commercial uses and prone to breaking, making TOH dangerous in recreation areas and roadsides. It also produces several bitter chemicals or quassinoids, which have phytotoxic activity to many native forest trees (Mergen 1959). Tree of heaven often grows along roadsides, newly harvested forest areas, agricultural land and other disrupted habitats. It is shade intolerant and can grow in dry, poor soils (Ding et al. 2006). Currently, TOH is present in 41 of the lower 48 states and has been declared a noxious weed in Connecticut, Massachusetts, New Hampshire and Vermont (USDA-NRCS 2011). In Virginia TOH is dominant along Interstate-81 and occupies hundreds of acres in the Shenandoah National Park (Marler 2000).

Mechanical control of TOH is expensive and labor intensive because the entire root system has to be removed. Physical removal will cause an increase in root sprouts and stand density. Therefore, additional herbicide treatment is needed to achieve complete eradication. However, all chemical treatments available are general-use herbicides that often kill more plant species than the target, leaving the treated area barren and ideal for TOH re-intrusion. Long-term

removal of TOH is taxing, expensive, and requires constant follow-up monitoring and additional treatments for new growth (Kok et al. 2008).

Verticillium wilt is a vascular wilt and root disease that can be caused by seven different species of *Verticillium*. Of these seven species, *V. albo-atrum* and *Verticillium dahliae* Kleb. are the two most important pathogens affecting agronomic plants (Pegg and Brady 2002) and both can cause disease in TOH. *Verticillium albo-atrum* has been a documented pathogen of TOH in Virginia, Pennsylvania, and New York since the early 1950's (Weiss and Muriel 1950-1953).

Verticillium albo-atrum is distinguished by the presence of specialized hyphae, which melanize to become dark, thick-walled resting structures. The phialides are arranged in verticillate whorls that contain a mass of oval shaped conidia (Pegg and Brady 2002). *V. albo-atrum* conidia and hyaline hyphae can survive in the soil for 3–4 weeks while the melanized mycelium can remain dormant from 9 months to 4 years depending on soil conditions (Pegg and Brady 2002). Germination occurs when propagules become stimulated by host root exudates (Pegg and Brady 2002, Tjamos 1993). *V. albo-atrum* hyphae can directly invade actively growing host plant root tips or enter through older roots or xylem through mechanical injury (Tjamos 1993). Once the host is penetrated, conidia move rapidly through the vascular tissue. Conidial development appears to be dominant over hyphal growth when the plant is alive and water is present in the vascular tissue. However after plant death, conidial growth is replaced by hyphal growth until the remaining host tissue is colonized (Heinz et al. 1998, Sewell and Wilson 1964). In addition, conidial and mycelial development forces the host to produce gums, which clog the xylem, resulting in wilt symptoms (Pegg and Brady 2002).

Other symptoms of *Verticillium* wilt include wilting of leaves, branch death sometimes in irregular patterns, and eventually death of the entire tree. A characteristic symptom is brown or greenish streaking in the xylem of branches, roots, and stems. Correct identification of this disease can only be verified by culturing the fungus (Manion 1990).

Several wilting stands of TOH have been observed in south-central Pennsylvania. Schall and Davis (2009a) concluded that *Verticillium albo-atrum* Reinke & Berthold PSU 140 (GenBank accession # FJ424082) and *Verticillium dahliae* Kleb. PSU 154 (GenBank accession # FJ424083) were the causal agents of the wilt. From 2000 to 2008, nearly 10,000 TOH hosts died as a result of the two species. Inoculation studies have shown that TOH is susceptible to both *V. albo-atrum* and *V. dahliae* in both greenhouse and field canopy studies. However, *V. albo-atrum* PSU 140 is much more aggressive and pathogenic, making it more suitable as a potential biocontrol agent (Schall and Davis 2009a). Host-range susceptibility testing suggests *V. albo-atrum* PSU 140 may be a low-risk biological control agent for TOH (Schall and Davis 2009b).

The purpose of this study was to conduct a preliminary survey to see if *V. albo-atrum* was present in TOH stands in Virginia, to examine the degree of pathogenicity on TOH seedlings using isolates, to quantify the level of root colonization, and to examine the origin of the colonies. The discovery of *V. albo-atrum* killing TOH in Virginia provides evidence that biological control using this fungus may be possible in Virginia.

2.2. Materials & Methods

2.2.1. Isolation of *V. albo-atrum* from TOH.

Samples of TOH explained throughout this chapter were surface disinfested by soaking in 70% ethanol for 1 min and isolated onto Komada's selective medium (KSM) [1000 ml distilled water, 2 g L-sorbose, 2 g L-asparagine, 1 g K₂HPO₄, 0.5 g KCl, 0.5 g MgSO₄·7H₂O, 0.01 g Fe-Na-EDTA, 1 g pentachloronitrobenzene (75% a.i.), 0.5 g oxgall, 1 g NaB₄O₇·10H₂O, 0.3 g streptomycin sulfate, pH 5.7] used to isolate *V. albo-atrum* from soil and other substrates (Christen 1982). Plates were sealed with parafilm and placed in the dark at 20–25°C for 7–14 d to allow adequate time for colony development (Christen 1982). However, difficulty was experienced in correctly identifying *V. albo-atrum* colonies on KSM because distinct fungal characteristics fail to develop satisfactorily (Talboys 1960). One difficulty was species colony characteristics on KSM were hard to distinguish from undesirable organisms. Highly populated plates were recognized as having smooth or floccose, hollow and hemispherical, cream-colored colonies and colonies on sparsely populated plates occasionally developed brownish centers (Christen 1982).

This problem was solved by transferring all potential *V. albo-atrum* colonies using sterilized flat toothpicks to plum-extract agar (PEA) (900 ml distilled water, 20 g agar, 100 ml concentrated plum extract, 1 g yeast extract, 1 g lactose monohydrate, pH 5.6–6.0), a semi-selective medium for *Verticillium* spp. (Talboys 1960). To reduce bacterial contamination, PEA was amended with streptomycin and neomycin (Schall and Davis 2009b). *Verticillium albo-atrum* isolates on PEA medium have distinct macroscopic species characteristics: the aerial mycelium is usually sparse

with numerous conidiophores giving the colony surface a gray, mealy appearance and after 6+ d hyphal walls on the older section of the colony become greenish-black pigmented while the reverse side of the colony is characteristically fibrous (Talboys 1960). Plates were sealed with parafilm and placed in the dark at 22°C (Talboys 1960) for 7–14 d to allow adequate time for characteristic pigment development. Verification of *V. albo-atrum* was confirmed morphologically.

2.2.2. Making conidial suspensions.

Separate conidial suspensions of *V. albo-atrum* PSU 140 (GenBank accession # FJ424082) and VA 100 (Virginia isolate) were made by washing five, 7-d old PEA plate cultures with 30% glycerol solution. Serial dilutions were performed in triplicate and the suspension was adjusted to 1×10^8 colony forming units (CFU) mL⁻¹. Suspensions were maintained at -20°C until needed. Inoculum viability was confirmed before inoculation if at least 75% of the propagules germinated on PEA (Schall and Davis 2009b).

2.3. Experiments

2.3.1 Preliminary survey for *V. albo-atrum* in Virginia.

The purpose of this survey was to see if *V. albo-atrum* was present in TOH stands in Virginia. A windshield survey was conducted on selected primary and secondary roads by visual observation of symptomatic stands. Symptoms included large areas of rapid, declining or dead TOH. When a stand met this criterion, individual trees were examined for vascular discoloration by peeling back the bark using a sterile pocketknife. If the exposed xylem contained greenish or brown-

streaks (Manion 1990) compared to white or cream colored healthy xylem, a wedge of xylem was removed, placed in individual freezer bags, and placed on ice. Several trees per symptomatic stand were sampled. The pocketknife was flame-sterilized between samples. Samples were returned to the laboratory within 5 h for isolation of *V. albo-atrum* using the method described above. In addition to the windshield survey, potential locations described by citizens from previous outreach efforts were also examined. Locations of sites were recorded using a global positioning system (GPS). If *V. albo-atrum* was isolated from the samples, the site was considered infested. For characterization of forest stand composition, total basal area (BA) was calculated for each infected stand.

2.3.2 Virulence testing.

An evaluation of virulence was made for two *V. albo-atrum* isolates, PSU 140 from the Tuscarora State Forest, Pennsylvania (Schall and Davis 2009a), and VA 100 from the previous study in Montvale, Virginia. The purpose of this experiment was to examine the degree of pathogenicity for the two isolates. It was hypothesized that degree of pathogenicity between the two isolates will be the same.

Tree of heaven seeds were collected in Blacksburg, Virginia from healthy trees and planted in 13-cm dia pots filled with Sta-Green® Nursery Blend Tree and Shrub Planting Mix (0.09:0.06:0.05% N:P:K). All seedlings were grown in a controlled environmental chamber set at 24°C, 60% RH, and a 12-h photoperiod using ultraviolet grow lights. After 10 weeks, seedlings were removed from the chamber. Three groups of nine seedlings were inoculated using a sterile syringe with 0.1 mL distilled water, conidial suspension VA 100 or PSU 140 using the method

described above, at two points located on the stem 5 cm above the soil line (Presley 1966). All plants were watered twice a week.

Seedlings were evaluated for Disease Severity Index (DSI) once a week until plant death. DSI was slightly modified from Bejarano-Alcazar et al. (1996) and represented natural progression of disease symptoms. The rate scale ranged from 0–4, where 0 = non-symptomatic leaves, 1 = wilting leaflets, 2 = chlorotic/necrotic leaflets, 3 = defoliating leaves, and 4 = seedling mortality. This experiment was replicated twice for a total of 18 *V. albo-atrum* VA 100 stem-inoculated seedlings, 18 *V. albo-atrum* PSU 140 stem-inoculated seedlings, and 18 sterile water stem-inoculated seedlings (controls).

