Biological control of the invasive *Ailanthus altissima* (tree-of-heaven) in Virginia using naturally occurring Verticillium wilt fungi

Rachel Keys Brooks

Dissertation submitted
to the faculty of the School of Plant and Environmental Sciences at
the Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Plant Pathology, Physiology, and Weed Science

Anton B. Baudoin, Chair
Scott M. Salom, Co-Chair
Matt T. Kasson
Jacob N. Barney

May 11, 2020
Blacksburg, Virginia

Keywords: *Ailanthus altissima*, tree-of-heaven, biological control, biocontrol, biopesticide, *Verticillium nonalfalfae*, *Verticillium dahliae*, nonnative forest tree, invasive plant, management, restoration

Biological control of the invasive *Ailanthus altissima* (tree-of-heaven) in Virginia using naturally occurring Verticillium wilt fungi

Rachel Keys Brooks

Abstract

The invasive tree-of-heaven, *Ailanthus altissima* (Miller) Swingle*,* is widespread and damaging throughout North America. Verticillium wilt disease is emerging as a potentially exciting biological control option for this difficult to control tree. In Virginia, *Verticillium nonalfalfae* has been confirmed causing significant mortality to *A. altissima,* while *V. dahliae* is suspected to be present and causing lower levels of disease. Little else is known regarding these two fungal species in this state. The purpose of this research was to gain a better understanding of how Verticillium wilt impacts *A. altissima* and its potential as a biological control agent. We first confirmed *V. dahliae*'s presence in Virginia and its pathogenicity to *A. altissima* using Koch's postulates. We then completed a regional field-inoculation experiment to show that *V. nonalfalfae* effectively kills and spreads to adjacent *A. altissima,* regardless of *V. dahliae* presence or other climate and stand variables. Additionally, we showed that *V. dahliae* causes lower levels of disease than *V. nonalfalfae,* and does not spread rapidly. Next, we surveyed all Virginia *A. altissima* stands known to be naturally infected with *V. nonalfalfae* to determine whether *V. nonalfalfae* persists long-term, that it considerably reduces *A. altissima* numbers, and that its local prevalence may be higher than initially suspected. However, we were unable to infect *A. altissima* seedlings using soil collected at these infested sites, suggesting that *V. nonalfalfae's* survival within field soil may be limited. Lastly, using paired *A. altissima* invaded-uninvaded sites, we found that *A. altissima* presence is associated with a decreased proportion of native plants and species in the woody and herbaceous understory, but not the germinable seedbank. Furthermore, we found that this impact on the woody understory appears to increase over time, supporting early management actions and helping us predict post-management restoration needs. We conclude that *V. nonalfalfae* has a high potential of successfully limiting *A. altissima* throughout Virginia, supporting its registration as a biopesticide.

Biological control of the invasive *Ailanthus altissima* (tree-of-heaven) in Virginia using naturally occurring Verticillium wilt fungi

Rachel Keys Brooks

General audience abstract

Commonly called the tree-of-heaven, the nonnative invasive forest-tree *Ailanthus altissima*, is extensive, damaging, and spreading throughout North America. After finding large areas of declining tree-of-heaven being killed by two different fungal species (*Verticillium nonalfalfae* and *V. dahliae*), research has been focused on how to use these fungi to help us manage the tree-of-heaven. In Virginia, *V. nonalfalfae* has been confirmed killing large numbers of tree-of-heaven, while *V. dahliae* is suspected to be present in areas with lower levels of decline. The purpose of our research was to gain a better understanding of how these pathogens impact tree-of-heaven and their potential as biological control agents in Virginia. We first confirmed that *V. dahliae* is present in Virginia and can cause disease on tree-of-heaven. We then inoculated tree-of-heaven stands throughout the state to confirm that *V. nonalfalfae* effectively kills and spreads to adjacent tree-of-heaven regardless of *V. dahliae* presence or other climate or site variables. In contrast, we found that *V. dahliae* only causes low levels of disease and does not spread effectively. Next, we surveyed all known naturally infected *V. nonalfalfae* sites in Virginia and demonstrated that *V. nonalfalfae* persists long term within these stands, considerably reducing but not eradicating the tree-of-heaven, and that *V. nonalfalfae*'s local prevalence may be higher than initially suspected. However, when tree-of-heaven seedlings were planted into soil collected from these infested sites, no disease developed, suggesting that *V. nonalfalfae*'s survival within the soil may be limited. Lastly, by looking at tree-of-heaven stands, we found that the tree-of-heaven's presence is associated with a lower percentage of native plants and species in the understory, but not in the seeds present in the soil. In addition, we found that this impact on the woody plants in the understory appears to become more severe over time, supporting managing the tree-of-heaven as soon as possible. We conclude that *V. nonalfalfae* used as a biological control has a good potential of successfully limiting the tree-of-heaven in Virginia and support its registration as a biopesticide.

Dedication

To Andy, who moved across the country in support of my career. And to my parents, who always encouraged my interests and backed my decisions, regardless of the number of miles it put between us.

Acknowledgments

I sincerely thank the following people for making this research happen:

The US Forest Service for their financial support (US Forest Service Grant 15-CA-11420004-161).

My co-advisors, Dr. Anton Baudoin and Dr. Scott Salom, for entrusting me with this research, for their good advice, and for dealing with the intradepartmental paperwork.

My other committee members: Dr. Matt Kasson, for his unending enthusiasm of forest pathology and his system specific knowledge, and Dr. Jacob Barney, for letting me join his lab group and sharing his invasion biology knowledge.

My fellow graduate students: for keeping me sane and always providing realistic advice. Especially my forest entomology lab for embracing a forest pathologist, my lone plant pathology lab-mate Caroline for helping me with molecular work, and my adopted invasive biology lab mates for not letting me forget about theoretical research.

The PPWS Plant Disease Clinic personnel: Elizabeth Bush and Mary Ann Hansen, for letting me use their facilities and tag along with them to meetings.

Table of contents

Chapter 1: Literature review

Abstract

Ailanthus altissima (Miller) Swingle, known most commonly as the tree-of-heaven, is an invasive tree that has spread globally from its native Chinese range. In its invaded range, it grows aggressively, spreads rapidly, and causes a variety of impacts to human health, agriculture, and the environment. Current control options are limited to a combination of chemical and mechanical tactics which are unpractical over large scales. Since 2002, areas of declining *A. altissima* have been identified in three US states and two European countries, with severe outbreaks caused by *Verticillium nonalfalfae* and smaller outbreaks caused by *V. dahliae.* Out of these two fungal vascular wilt pathogens, *V. nonalfalfae* in particular appears to rapidly kill *A. altissima* and spread to adjacent *A. altissima*. Initial studies show that *V. nonalfalfae* isolates are host limited, are easily inoculated, reliably cause disease, and effective at controlling *A. altissima*. The biological control potential of *V. nonalfalfae* is promising, and biopesticide registration efforts are ongoing.

1.0 *Ailanthus altissima*

1.1 Background

Ailanthus altissima (Miller) Swingle, commonly known as the tree-of-heaven, paradise tree, Chinese sumac, stink tree, or merely Ailanthus, is a deciduous tree in the order Sapindales and the family Simaroubaceae (Asaro et al., 2009). It is one of five species within the *Ailanthus* genus, which are all native to Southeastern Asia or the Pacific Islands (Hu, 1979; Kowarik and Säumel, 2007). Out of these, *A. altissima* uniquely grows in the subtropical zone (Kowarik and Säumel, 2007) and is native to most regions of China (Wu et al., 2008). Within its native range, *A. altissima* grows in broadleaf forests at low abundances as a canopy tree (Hu, 1979; Kowarik and Säumel, 2007).

1.2 Growth

Ailanthus altissima can grow for well over 100 years, have a diameter at breast height (DBH) approaching 2 m (Kasson et al., 2013a), and reach up to 20 m in height (Wu et al., 2008). Seedlings can reach a height of up to two meters during their first growing season (Hu, 1979) with clonal growth surpassing 4.5 m in added height per year (Swingle, 1916).

Ailanthus altissima can tolerate a wide range of growing conditions. It successfully grows in arid, humid, subtropical, or temperate climates (Miller, 1990) and has high drought resistance

(Knüsel et al., 2015). It can tolerate a broad range of soil types, including harsh sites with nutrient-poor or alkaline soils, and can tolerate dust, salt spray, and air pollution (Hepting, 1971; Kowarik and Säumel, 2007; Miller, 1990). *Ailanthus altissima* can be found in areas with high levels of disturbance (Fotiadis et al., 2011; Kasson et al., 2013a; Kowarik and Säumel, 2007; McAvoy et al., 2012; Motard et al., 2011) and is typically described as a shade-intolerant, gap-obligate, early-successional species (Carter and Fredericksen, 2007; Kasson et al., 2013a; Knapp and Canham, 2000). Despite this, *A. altissima* can survive in the understory as clonal ramets (Kowarik, 1995) and *A. altissima* saplings can grow in relatively low light conditions that are sufficient for survival and even substantial growth in closed canopy forests for at least seven years (Knusel et al., 2017).