The experiment was conducted in a completely randomized block design, blocked by replication date. Differences in disease severity values among species were evaluated using area under the disease progress curve (AUDPC): $[(y_i + y_{i+1})/2](t_{i+1} - t_i)$ where y_i is the disease severity rating, t_i is the time of the i th rating, and $i = 1, 2, 3 \dots n - 1$ (Jeger and Viljanen-Rollinson 2001). A Wilcoxon/Kruskal-Wallis test was used to analyze data (JMP 1989–2009) and pairwise comparisons using Wilcoxon rank sum tests were used to determine significant ($\alpha = 0.05$) differences among treatments (R-Development-Core-Team 2011). Temporal trends in weekly DSI ratings for inoculated seedlings are illustrated graphically (Schall and Davis 2009b).

2.3.3 Colonization of TOH roots by *V. albo-atrum*.

The purpose of this experiment was to quantify the level of root colonization by *V. albo-atrum* from infected TOH saplings, after overwintering, and to examine the origin of the colonies.

In March 2010, 30-cm stem cuttings were made from healthy TOH and immediately moved to a greenhouse for propagation. The cuttings were potted in 25-cm pots filled with Sta-Green® Nursery Blend Tree and Shrub Planting Mix, and were watered three times a week. By June the cuttings had established root systems and three plants were moved to a quarantine laboratory. These plants were stem inoculated with 0.5 mL of *V. albo-atrum* PSU 140 conidial suspension using the method stated above using a sterile syringe at two points, both 5 cm above the soil line.

By 4 months after inoculation (September 2010) all plants had died from *V. albo-atrum*, and rhizosphere potting mix samples were taken from each pot to determine the quantity of *V. albo-atrum*. Samples were collected (0.1 g) using a sterile scoopula near roots of each plant. Serial dilutions were performed in triplicate on KSM for each sample and mean CFU g⁻¹ were recorded for each potting mix.

Pots were kept in a controlled environmental chamber for overwintering. The soil was kept moist by adding water through bottom saucers once a week. The pots were placed in the chamber at an initial temperature of 20°C, 60% RH, and a 12-h photoperiod. The initial temperature and photoperiod corresponded to the average monthly conditions in Blacksburg, Virginia in September. These conditions were changed weekly to simulate mean local conditions over the winter season (Herrick et al. 2011). Every week the temperature was reduced by 2°C and every 4 weeks the light period was decreased by 1 h until week 10 where the lowest exposure conditions reached 2°C and continued through week 11. At week 12, temperature was then increased by 2°C and photoperiod increased by 1 h every 4 weeks. By February, conditions were the same as

initially set and remained constant through March. Roots from each plant were carefully recovered from the soil and rinsed with tap water.

Several primary and secondary roots from each plant were removed and cut into 1-cm sections from oldest to youngest growth. Confirmation of infection and colonization was determined by plating root sections and isolating *V. albo-atrum* using the method described above. This experiment evaluated the quantity of root sections colonized by *V. albo-atrum* and also provided a location to predictable colonized areas. These roots were cut in similar sections and were soaked in chloral hydrate for 24 h, to clear root tissues (Wick 2009). After 24 h, roots were sliced into thin sections and examined for the origin of *V. albo-atrum* propagules present in the roots.

2.4. Results

2.4.1 Preliminary survey of *V. albo-atrum* in Virginia.

Seven sites were sampled in Virginia for *V. albo-atrum*. Samples from two of these sites were collected by local foresters, and two sites were reported by email from local arborists. All samples at these four sites, tested negative for *V. albo-atrum*. At one site, *V. dahliae*, was isolated. The cause of symptoms from the other three sites was unknown. Three other sites were located from surveys of over 400 km primary and secondary roads in southwest Virginia. One site was positive for *V. dahliae* and two were positive for *V. albo-atrum* (Figure 2.1), which were measured for forest stand composition.

One positive site for *V. albo-atrum* was located at 37°24'158" N latitude and 080°42'158" W longitude, along Interstate-81 North between mile markers 125.7–125.8. The area is 36.5 m x

18.3 m (l x w) with approximately 305 canopy TOH, mean 7.5 cm diameter at breast height (DBH). Most of the TOH in this stand were severely defoliated. Other trees species in the stand included: one paulownia [*Paulownia tomentosa* (Thunb.) Steud.] 5 cm DBH, three boxelder (*Acer negundo* L.) mean 10 cm DBH, four redbud (*Cercis canadensis* L.) mean 7 cm DBH, and two black locust (*Robinia pseudoacacia* L.) mean 7 cm DBH. The total BA of TOH was 20.2 m² hectare⁻¹. Six vascular samples were collected and returned to the laboratory, where *V. albo-atrum* was isolated from all trees.

The second *V. albo-atrum* site was located at 37°37'947" N latitude and 079°70'853" W longitude near a railroad crossing in Montvale, Virginia. The area is 76 m x 15 m (l x w) and included approximately 91 symptomatic canopy TOH and 76 dead TOH (mean 15 cm DBH). Five vascular samples were collected at this site, and *V. albo-atrum* was isolated from all samples. Other tree species were growing in this stand, but TOH was the dominant or co-dominant species. Other tree species included: 10 Eastern cottonwood (*Populus deltoides* Bartr. ex Marsh.) mean DBH 15 cm, 25 black cherry (*Prunus serotina* Ehrh.) mean 19 cm DBH, 2 black locust mean 20 cm DBH, 1 pignut hickory (*Carya glabra* Mill.) 3 cm DBH, and 177 boxelder mean 5 cm DBH. The total BA of TOH was 26.3 m² hectare⁻¹.

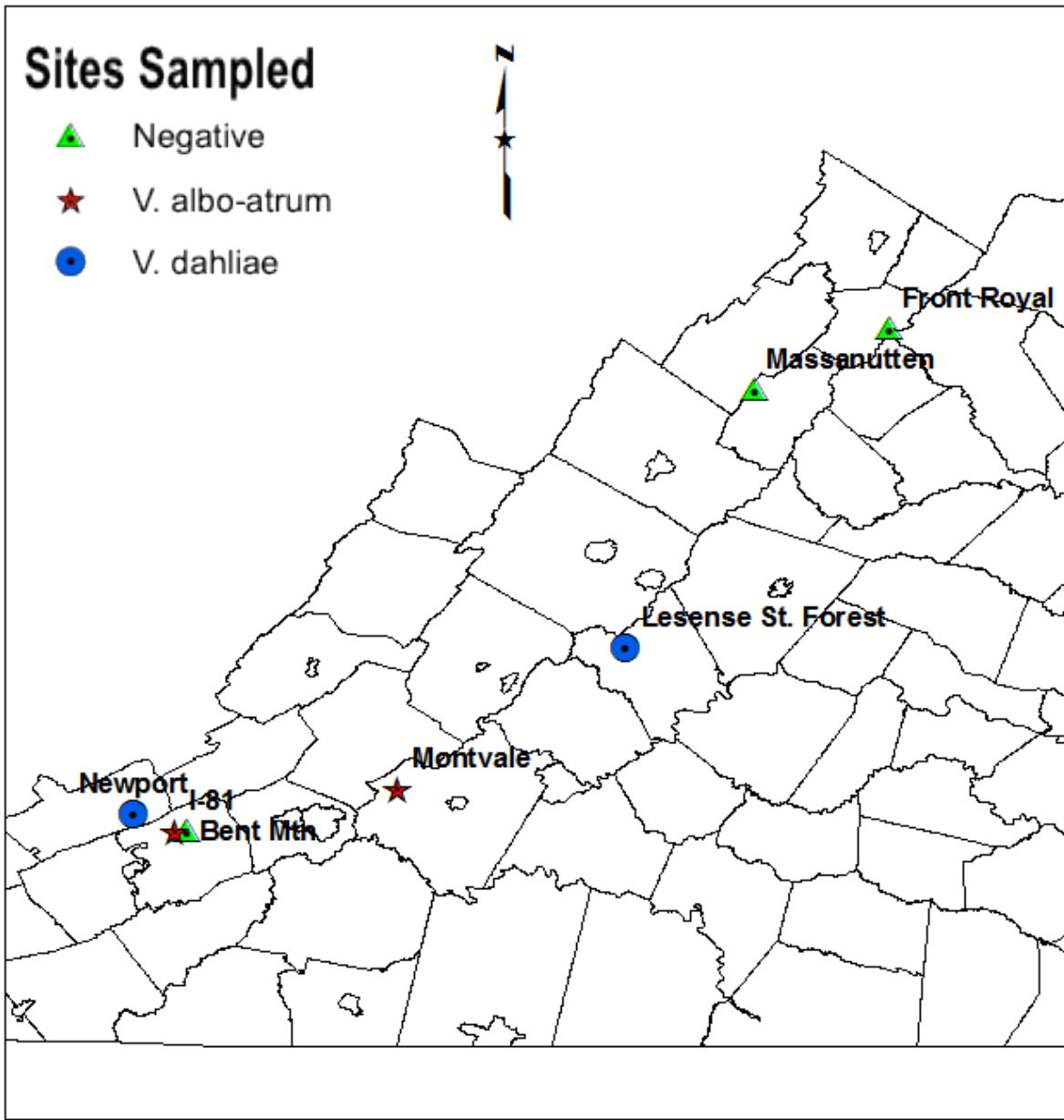


Figure 2.1 Potential *V. albo-atrum* infected stands sampled in Virginia (ArcGIS 10 and ArcMap 10 software from ESRI 2011).

2.4.2 Virulence testing.

The overall rank test was significant ($\chi^2 = 36.97$, $df = 2$, $P < 0.0001$) and isolates, PSU 140 and VA 100 had a significantly greater disease severity compared with the control ($W = 324$ $df = 2$; $p < 0.0001$). However, there were no significant differences found in disease severity between the two *V. albo-atrum* isolates ($W = 140.5$ $df = 2$; $p = 0.51$). Inoculated PSU 140 and VA 100 seedlings quickly developed symptoms at 2 weeks post inoculation with a rapid increase in disease severity between 4 and 7 weeks. Defoliation occurred at week 6, and all seedlings were dead by week 9. All control plants were found non-symptomatic each week (Figure 2.2). These results indicate the degree of pathogenicity between the two isolates was similar.

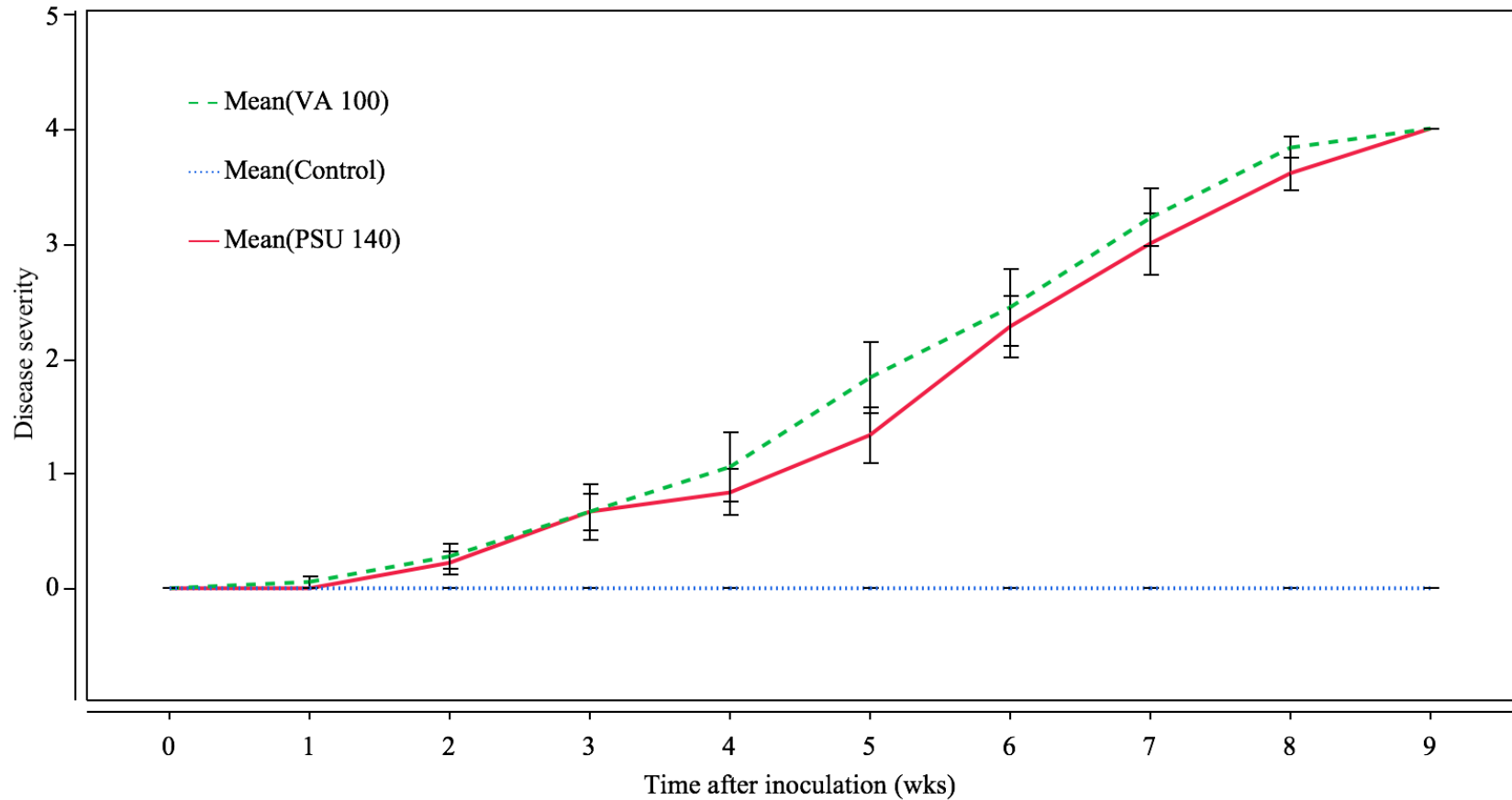


Figure 2.2 Mean disease severity \pm standard error of potted TOH seedlings at various times after 0.2 mL stem inoculations with sterile distilled water, PSU 140, or VA 100 *V. albo-atrum* suspensions adjusted to 1×10^8 propagules mL^{-1} . Disease severity was rated based on the natural progression of disease symptoms; 0 = non-symptomatic leaves, 1 = wilting leaflets, 2 = chlorotic/necrotic leaflets, 3 = defoliating leaves, and 4 = seedling mortality.

2.4.3 Colonization of TOH roots by *V. albo-atrum*.

Four months after inoculation (September) all stem sections tested positive for *V. albo-atrum*, confirming that TOH saplings had become infected. Rhizosphere soil samples for each infested potting mix averaged 1.3×10^2 , 6.7×10^2 , and 5.2×10^3 *V. albo-atrum* CFU g⁻¹. Four (11%) of 37 root sections plated for colonization were found to be positive for *V. albo-atrum*. Primary roots removed from the oldest growth were most commonly colonized; 15 root sections were taken from this location for examination. However, *V. albo-atrum* was not observed in any of the mounted sections.

2.5. Discussion

Tree of heaven can increase nutrient availability (pH, exchangeable Ca, exchangeable K, total N) and cycle rate (net nitrification) in surface soil. This is most likely due to extraordinarily high nutrient concentration in its leaf litter, estimated to be four times higher than native tree species (Gómez-Aparicio and Canham 2008). As the leaf litter from TOH increases the available nutrients in the surrounding soils, ideal areas for new generations of TOH through prolific seed production and root sprouting are created. In addition, only trees resistant to the phytotoxic quassinoids produced by TOH, such as ailanthone, are able to utilize these fertile soils. Gómez-Aparicio and Canham (2008) found *Acer rubrum* L. to be one of these species. Interestingly, the increase in abundance of *A. rubrum* in the eastern United States has been one of the most widespread changes in forest composition in the last century (Abrams 1998, Larsen 1953). However, ailanthone is quickly degraded in the soil. Heisey (1996) found ailanthone applied to non-sterile soils lost its toxic effects on plant growth after just 5 d and Gómez-Aparicio and Canham (2008) did not find any allelopathic effects on native seedling survival after 2 years in

forest plots. This implies that removal of a TOH stand, followed by degradation of ailanthone, could have a positive effect on native seedlings due to increases in soil fertility.

This may explain the characterization results at the Montvale site. It appears that before *V. albo-atrum* infested the site, the growth of boxelder was suppressed. After the infestation and recent mortality of canopy TOH, numerous boxelder seedlings as well as many TOH seedlings were beginning to re-establish. Schall (2008) found similar re-establishment at a *V. albo-atrum* infested TOH site in Pennsylvania. As canopy TOH died from wilt at the site, the canopy gaps were filled with TOH seedlings, which lived for only 1–2 years before they were killed by soil-borne *V. albo-atrum*. Tree species tolerant to natural infections by *V. albo-atrum* included American basswood (*Tilia americana* L.), American elm (*Ulmus americana* L.), black ash (*Fraxinus nigra* Marsh.), black gum (*Nyssa sylvatica* Marsh.), black locust, butternut (*Juglans cinerea* L.), chestnut oak (*Quercus prinus* L.), northern red oak (*Quercus rubra* L.), red maple, sassafras [*Sassafras albidum* (Nutt.) Nees.], shellbark hickory [*Carya laciniosa* (F. Michx.) Lould.], sweet birch (*Betula lenta* L.), white ash (*F. americana* L.), witch-hazel (*Hamamelis virginiana* L.), and yellow-poplar (*Liriodendron tulipifera* L.). These would likely fill the niche left by the dying TOH seedlings (Schall and Davis 2009b).

The discovery of *V. albo-atrum* at two infested TOH sites in southwestern Virginia was unexpected. Although *V. albo-atrum* was reported on TOH in Virginia over 60 years ago (Weiss and Muriel 1950-1953), little information was collected at the time. Fowler (1937) reported *Verticillium* wilt on TOH in Roanoke, Virginia, however, the species of *Verticillium* was not identified. Gravatt and Clapper (1932) reported *V. albo-atrum* infecting a prized smoke tree

(*Cotinus coggyaria* Scop.) in Mount Vernon, Virginia. However, at this time (1932) many researchers did not distinguish *V. albo-atrum* from *V. dahliae*. As a result of this discovery of *V. albo-atrum* on TOH in southwestern Virginia, it is important to determine how widespread this pathogen is on TOH in the rest of Virginia and the surrounding states.

With cooperative efforts from the Virginia, South Carolina and North Carolina Departments of Forestry and Virginia Tech, starting in June 2011, there will be a multi-state survey conducted to document naturally-occurring *V. albo-atrum* infections on TOH stands. The survey will include initial sampling from symptomatic stands in these states to locate isolated pockets of infection. The following year, 2012, a systematic survey will be conducted to identify the spatial distribution of each pocket. By understanding the natural distribution and location of *V. albo-atrum* on TOH, we can better understand this pathogen's potential as a biological control agent (Snyder 2010).

During a preliminary seedling inoculation trial, wilt of the plant was always observed as the first symptom, sometimes followed by chlorosis or necrosis, or proceeding directly into defoliation, and eventually death. There were slight differences in symptom order from Bejarano-Alcazar et al. (1996) and Schall and Davis (2009a) possibly due to a difference in facilities used to grow the seedlings. Regardless of symptom order, the high pathogenicity of *V. albo-atrum* TOH seedling inoculations was consistent with results of Schall and Davis (2009a). It is likely that PSU 140 and VA 100 are the same strain of *V. albo-atrum*, which is highly pathogenic on TOH. However, more work including genetic sequencing of the DNA of each isolate is needed to verify this conclusion.