1.3 Reproduction and dispersal

Ailanthus altissima produces panicles with light-green flowers on both sexes in the spring, with female plants developing winged samaras that persist well into the winter (Hu, 1979; Wu et al., 2008). Female trees can produce viable seeds at ages as young as three to four years through 100 years of age (Kasson et al., 2013a; Kowarik and Säumel, 2007; Wickert et al., 2017). A single large female *A. altissima* can produce around one million seeds a year, with calculations indicating that a single female tree can produce 10 million seeds (Wickert et al., 2017). These samaras are dispersed primarily by wind, and have been shown to regularly travel 100 m over a field and over 450 m on paved ground (Kowarik and Von der Lippe, 2011; Landenberger et al., 2007). Furthermore, water is a secondary vector of these seeds, significantly increasing their dispersal potential (Kowarik and Säumel, 2008; Planchuelo et al., 2016).

Ailanthus altissima seed germination rates can range between 1 to 95% (Rebbeck and Jolliff, 2018; Wickert et al., 2017). Over a five-year time period, germination rates of seeds incubated outdoors can remain high (up to 75%) (Rebbeck and Jolliff, 2018), contradicting an earlier study that indicated that *A. altissima* seeds do not remain viable for more than one year (Kota et al., 2007). *Ailanthus altissima* seeds have been shown to germinate well in high-light conditions (Kota et al., 2007) and poorly in low-light conditions (Kowarik, 1995).

Ailanthus altissima is also well known for its vegetative reproduction, in which it sends up numerous sprouts from both stumps and roots (Hu, 1979). In fact, even just stem fragments are able to develop both shoots and roots when buried (Kowarik and Säumel, 2008).

1.4 Uses

Though the wood of live *A. altissima* is known to be brittle and weak (Hepting, 1971), in the United States, *A. altissima* may be accepted at pulp mills, can be burned as firewood if dried well before use, can be processed into natural lump charcoal, and can be used to produce lumber, though warping, cracking, stiffness, and poor strength can be problematic (Asaro et al., 2009). In China, *A. altissima* wood is used for the construction of wooden steamers for cooking food (Hu, 1979). The Ailanthus silkworm (*Samia cynthia* Drury*)*, which feeds on *A. altissima*, is still used to a limited extent to produce silk in China (Chuanhui et al., 2010). In traditional Chinese medicine, almost every part of *A. altissima* is utilized (Hu, 1979; Laskar, 2010), and modern research looking into the medicinal properties of *A. altissima* has shown promising results (Laskar, 2010).

2.0 *Ailanthus altissima* as an invasive species

2.1 Current distribution

Through both human dispersal (Hepting, 1971; Kasson et al., 2013a; Miller, 1990) and aggressive seed production and sprouting (Wickert et al., 2017), *A. altissima* is now found on all continents (except Antarctica) in the temperate and subtropical zones and is considered one of the most invasive trees in the world (Kowarik and Säumel, 2007; Richardson and Rejmánek, 2011). Modeling indicates that *A. altissima* is likely to continue to expand its distribution throughout the world (Albright et al., 2010; Walker et al., 2017).

Ailanthus altissima's journey to North America began in 1751, when seeds were mailed from China to Europe, misidentified as the Chinese varnish tree (Hu, 1979; Swingle, 1916). These seeds grew into healthy trees in Europe, where their popularity increased rapidly even while their identity and taxonomy were being debated (Hu, 1979; Swingle, 1916). From Europe, *A. altissima* was introduced in 1784 to Pennsylvania, USA as a highly-prized ornamental tree (Del Tredici, 2017; Hu, 1979; Kasson et al., 2013a). Though *A. altissima* was initially grown and guarded by only a few people, it quickly became popular in the horticultural trade due to its tropical appearance, rapid growth, and ability to grow in low-quality sites (Hu, 1979; Kasson et al., 2013a). Additionally, Chinese miners brought *A. altissima* seeds with them directly from China during the California gold rush in the 19th century, establishing new populations on the west coast of North America (Rohe, 1984). Throughout North America, *A. altissima* has shown no sign of inbreeding depression and has high levels of genetic diversity (Feret et al., 1974; Kowarik and Säumel, 2007; Petruzzellis et al., 2018).

Today, *A. altissima* can be found in over 40 US states (EDDmapS, 2019), where it grows in form as a tree, a hedge, or a bush and is easily recognized by its large pinnately-compound leaves and smooth light-gray bark (Hu, 1979). In Virginia, USA the US Forest Service's Forest Inventory and Analysis (FIA) estimates that measurable quantities of the tree can be found in over half of Virginia's counties, with total volumes adding up to 1.9 million cubic meters (67 million cubic feet), making it the 42nd most abundant tree in Virginia (Asaro et al., 2009).

2.2 Impacts

Though *A. altissima* was once widely planted on purpose (Kasson et al., 2013a), it is no longer favored. Its flowers and damaged vegetation produce an unpleasant odor, it is a messy yard tree, and its constant sprouting is undesirable (Albright et al., 2010; Hepting, 1971; Swingle, 1916). Additionally, the aggressive growth of *A. altissima* can cause structural problems to infrastructure (Hu, 1979; Kowarik and Säumel, 2007), takeover agricultural fields (Hepting, 1971), and obstruct lines-of-sight along rights-of-way (Burch and Zedaker, 2003).