There have been numerous publications regarding host colonization and pathogen dissemination of a similar *Verticillium* species, *V. dahliae*, and it is likely *V. albo-atrum* characteristics could be similar. Menzies (1970) reported that a 2.5-cm potato stem section contained 20,000 to 50,000 viable *V. dahliae* microsclerotia, which do not reach their full inoculum potential until host tissue decomposes, freeing them from tissues. It is estimated that soil counts could increase 1–2 years after death of the host plant, due to inoculum becoming individual units rather than remaining in clusters (Bruehl 1987). Colony clustering was also identified by Huisman (1988) in distribution studies of *V. dahliae* in cotton (*Gossypium hirsutum* L.) roots. Huisman also reported that inoculum densities in soil samples decreased with soil depth from 32.0, 7.9, 1.8, 1.5 and 0.9 microsclerotia g⁻¹ of soil at 0–15, 15–30, 30–60, 60–90, and 90–120 cm depths, respectively, and found a significant decrease in colonization of cotton roots as temperature increased, 3.8, 2.7, 5.2, 5.1 colonies cm⁻¹ of root (x 10⁻²) at 31°, 28°, 23°, 20° C, respectively. In another experiment, Huisman (1988) found 115 positive *V. dahliae* root sections from a total of 795 root sections (14.5%) collected from the area of highest inoculum density (top 30 cm of soil). Our results of 4 positive root sections from of a total of 37 (11%) were similar to Huisman (1988) even when temperature influence and soil depths were not considered.

Termorshuizen et al. (1998) found naturally infested soil samples differed widely in the content of *V. dahliae*, averaging 1.1 to 120 CFU g⁻¹ of soil. Other studies found *V. dahliae* could persist in soil in quantities of 100–300 (Evans et al. 1967) and 100–1200 (Menzies 1970) CFU g⁻¹ of soil. Our potting mix samples were higher for *V. albo-atrum* propagules compared with previously studied *V. dahliae* soil samples. One reason may be related to the fact that we

collected rhizosphere potting mix samples, which contained high amounts of roots. Schroth and Hendrix Jr. (1962) found *Fusarium solani* f. sp. *phascoli* propagules were nearly two times greater in rhizosphere soil samples compared with nonrhizosphere samples.

Most vascular pathogens initiate infection in the plant by entering through various natural openings or artificial wounds. It is estimated that more than 20% of the smaller parts of the root system of a healthy plant may be damaged *in situ* by mechanical damage from rock particles or soil microorganisms. This allows direct entry of the pathogen into the vascular system (Pegg 1985). After entry, *Verticillium* spp. are confined to the fluid environment of the xylem vessel and rarely leave until death of the surrounding tissues (Pegg and Brady 2002). Because high quantities of propagules were observed existing in the potting mixes, new infections within a stand could result from close root-to-root contact or root grafting of TOH (Lewis and McCarthy 2008). It has been documented that *V. albo-atrum* is capable of overwintering in some perennial hosts (Fradin and Thomma 2006), and in infected TOH canopy trees (Schall 2008). This study also confirms *V. albo-atrum* is able to overwinter in potting mix and remain viable in roots.

The discovery of *V. albo-atrum* killing TOH in Virginia provides evidence that biological control using this fungus may be possible in Virginia. These small sites found may eventually spread, acting similarly as the sites observed in Pennsylvania (Schall and Davis 2009b). The high pathogenicity on TOH of the two isolates from Virginia and Pennsylvania, further suggests similar biocontrol results may be achieved. In lab studies, we found that *V. albo-atrum* colonizes TOH roots and can overwinter in potting mix. In addition rhizosphere potting mix samples contained high amounts of propagules. This suggests dissemination could have been achieved at

the Virginia sites through root grafting or contact. After TOH was killed by the pathogen at one Virginia site, canopy gaps formed and otherwise suppressed native species were able to take advantage of the fertile soils left by TOH and begin to re-establish. Due to the success and high host specificity seen with *V. albo-atrum* PSU 140 in Pennsylvania and the wide distribution of TOH in Virginia, the release of a second biological control agent in the form of an herbivorous insect, to aid in fungal dissemination may lead to a novel solution for control of TOH in Virginia.

Chapter 3. Assessing *Eucryptorrhynchus brandti* as a potential carrier of *Verticillium albo-atrum* to *Ailanthus altissima*, in laboratory assays

Abstract

Two potential biological control agents for the invasive tree of heaven (TOH), *Ailanthus altissima* (Mill.) Swingle, have been extensively studied: a vascular wilt fungus, *Verticillium albo-atrum* Reinke & Berthold, and a host-specific weevil from China, *Eucryptorrhynchus brandti* Harold (Coleoptera:Curculionidae), which is currently pending quarantine release. In 2002, *V. albo-atrum* was observed in Pennsylvania causing significant mortality to TOH. This fungus is highly pathogenic to TOH. Quarantine experiments were conducted to test different forms of transmission with *E. brandti* and *V. albo-atrum* simultaneously. In one experiment, 75% (n = 32) of adult *E. brandti* transmitted *V. albo-atrum* to TOH seedlings after walking on an actively growing culture and feeding on infected plant material. In another study 16.7% (n = 120), 15.0% and 12.5% of adult *E. brandti* ingested and passed viable *V. albo-atrum* propagules into feces after feeding on infected TOH stems for 24, 48 and 72 h, respectively. Surviving weevils (83%, n = 20) overwintering in naturally infested potting mix carried viable *V. albo-atrum* propagules externally. In addition, all weevil progeny that emerged from infected TOH billets appeared to be as healthy as weevils reared from non-infected billets and wild parents from China. Results from these laboratory studies indicate *E. brandti* has the ability to spread *V. albo-atrum* from tree to tree in a laboratory setting.

3.1. Introduction

Ailanthus altissima (Mill.) Swingle is native to China, Taiwan, Japan, and India (CSIR 1985) and is a widespread invasive species found throughout much of the continental United States. Tree of heaven was imported to the United States as a shade tree from Europe in the 18th century and again in the mid-19th century when it was planted for medicinal purposes by Chinese laborers during the California gold rush (Tellman 1997). Tree of heaven is a serious threat to native ecosystems because it is a strong competitor as it produces allelopathic chemicals that inhibit native tree growth. In addition, there are few herbivores that impact its health and reproduction. Control of TOH is a large economic and agricultural concern because of its invasiveness and lack of conventional control methods to combat it (Kok et al. 2008).

Tree of heaven is a fast growing, dioecious tree capable of producing as many as 300,000 viable seeds per year (Sheppard et al. 2006). Each seed is light and ideal for wind or water dispersal for great distances from the parent tree (Landenberger et al. 2007). Tree of heaven also spreads vigorously through asexual sprouting from roots and stumps (Hu 1979), often resulting in dense thicket growth. The tree produces weak wood unsuitable for most commercial uses and prone to breaking, making TOH dangerous in recreation areas and roadsides. It also produces several bitter chemicals or quassinoids, which have phytotoxic activity to many native forest trees (Mergen 1959). Tree of heaven can often be seen growing along roadsides, newly harvested forest areas, agricultural land and other disrupted habitats. It is shade intolerant and can grow in dry, poor soils (Ding et al. 2006). Currently, TOH is present in 41 of the lower 48 states and has been declared a noxious weed in Connecticut, Massachusetts, New Hampshire and Vermont

(USDA-NRCS 2011). In Virginia TOH is common along Interstate-81 and occupies hundreds of acres in the Shenandoah National Park (Marler 2000).

Mechanical control of TOH is expensive and labor intensive because the entire root system has to be removed to achieve complete control. Physical removal alone will cause an increase in root sprouts and stand density. Therefore, additional herbicide treatment is needed to achieve complete eradication. However, all chemical treatments available are general-use herbicides that often kill more than the target plant species, leaving the treated area barren and ideal for TOH re-intrusion. Long-term removal of TOH is taxing, expensive and requires constant follow-up monitoring and additional treatments for new growth (Kok et al. 2008). These reasons make TOH a suitable species for biological control.

There were 46 phytophagous arthropod species associated with TOH in China (Ding et al. 2006). Most are defoliators and do not impact the growth of TOH, and few attack trunks and branches. Two Curculionidae species, *E. brandti* and *Eucryptorrhynchus chinensis* Oliver (Coleoptera), are major pests of TOH in China and are thought to feed exclusively on TOH (Ge 2000). In some areas of China, 80–100% of TOH were attacked by *E. brandti* and *E. chinensis* and 12–37% of those trees died (Ding et al. 2006, Ge 2000). Although the insects could have caused enough damage to kill entire trees, additional secondary factors could have been involved such as stress and disease.

Importation of *Eucryptorrhynchus* spp. from China to the United States have provided material for the studies of general biology, development, and most efficacious rearing method at Virginia

Tech Beneficial Insect Quarantine laboratory. *E. brandti* seems to be highly host-specific in feeding and adult oviposition tests (Herrick 2011). Adult *E. brandti* feeds on leaves, stems and petioles and larvae feed under the bark, destroying cambial tissues (Ding et al. 2006). Larvae overwinter under the bark and adults overwinter in soil near the host plant (Ge 2000). Females oviposit eggs under the bark in the basal area of the trunk (≤ 3 m) (T. McAvoy, personal communication, Department of Entomology, Virginia Tech) where the larvae feed until they become adults and emerge through small exit holes on the bark (Kok et al. 2008). In addition to causing cambial damage, adult exit holes may increase tree susceptibility to pathogen infection.

Several wilting stands of TOH have been observed in south-central Pennsylvania. Schall and Davis (2009a) concluded that *V. albo-atrum* PSU 140 (GenBank accession # FJ424082) and *Verticillium dahliae* Kleb. PSU 154 (GenBank accession # FJ424083) were the causal agents of the wilt. Inoculation studies have shown that TOH is susceptible to both *V. albo-atrum* and *V. dahliae* in greenhouse and field canopy studies. However, *V. albo-atrum* PSU 140 is much more aggressive and pathogenic, making it more suitable as a potential biocontrol agent (Schall and Davis 2009a). From 2000 to 2008, nearly 10,000 TOH host trees died mainly as a result of *V. albo-atrum*. Host-range susceptibility tests suggests that *V. albo-atrum* PSU 140 may be a low-risk biological control agent for TOH (Schall and Davis 2009b).

Verticillium albo-atrum conidia and hyaline hyphae can survive in the soil for 3–4 weeks while the melanized mycelium can remain dormant from 9 months to 4 years depending on soil conditions (Pegg and Brady 2002). Insects are able to transport spores from one place to another directly using specialized structures or indirectly through contact by legs or snout (Kok and Abad

1994). Fungal associations with wood-boring weevils are closely connected with inner bark feeding and are often contaminated with fungal spores internally from ingestion and externally after emerging from pupal galleries (Lieutier 2004).

If an herbivorous insect carrying a pathogen can breach barriers of a host plant such as intact bark, it may be able to inoculate the plant and cause a new infection. Since *V. albo-atrum* occurs naturally on TOH, releasing a possible long-range insect carrier may spread the pathogen to stands that are unreachable for short-range transmission. Although no studies have been conducted on the dispersal and carrying capabilities of *E. brandti*, it is likely that it would act like other curculionids. Due to the aggressiveness and high pathogenicity already seen with *V. albo-atrum* PSU 140 and the wide distribution of *E. brandti* on TOH in China, the manipulation of an insect-pathogen combination might lead to a novel solution for the control of TOH.

The purpose of this study was to attempt to predict the relationship by incorporating a second biological control agent, *E. brandti*, to aid in *V. albo-atrum* dissemination for control of TOH. Experiments were conducted to test different forms of transmission with *E. brandti* and *V. albo-atrum* simultaneously.

3.2. Materials & Methods

Any experiment that required the use of *V. albo-atrum* was performed using the Pennsylvania isolate, PSU 140 GenBank accession # FJ424082 (D. Davis, personal communication, Department of Plant Pathology, Penn State) and were conducted under controlled conditions at the Virginia Tech Beneficial Insects Quarantine Laboratory, Price's Fork Research Station.

3.2.1 Isolation of *V. albo-atrum* from TOH.

(See Chapter 2)

3.2.2 Infecting TOH stems.

All TOH saplings approximately 2-cm diameter at breast height (DBH) were removed from healthy TOH stands near Radford, Virginia. Saplings were replanted in 25-cm dia pots filled with Sta-Green® Nursery Blend Tree and Shrub Planting Mix (0.09:0.06:0.05% N:P:K) and grown under greenhouse conditions for a minimum of 3 weeks. Plants were transported to the Virginia Tech Beneficial Insects Quarantine Laboratory, where they were maintained in a growth room at 25°C, 60% RH, under a 14-h photoperiod using natural and fluorescent lights. Saplings were inoculated using a sterile syringe with *V. albo-atrum* conidial suspension PSU 140 (Chapter 2) at two points on the stem, both 5 cm above the soil line (Presley 1966). Wounds were sealed with parafilm. Inoculated plants were watered twice a week for at least 8 weeks post-inoculation. Confirmation of infection was determined by isolating *V. albo-atrum* from a 1 cm stem section as stated above.

3.2.3 Measuring carrying ability and transmission of *V. albo-atrum* by *E. brandti*.

Tree of heaven seeds were collected from healthy TOH in Blacksburg, Virginia and potted in 15-cm pots with Sta-Green® Nursery Blend Tree and Shrub Planting Mix and kept in the University greenhouse for at least 12 weeks. Seedlings were watered three times a week and moved to the quarantine lab when needed. A cage was created for each weevil by stapling window screen (46 x 41 mesh count cm⁻¹, wire diameter 0.279 mm) around a 5-cm stem section on a potted TOH seedling. Individual weevils were placed in each cage to feed and secured with two plastic-

coated wires on each side. After 72 h the weevils were removed to prevent plant girdling from excessive feeding. The seedlings were kept in a room at 25°C, 60% RH, under a 14-h photoperiod using natural and fluorescent lights. All seedlings were watered twice a week for 3 weeks until harvest. At harvest, the cage was removed, each stem section cut in half longitudinally, and plated for isolation of *V. albo-atrum* using methods described above. After the weevil was removed from each seedling, it was placed in a sterile 1.5 µL centrifuge tube with 500 µL of sterile distilled water and agitated with a vortex mixer for 30 s. The weevil was removed from the tube using sterile forceps and the water solution was spread evenly on a KSM plate and air-dried in a biological safety cabinet. Fungal colonies were transferred to PEA, and subcultures were examined for *V. albo-atrum*. Komada's selective medium plates were then recounted for number of *V. albo-atrum* colony forming units (CFU) per weevil.

3.3. Experiments

3.3.1 Assessing transmission of *V. albo-atrum* from feeding and tarsal contact of *E. brandti*.

This experiment was performed to determine if *E. brandti* could carry and infect healthy TOH seedlings with *V. albo-atrum* using two passive transmission dispersal methods. The success of these methods would provide valuable information for future releases of *E. brandti* and *V. albo-atrum* simultaneously. It was hypothesized that transmission is independent of dispersal method and sex.

Adult *E. brandti* were lab-reared from TOH billets using the *E. brandti* rearing method developed by Herrick et al. (2011), where the most efficient adult rearing involved caging 12 males and 12 females for 7 d on 23–92 cm long TOH billets. Seventy-six adult *E. brandti* were

separated into four 0.5-L containers: container I (18 males), container II (18 females), container III (20 males) and container IV (20 females). The bottom of each container was amended with a 5-cm radius fine polyester mesh (Sefar™) to increase ventilation. *Verticillium albo-atrum*-infected TOH stems, using the method described above, were provided as food for container I (males) and container II (females) for 72 h. Container III (males) and container IV (females) included non-infected TOH stems as food. Dead weevils were removed from each container after 72 h. After feeding, 16 live weevils from each container were randomly selected and each one was placed on a 7-d old, actively growing culture of *V. albo-atrum* on a PEA plate. This type of handling usually induced thanatosis, and once broken, the weevil would immediately begin walking. The weevil was allowed to walk across the culture for 5 min total. If the weevil walked off the plate, it was picked up with sterile forceps and placed back on the culture until 5 min was complete. Transmission of *E. brandti* to TOH seedlings was measured for each weevil using the method described above. The remaining live weevils from container III (males) and container IV (females) (six total) that fed on non-infected TOH stems for 72 h were used as controls. The controls served only to confirm that *V. albo-atrum* was not transmitted to the seedlings by an extraneous source and was not analyzed statistically. Confirmation of *V. albo-atrum* adherence to tarsi was conducted by allowing additional weevils to walk across the culture. Their legs were then removed, mounted, coated with gold–palladium and examined using a FEI Quanta 600 FEG Scanning Electron Microscope.

The experiment used a completely randomized design and consisted of male (n = 3) and female (n = 3) controls, male (n = 16) and female (n = 16) tarsal contact/infected feeding, and male (n = 16) and female (n = 16) tarsal contact/non-infected feeding. *Verticillium albo-atrum* transmission

was recorded for each individual as a binomial response (non-infected seedling = 0, infected seedling = 1). Fisher's exact tests were used to test independence between sex for each dispersal method ($\alpha < 0.05$). If sex was found to be independent, data were combined and Pearson's Chi squared test used to determine independence between dispersal methods ($\alpha = 0.05$) (JMP 1989–2009). Percent successful TOH transmission was recorded for each dispersal method.

3.3.2 Assessing carrying ability and propagule quantity from *E. brandti* feces in *V. albo-atrum* feeding tests.

The purpose of this objective was to test the interior transport capabilities of *E. brandti* after feeding on *V. albo-atrum*-infected TOH stems using two experiments. The first experiment was conducted to assess the percentage of weevils able to ingest *V. albo-atrum* through infected TOH stems and pass viable propagules into fecal pellets at different feeding durations. It was hypothesized that carrying ability will be independent of feeding duration and sex. The second experiment examined the quantity of viable *V. albo-atrum* propagules present in feces after different feeding durations. It was hypothesized that the quantity of *V. albo-atrum* propagules recovered from feces will be the same for each feeding duration.

Experiment 1: Proportion of individuals passing *V. albo-atrum* through feces.

All adult *E. brandti* used were collected in China by Dr. Du Yuzhou, Yangzhou University, the People's Republic of China (PRC) and shipped to the Virginia Tech Beneficial Insects Quarantine Laboratory. One TOH sapling was infected with *V. albo-atrum* using the method described above. The stem of the sapling was cut into 10 cm sections and divided evenly into two 0.5 L containers. Forty male and forty female adult *E. brandti* were starved for 24 h, and placed into the containers by sex to feed for 24 h. Ten live weevils were randomly chosen from

each of the two containers, surface disinfested for 10 s using 10% bleach, and immediately flushed for 30 s with sterile distilled water to ensure transport by exterior means would be excluded. Each weevil was transferred into a separate sterile Petri dish with no food for 24 h to defecate. This process was repeated twice to allow the remaining weevils in the containers to feed on the same infected TOH stem sections for 48 and 72 h. Extra weevils were included in the containers to account for mortality. Fecal pellets from each Petri dish were collected using sterile forceps and plated directly on KSM, subcultured to PEA, and examined for *V. albo-atrum*. *V. albo-atrum* carrying ability was recorded for each individual as a binomial response (*V. albo-atrum* not present in any fecal pellets = 0, *V. albo-atrum* present in at least one fecal pellet = 1).

The experiment used a randomized complete block design, blocked by food (TOH infected saplings) and consisted of 24, 48 and 72 h feeding duration treatments for males and females. The experiment was replicated six times for a total of 60 male and 60 female weevils for each feeding duration. A generalized linear model was used to analyze the binomial data. Pearson's Two-tailed Chi Square test ($\alpha = 0.05$) was used to determine independence (JMP 1989–2009). Feeding duration and sex were independent variables, and number of carriers was the dependent variable. Percent of *E. brandti* carriers was recorded.

Experiment 2: Quantity of *V. albo-atrum* in feces.

The quantity of *V. albo-atrum* CFU g⁻¹ of feces was assessed for each time increment. This experiment followed a similar experimental design as stated earlier, except each group of 10 weevils removed from the container at specified feeding durations was placed collectively in one Petri dish. This allowed for a larger fecal sample to be collected and quantified. In addition, male and female control groups were included that fed on non-infected plant material during the same

feeding durations. The controls served only for confirmation that *V. albo-atrum* was not present in feces by an extraneous source and were not analyzed statistically. Fecal pellets from each Petri dish were collected using sterile forceps, placed into a sterile 1.5 mL centrifuge tube, and dried for 24 h in a biological safety cabinet. Each sample was weighed (dry weight), 500 μ L of sterile distilled water added, and crushed using a sterile micropipette tip. The sample was then agitated using a vortex mixer for approximately 3 min to obtain a semi-homogenous solution. The solution was evenly spread on a KSM plate and colonies transferred to PEA. Colonies on KSM plates were then recounted for *V. albo-atrum* and calculated for CFU g^{-1} of feces. Any sample with zero propagules recovered was recorded separately and not used in analysis.

This experiment used an unbalanced randomized block design, blocked by food (infected TOH saplings) and consisted of feeding duration treatments of 24, 48 and 72 h for both male and female weevils. This experiment was replicated six times for a total of six male fecal samples and six female fecal samples per feeding duration. The control was replicated three times, for a total of three male fecal samples and three female samples for each duration. Independent variables were sex and feeding duration, and the dependent variable was quantity of CFU g^{-1} of feces. Quantity of feces was normalized using the logarithmic transformation, which was confirmed by Shapiro–Wilk W goodness–of–fit test. Variances were tested for equality using Levene’s test. Maximum likelihood estimations (standard least squares fit) were used to analyze data. Analysis of variance (ANOVA) and Tukey’s HSD comparisons were used to detect significant differences ($\alpha = 0.05$) due to treatment means (JMP 1989–2009)

3.3.3 Assessing emerging adult *E. brandti* health, carrying ability and transmission of the fungus from *V. albo-atrum*-infected billets.

The weight of emerging adult *E. brandti* from *V. albo-atrum* infected billets was determined by measuring weights of male and female progeny and comparing them with randomly selected parent *E. brandti* from China and adult progeny reared on non-infected billets. In addition, the quantity of *V. albo-atrum* propagules present on the exoskeleton and transmission to a TOH seedling were measured for *V. albo-atrum* reared progeny only. It was hypothesized that male and female parent weevils from China and weevils reared from non-infected billets will have the same weight as male and female progeny weevils reared on *V. albo-atrum* infected billets.

In late summer 2010, three non-infected and four *V. albo-atrum*-infected TOH canopy trees (mean DBH=19 cm) were felled. Three of the infected trees were cut from Michaux State Forest, Pennsylvania and the other was cut in Montvale, Virginia. All trees were naturally infected with *V. albo-atrum*, were alive at the time of felling, and were at least 50% defoliated. Non-infected trees served as controls and were cut in Dublin, Virginia. Each infected tree was cut into 5 billets and control trees were cut into two billets, 46-cm long and were placed in the quarantine laboratory. Isolation of *V. albo-atrum* was verified by plating a wedge of discolored vascular tissue from each tree using the method described above. Billet tops were waxed with paraffin and billet bottoms were left unwaxed and were kept in a 120 x 35 x 2.54 (l x w x d) cm galvanized metal water trough to prevent desiccation. All billets were stored in a cold room at 12°C for no more than 30 d until needed. One billet from each tree was set-up in a separate fiberglass, rearing chamber approximately 61 x 30.5 x 30.5 (l x w x d) cm every 7 d. The rearing chamber was designed for rearing *E. brandti* from TOH billets in quarantine (Herrick et al. 2011).

Eucryptorrhynchus brandti collected in China in June 2010 were delivered to the quarantine laboratory. Fifty live weevils were randomly chosen, weighed, and identified to sex. These weevils represented the P₁ comparison cohort. Random groups of 10 female and 6 male weevils of varying ages were placed on each billet in the rearing chambers for 7 d to oviposit. Adult density was based on maximum number of weevils available from the shipment and laboratory. Tree of heaven leaves and stems were provided as the food source in each chamber bi-weekly. After 7 d, all weevils were removed and placed into two 0.5-L containers based on their exposure to infected or non-infected billets. Deceased weevils were replaced by live ones of the same sex. Weevils were then re-randomized into groups at the same density to be used as parents on the next set of billets. This process was repeated for all 26 billets.

After billets were exposed to *E. brandti*, they were removed from the chamber and loosely wrapped with window screen (46 x 41 mesh count cm⁻¹, wire diameter 0.279 mm), which was stapled to create a closed sleeve for emerging adults. The bottom of the sleeve was secured around the base of the billet with a cord and the top of the sleeve was gathered and secured with a plastic-coated aluminum wire above the billet. Billets were placed in similar galvanized metal water troughs as described earlier and maintained in an emergence room. The room was kept at 25°C, 60% R.H., under a 14-h photoperiod, using natural and fluorescent lights. These conditions were chosen because they were successful in rearing the initial shipment of weevils from China and similar to local ambient conditions (Herrick et al. 2011). Water troughs were cleaned bi-monthly and water replaced as needed. Billets were checked daily for emerged adult progeny.

Each emerged adult was weighed, and sex was recorded. Weevil progeny from infected billets were also measured for carrying ability and transmission using the method described above. *Verticillium albo-atrum* transmission to a TOH seedling was recorded for each individual as a binomial response (non-infected seedling = 0, infected seedling = 1) and carrying ability of *V. albo-atrum* on the exoskeleton of each weevil was recorded as a quantity of CFU per weevil using the method described above.

This experiment used a completely randomized design and consisted of male and female adult *E. brandti*, parent weevils from China, progeny from infected *V. albo-atrum* billets and progeny from non-infected billets. The dependent factor was weight. Data were normalized using the logarithmic transformation, which was confirmed by Shapiro–Wilk W goodness–of–fit test and variances were tested for equality using Levene’s test. Analysis of variance and Tukey’s HSD test was used to detect significant differences ($\alpha = 0.05$) due to treatment mean (JMP 1989–2009). Sex ratios were recorded for both parents and progeny.

3.3.4 Measuring transmission of *V. albo-atrum* by *E. brandti* overwintering in infested potting mix.

Fungal transmission and carrying ability were measured for overwintering live and dead *E. brandti* placed in naturally infested potting mix. It was hypothesized that carrying ability and transmission by live and dead overwintering *E. brandti* in infested potting mix will be the same.

Infested potting mix used for this experiment was described in Chapter 2. Weevils were added to the potting mix prior to overwintering. Adult *E. brandti* were collected in China in September

2010 and shipped to the quarantine laboratory. Ten male and ten female weevils were randomly selected, separated by sex, and placed in each of the three pots. All stems from infected plants were cut to the soil line so weevils could not feed on them, eliminating the possibility of internal exposure. Pots were caged with window screen, secured with tape around the middle of the pot, and a plastic-coated wire around the gathered material above the top of the pot. Tree of heaven stems were provided as the food source for the weevils and the soil was kept moist by adding water through the bottom saucers once a week.

As weevils broke quiescence, they were removed from the pot and measured for *E. brandti* fungal carrying ability and transmission using the method described above. After all live weevils broke quiescence, pots were examined for remaining deceased weevils that were also measured for carrying ability. Carrying ability of *V. albo-atrum* on the exoskeleton of each weevil was recorded as quantity of CFU per weevil and any sample with zero propagules recovered was recorded separately and not used in analysis. *V. albo-atrum* transmission to a TOH seedling was recorded for each individual as a binomial response (non-infected seedling = 0, infected seedling = 1).

This experiment used an unbalanced randomized block design, blocked by infested potting mix, and consisted of 60 weevils. Live/dead weevil was the independent factor, and carrying ability was the dependent factor. Carrying ability was normally distributed, confirmed by Shapiro–Wilk W goodness-of-fit test. Variances were tested for equality using Brown–Forsythe test.

Maximum likelihood estimations (standard least squares fit) were used to analyze data. A

Student's t-test was used to detect significant differences ($\alpha = 0.05$) due to treatment mean (JMP 1989–2009)

3.4. Results

3.4.1 Assessing transmission of *V. albo-atrum* from feeding and tarsal contact of *E. brandti*.

Examination using SEM confirmed weevils were able to acquire conidia on the setae of their tarsi after walking for 5 min across an actively growing plate of *V. albo-atrum* (Figure 3.1). *Verticillium albo-atrum* was not recovered from any seedlings from the control groups, confirming no transmission occurred by extraneous sources. There was no significant difference found for sex for either, tarsal contact/infected feeding ($\chi^2 = 0.672$, $df = 1$, $P < 0.6851$) or tarsal contact/non-infected feeding ($\chi^2 = 0.501$, $df = 1$, $P < 0.7244$). As a result, data for each sex were pooled. Of the two dispersal methods, tarsal contact/infected feeding was found to be significantly greater at 75% ($n = 32$) transmission than that of tarsal contact/non-infected feeding at 50% ($n = 32$) transmission (chi-squared test, $\chi^2 = 4.267$, $df = 1$, $P < 0.0389$). Success of *E. brandti* transmitting *V. albo-atrum* to a non-infected TOH seedling is dependent on infected TOH feeding.

3.4.2 Assessing carrying ability and propagule quantity from *E. brandti* feces in *V. albo-atrum* feeding tests.

Experiment 1: Proportion of individuals passing *V. albo-atrum* through feces.

Overall, a mean of 14.7% of weevils passed *V. albo-atrum* into feces after feeding on infected TOH. The model did not identify a significant interaction ($\chi^2 = 1.177$, $df = 2$; $P < 0.5551$), a significant main effect for feeding duration ($\chi^2 = 1.112$, $df = 2$; $P < 0.5734$), or a significant

main effect for sex ($\chi^2 = 0.213$, $df = 1$; $P < 0.6444$); as a result data for each sex were pooled.

Overall, the number of weevil carriers decreased as feeding duration increased but did not differ significantly (Table 3.1); therefore carrying ability was independent of feeding duration and sex.

Experiment 2: Quantity of *V. albo-atrum* in feces.

There were no propagules recovered in any control sample, confirming no transmission occurred by extraneous sources. The model did not identify a significant interaction ($F = 1.731$ $df = 2$; $P = 0.2372$), or a significant main effect for sex ($F = 0.493$, $df = 1$; $P = 0.5024$); as a result data for each sex were pooled. The model did identify a significant main effect for feeding duration ($F = 11.246$, $df = 2$; $P = 0.0047$). The quantity of *V. albo-atrum* propagules present in each fecal sample increased with each feeding duration and differed significantly at 72 h (Table 3.1). When results from the two experiments are combined, it is estimated that each carrier weevil would be able to carry approximately 145, 339, and 2,600 propagules, respectively, for each feeding duration.

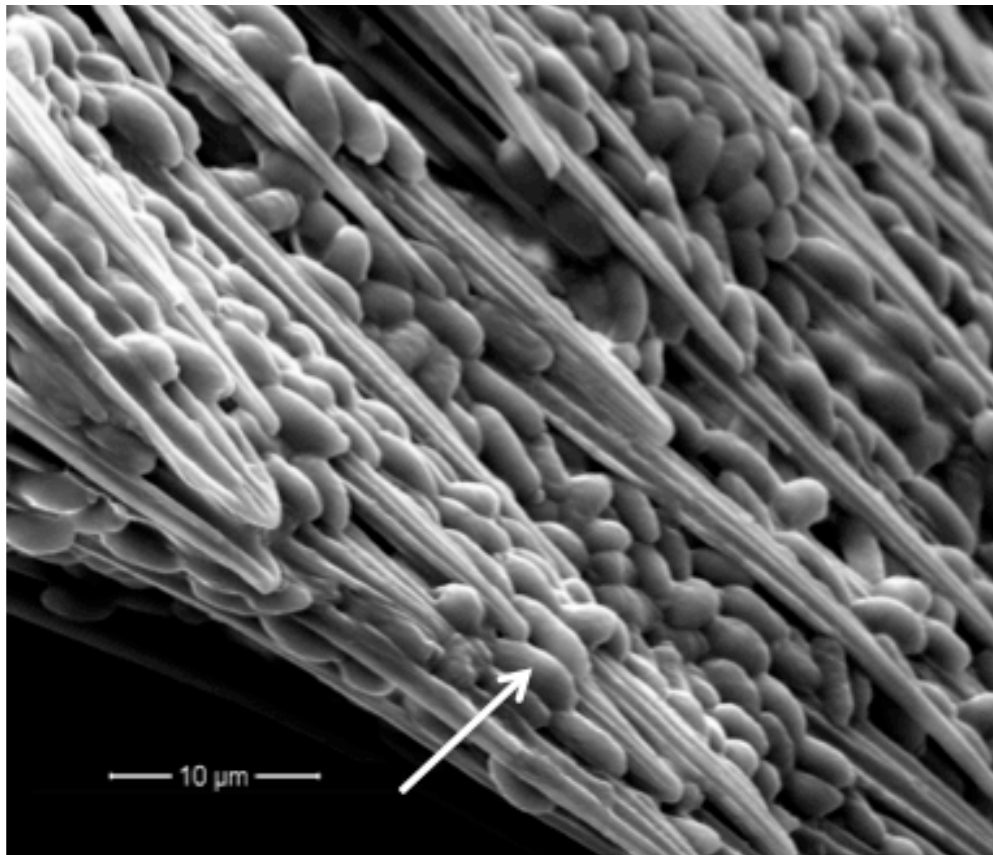


Figure 3.1 Scanning electron microscopy photograph of *V. albo-atrum* conidia (arrow) on *E. brandti* tarsal setae after contact with an actively growing culture.

Table 3.1 Percent of *Eucryptorrhynchus brandti* carriers and mean quantity \pm standard error (SE) of *Verticillium albo-atrum* propagules recovered from fecal pellets after 24, 48 and 72 h of feeding on infected *Ailanthus altissima* in two experiments.

Feeding duration (h)	Experiment 1		Experiment 2	
	n	Percent <i>E. brandti</i> carriers	n	Mean \pm SE (CFU g ⁻¹) from positive samples
24	120	16.7 a ¹	4	8826 \pm 13992 a ²
48	120	15.0 a	6	12248 \pm 11218 a
72	120	12.5 a	9	52024 \pm 8467 b

¹ Counts followed by different letters are significantly different (Pearson's Chi squared test; $\alpha = 0.05$).

² Data were transformed using the logarithmic transformation prior to analysis. Means followed by different letters in the column are significantly different (Tukey's HSD test; $\alpha = 0.05$).

3.4.3 Assessing emerging adult *E. brandti* health, carrying ability and transmission of the fungus from *V. albo-atrum*-infected billets.

All xylem samples were positive for *V. albo-atrum* for infected trees and negative for control trees. Weight of weevil progeny reared on *V. albo-atrum*-infected billets was not significantly different compared with the same sex of weevil progeny reared on control billets or parent weevils from China. However, female parents, female progeny and female control weevils were significantly heavier than male parent, male progeny and male control weevils ($F = 26.06$; $df = 5$; $P = 0.0001$) (Table 3.2). Sex ratios were approximately 1:1 (male:female) for control, parent and progeny weevils. No progeny weevils were found to carry *V. albo-atrum* on their exoskeletons, limiting their ability to transmit the pathogen to seedlings.

Table 3.2 Mean weight \pm standard error (SE) of field collected and emerged adult *Eucryptorrhynchus brandti* from *Verticillium albo-atrum* infected and non-infected *Ailanthus altissima* billets and transmission from progeny to *A. altissima* seedlings.

Measured <i>E. brandti</i>		n	Mean weight (g) \pm SE	Quantity from exoskeleton mean \pm SE CFU per weevil	Transmission to seedling
Sex (Treatment)	Reared from:				
Male (Parents)	Field collected	28	0.0600 \pm 0.0022 a ¹	N /A	N /A
Female (Parents)	Field collected	23	0.0834 \pm 0.0024 b	N /A	N /A
Male (Control)	Non-infected billets	15	0.0692 \pm 0.0025 a	N /A	N /A
Female (Control)	Non-infected billets	22	0.0963 \pm 0.0034 b	N /A	N /A
Male (Progeny)	<i>V. albo-atrum</i> billets	17	0.0686 \pm 0.0034 a	0 – 0 (0)	0
Female (Progeny)	<i>V. albo-atrum</i> billets	24	0.0862 \pm 0.0031 b	0 – 0 (0)	0

¹ Data were transformed using the logarithmic transformation prior to analysis. Means followed by different letters are significantly different (Tukey's HSD test; $\alpha = 0.05$).

3.4.4 Measuring transmission of *V. albo-atrum* by *E. brandti* overwintering in infested potting mix.

Rhizosphere soil samples taken in triplicate for each infested potting mix averaged 1.3×10^2 , 6.7×10^2 and 5.2×10^3 *V. albo-atrum* CFU g⁻¹ respectively. Twenty weevils (33%) survived the overwintering process, of which 15 carried the fungus externally. There were 40 deceased weevils (67%) and 11 carried the fungus externally. The model did identify a significant main effect for live/dead weevils ($F = 6.498$ $df = 1$; $P = 0.0183$). The quantity (mean \pm standard error) of fungus found on the exoskeleton of live weevils, 175.46 ± 29.87 CFU per individual, was significantly greater than on dead weevils, 27.80 ± 31.75 CFU per individual ($t = 2.549$ $df = 22$; $P = 0.0183$). No live weevils transferred *V. albo-atrum* to a TOH seedling.

3.5. Discussion

Predicting a relationship between two potential biological control agents is important for future field releases. There are few publications on predicting such a relationship especially in a quarantine setting before releases have occurred. In one study by Kok and Abad (1994), transmission of *Puccinia carduorum* Jacky (Uredinales), using three herbivore species resulted in 53–73% musk thistle infection. They also observed all three species consistently acquiring spores on the setae on the legs and occasionally on the rostrum. In our study, SEM techniques allowed us to confirm *E. brandti* adults were able to acquire *V. albo-atrum* on the setae of the tarsi after walking across an actively growing culture, thus allowing them to carry the fungus. In addition, weevils that acquired the fungus by tarsal contact and after feeding on infected TOH stems were able to infect more TOH seedlings than weevils that acquired the fungus by tarsal

contact only. No *V. albo-atrum* was recovered from plants in the control group, which confirms the pathogen was not transmitted by an extraneous source.

Animal transmission is an important source for both short and long-range pathogen transmission. In fecal transmission studies, *V. albo-atrum* can survive passage through the digestive tract of sheep (Huang et al. 1986), as well as many leaf-chewing insects including two species of grasshoppers, *Melanoplus bivittatus* Say and *Melanoplus sanguinipes* Fabricius, the alfalfa weevil, *Hypera postica* Gyllenhal, and the woolly bear larvae, *Apantesis blakei* Grote (Huang and Harper 1985). They also adult found alfalfa weevils were able to transfer *V. albo-atrum* to 12.8, 4.6 and 7.8% of their fecal pellets after 24, 48 and 72 h of feeding on infected alfalfa plants, respectively. Even though our study measured the number of *E. brandti* able to transfer the pathogen into feces in contrast to number of infested fecal pellets, our numbers are similar at 16.7, 15.0 and 12.5% (n = 120) for the time periods, respectively. Overall, there was little change in the number of *E. brandti* carriers with increased feeding time; however, the quantity of propagules recovered from feces did increase over time.

Huang and Harper (1985) found *V. albo-atrum* persisted for approximately 1.6 days in leaf-feeding insects that defecate less than 25 fecal pellets per day. This persistence could explain the increase in propagule quantity. Another possibility explaining these trends may be related to use of the same infected plant material as food. The weevils may have been less inclined to feed on older plant material after feeding on fresh material. In addition, *V. albo-atrum* colonization may have increased in the aging plant material contributing to a larger propagule intake. Heinz et al (1998) and Sewell and Wilson (1964) found that conidial development appears to be dominant

over hyphal growth until plant death, when hyphal growth replaced conidial growth until all remaining host tissue was colonized. At this time, the type of propagules present in the feces, and quantity of propagules needed to initiate a new infection in TOH are currently unknown. In another experiment, *V. albo-atrum* contaminated fecal pellets from the grasshopper, *M. sanguinipes*, were buried near the tap root of alfalfa seedlings and were able to act as a source of inoculum (Huang and Harper 1985). In addition, when contaminated grasshopper fecal pellets were subjected to various temperatures, survival of *V. albo-atrum* increased with decreasing temperatures as low as -40°C (Harper et al. 1988). This indicates that it is possible for some leaf-chewing insects such as *M. sanguinipes* to consume, ingest and defecate viable *V. albo-atrum* propagules capable of overwintering and act as a source of inoculum and it is likely *E. brandti* can do the same. A similar experiment was performed that used infested *E. brandti* feces as a source of inoculum. However, the experiment resulted in no transmission to seedlings due to propagules not surviving the storage process (see Appendix A). With repeated efforts and an improved storage method, *E. brandti* contaminated feces may act similarly in a forest setting and serve as an inoculum source to begin new infections in TOH stands.

Weight and sex ratio of emerged progeny for *V. albo-atrum* infected billets, controls and parent weevils were similar between the same sex. Females were found to be significantly heavier than males, which was consistent with the report by Herrick et al. (2011). Total emerged progeny was lower than expected. This may be explained by a lower fecundity due to the late time of year *E. brandti* was collected in China. In lab assays, Herrick et al. (2011) found *E. brandti* fecundity to be low (3.4 eggs per female) with a peak oviposition time at day 33–39 post-emergence, which ceased on day 84. Since weevils were not collected until June, and have a short oviposition peak

with low fecundity in lab, only a short amount of time was available to have maximum progeny. In addition, adult density was lower (10:6) (male:female) than the most efficacious rearing method found by Herrick (12:12). Low progeny production was unlikely due to the presence of *V. albo-atrum*. Regardless of progeny produced, weight was found to be the same in controls and parents collected in China compared with progeny reared from *V. albo-atrum* infected billets. This study shows that *E. brandti* is capable of producing healthy progeny when *V. albo-atrum* is present in the host tree.

When progeny from *V. albo-atrum* infected billets were tested for carrying ability and transmission to a TOH seedling, no propagules were present on the exoskeleton of any weevil. Thus, no pathogen was transmitted. These results were unexpected since weevils are often contaminated externally with fungal spores after emerging from pupal galleries (Lieutier 2004). The black-stain root disease pathogen, *Leptographium wageneri* W.B. Kendr., can be transported by *Hylastes gracilis* LeConte and *Hylastes longicollis* Swaine at densities of 1×10^1 to 1×10^4 spores per individual (Schweigkofler et al. 2005). In another study, Piou (1993) found 3–47% of emerging pine weevils, *Hylobius abietis* L. can carry spores of the blue stain fungus, *Leptographium procerum* W.B. Kendr. on pronotal setae positioned in cuticular depressions on the anterior dorsal and lateral sides of the pronotum, and were able to transmit the pathogen to 18% of new seedlings by feeding.

One explanation of these results may be due to unnatural laboratory conditions. Billets from this study were placed in water for the duration of the experiment (June 2010 – March 2011) and kept at 25°C with 60% RH. By the end of this experiment, billets contained many saprophytic

fungi. The survivorship of a vascular wilt fungus such as *V. albo-atrum* under these conditions may be unfavorable. Menzies (1970) observed *V. dahliae* microsclerotia production being prevented in areas contaminated with *Penicillium* or *Aspergillus* on potato stems. Kerr (1961) reported tomato root-surface organisms were antagonistic to *V. albo-atrum*. Numerous saprophytic fungi that grow on plant debris in wet regions may competitively inhibit production of microsclerotia by *V. dahliae*. In addition, the method of measuring *E. brandti* for carrying ability from the billets occasionally resulted in a large amount of undesirable organisms even though a selective medium was used. The abundance of organisms may have contributed to a false-negative result of the plate. In dissemination studies, Schall (2008) was able to identify *V. albo-atrum* from leaves and leaflets, 12 % of seeds, and 28% of ambrosia beetles, *Euwallacea validus* Eichhoff, using a more sensitive technique, polymerase chain reaction (PCR). In a preliminary study, Herrick (personal communication- Virginia Tech) reared 36 weevils on infested TOH billets and Schall (personal communication- Penn State) found 11% of these insects emerged with the fungus using PCR. In future studies, an increased sample size and use of a more sensitive technique such as PCR may reveal that *E. brandti* can emerge and carry *V. albo-atrum* externally from infected TOH billets.

Live and dead overwintering weevils in infested potting mix carried the fungus externally. The quantities of propagules present on the exoskeleton of these weevils were variable, but at times were quite high. However, none of the surviving weevils were able to cause infection of a TOH seedling. This may be due to transmission testing of only a single weevil per seedling. Kok and Abad (1994) found more than 50% transmission of *P. carduorum* to musk thistles when each plant was exposed to at least 50 spore-carrying insects of the same species. In addition to

increasing the number of exposed weevils per plant, feeding time should also be increased. In preliminary experiments, some *E. brandti* were able to girdle a 12-week TOH seedling in more than 3 d, so a feeding time of 3 d was chosen. However, studies by Carraro et al. (2001) found *Cacopsylla pruni* Scopoli (Hemiptera, Psylloidea) were unable to transmit a phytoplasmid to *Prunus salicina* Lindl. after 1–2 d of feeding but were able to inoculate 30% of the plants after 4–7 d of feeding. This minimum period of acquisition should also be established for *E. brandti*. In future studies, larger diameter TOH seedlings should be used and time of feeding durations should be increased to enhance the probability of successful transmission to TOH.

Finding an efficacious dispersal method is important for future releases of *E. brandti* and *V. albo-atrum*. By applying these methods to future field releases of *E. brandti*, project leaders have the potential to release both organisms simultaneously. These studies demonstrate that where forest conditions may not be suitable for *V. albo-atrum* production, the pathogen may be carried by *E. brandti*. Studies have shown that *E. brandti* can carry the pathogen internally via feces, externally by overwintering and were able to begin new infections by spore transmission from tarsal contact. In addition, weevils were able to reproduce healthy generations on infected TOH billets. More research is needed on the dispersal behavior of *E. brandti* and survival of *V. albo-atrum* under field conditions. However, the results from these laboratory experiments suggest that *E. brandti* has potential for aiding in the spread of *V. albo-atrum* from tree to tree because they can carry propagules both externally and internally.

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Appendix A. *E. brandti* feces as an inoculum source.

Introduction

Two potential biological control agents for the invasive tree of heaven (TOH), *Ailanthus altissima* (Mill.) Swingle, have been extensively studied: a native vascular wilt fungus, *Verticillium albo-atrum* Reinke & Berthold, and a host-specific weevil from China, *Eucryptorrhynchus brandti* (Harold) (Coleoptera, Curculionidae), which is currently pending quarantine release. In 2002, *V. albo-atrum* was observed in Pennsylvania causing significant mortality to TOH. The pathogenicity of this pathogen suggests that it could be used together with host-specific insects for the biological control of TOH. Quarantine experiments were conducted to test different forms of transmission with *E. brandti* and *V. albo-atrum* simultaneously.

Animal transmission is an important source for both short and long-range pathogen transmission. In fecal transmission studies, *V. albo-atrum* can survive passage through the digestive tract of sheep (Huang et al. 1986), as well as many leaf-chewing insects including a grasshopper, *Melanoplus sanguinipes* Fabricius (Huang and Harper 1985). Feces from these animals were also able to act as a primary inoculum source to initiate new infections on alfalfa.

Materials and Methods

Growing seedlings. Tree of heaven seeds were collected from healthy TOH in Blacksburg, Virginia and planted in 13-cm dia pots filled with Sta-Green® Nursery Blend Tree and Shrub Planting Mix. All seedlings were grown in the Virginia Tech Beneficial Insects Quarantine Laboratory in an environmental chamber at 24°C, 60% RH, and a 12-h photoperiod using

ultraviolet grow lights. After 10 weeks, seedlings were removed from the chamber and kept in a room with similar conditions.

Experiment

This experiment tested whether *V. albo-atrum* infested adult *E. brandti* feces could be used as an inoculum source for TOH seedlings. It was hypothesized that *V. albo-atrum*-infested feces of *E. brandti* will not infect *A. altissima*.

Hundreds of *E. brandti* adults were fed infected TOH stems for 72 h using the method described in Chapter 2. The adults were kept in a 35.5 x 20 cm (l x w) container amended with fiberglass window screening (46 x 41 mesh count cm⁻¹, wire diameter 0.279 mm) to increase ventilation. Duration of feeding was based on results from Chapter 3 where 72 h of feeding yielded significantly more *V. albo-atrum* CFU g⁻¹ feces. After 72 h, weevils were removed and fecal pellets were collected. This collection process was repeated many times to increase the amount of feces. All feces were pooled in a sterile Petri dish sealed with parafilm and stored for 60 d at 20°C. Quantity of *V. albo-atrum* was measured by plating serial dilutions in triplicate on KSM. Control treatments included feces from weevils that were fed non-infected TOH stems. Feces (0.13 g) were buried in contact with the taproot of each potted TOH seedling (Huang and Harper 1985). All seedlings were watered twice a week. Eight weeks later, seedlings were harvested and two stem sections were isolated per plant for *V. albo-atrum* using the method described in Chapter 2.

This experiment used a completely randomized design and consisted of six *V. albo-atrum* fecal-inoculated seedlings and six control fecal-inoculated seedlings. Inoculation was recorded for each seedling as a binomial response (non-infected seedling=0, infected seedling=1).

Results and Discussion

All seedlings and plated dilutions were negative for *V. albo-atrum*. These results were not consistent with Huang and Harper (1985) who found *V. albo-atrum* infested feces from *M. sanguinipes* were able to act as a primary inoculum to new alfalfa seedlings. These positive isolations were most likely due to the short storage time (10 d) used for feces. This can be explained by the source of colonization in the feces. Weevils were fed live, recently infected TOH stems. Once infection occurs, conidia move rapidly through the vascular tissue. Presley (1966) noted conidia could colonize a 115-cm tall cotton plant in 24 h. Conidial development appears to be dominant over hyphal growth when the plant is alive and water is present in the vascular tissue. However after plant death, conidial growth is replaced by hyphal growth until the remaining host tissue is colonized (Heinz et al. 1998, Sewell and Wilson 1964). It is likely that the source of colonization of *V. albo-atrum* in the stems was abundant with conidia, which only remains viable for three to four weeks compared with melanized mycelium which can remain dormant for 9 months to 4 years (Pegg and Brady 2002). Thus, it is probable that *E. brandti* feces contained only short-lived conidia, which did not remain viable through the 60 d storage process. With repeated efforts and an improved storage method, it may be possible that *E. brandti* feces could act similarly as those of *M. sanguinipes*, which was used as a short-lived source of inoculum.