Regarding human health, it has been reported that the pollen of *A. altissima* is allergenic, and that direct contact with the tree may cause dermatitis of human skin (Kowarik and Säumel, 2007; Swingle, 1916). In *A. altissima*'s invaded range, its pollen is an emerging cause of respiratory allergy, with numerous extracts identified as allergens (Mousavi et al., 2017). Additional reports of more severe health impacts have been recorded, though confirmation that *A. altissima* was the cause was not included (Hu, 1979).

Ailanthus altissima also impacts the soil, with its presence increasing total nitrogen (N), increasing organic carbon (C), decreasing the C/N ratio, and increasing pH (Motard et al., 2015; Vilà et al., 2006). It has also been shown that its root exudates stimulate nodulation of other plant species, though the exact mechanism is not understood (Greer et al., 2016).

Ailanthus altissima also produces allelopathic chemicals, consisting mainly of the quassinoid compound ailanthone (Heisey, 1996; Mergen, 1959). These allelopathic properties have been shown to have strong herbicidal impacts on many other tree species (Heisey, 1996;

Heisey and Heisey, 2003; Lawrence et al., 1991; Mergen, 1959), leading to a reduction in competition, as *A. altissima* itself appears to be uninjured by its own compounds (Heisey, 1996).

A. altissima has been shown to influence the soil food web by altering several soil trophic levels (Motard et al., 2015). Motard et al. (2015) showed that high densities of *A. altissima* could result in the reduction of soil microbial activity and the increase (either in number or species diversity) of litter detritivores, above-ground predatory Coleoptera (beetles), terrestrial Gastropoda (snails and slugs), Lumbricidae (earthworms), and coprophagous Coleoptera.

Today, *A. altissima* is causing problems by interfering with the growth of other trees, including the regeneration of oaks in forest stands (Hubner and Rebbeck, 2014). In areas that *A. altissima* has invaded, it has been shown to cause a decrease in overall species richness (Gaertner et al., 2009; Vilà et al., 2006), a reduction in native plant species diversity (Vilà et al., 2006), a general decrease in the diversity of understory vegetation (Motard et al., 2011), and has the potential to significantly alter the functioning of the ecosystem by influencing species diversity and soil traits (Traveset et al., 2008). *A. altissima* has also been associated with the presence of other synanthropic species, which in turn can cause their own additional impacts (Fotiadis et al., 2011). For example, *A. altissima* also appears to limit vegetative growth of black locust (*Robinia pseudoacacia* L.) seedlings, while *R. pseudoacacia* appears to slightly facilitate *A. altissima'* growth (Nilsen et al., 2018).

2.3 Management options

When injured, *A. altissima* responds by re-sprouting, making mechanical control methods such as cutting, mowing, and girdling ineffective (Burch and Zedaker, 2003; Meloche and Murphy, 2006). Therefore, to successfully control *A. altissima*, herbicide is usually needed to prevent re-sprouting (Asaro et al., 2009; Burch and Zedaker, 2003; Hubner and Rebbeck, 2014). Herbicides are available for application as foliar sprays, basal stem sprays, stem injections (including hack-and-squirt), or cut-stump treatments (Asaro et al., 2009; Kok et al., 2008), though cut-stump treatments will only limit stump sprouts, not root sprouts (Gover et al., 2013). It is usually recommended that a systemic herbicide is applied during the second half of the growing season and to wait until the following dormant season to remove the tree to ensure the herbicide is translocated to the roots (Gover et al., 2013). With consistent follow up monitoring to identify any areas that need additional management, *A. altissima* can be eradicated at small spatial scales (Asaro et al., 2009; Hubner and Rebbeck, 2014). Chemical control however is impractical over large scales, as it is expensive, labor intensive, and potentially hazardous to the environment and applicator (Ding et al., 2006; Kok et al., 2008; O'Neal and Davis, 2015). Therefore, no large-scale management methods currently exist for *A. altissima,* and no publicly available biological control options are available (Asaro et al., 2009).

3.0 Diseases and pests of *A. altissima*

In *A. altissima's* native range, 46 arthropods are associated with this tree, of which three (*Eucryptorrhynchus brandti* Harold, *E. chinensis* Oliver, and *Orthopagus lunulifer* Uhler) cause significant damage only to *A. altissima* (Ding et al., 2006). Of these, *E. brandti* is currently in quarantine at Virginia Tech for biological control testing (Herrick et al., 2012; Snyder et al., 2012). In contrast, only ten insects have been reported feeding on *A. altissima* outside of its native range (Ding et al., 2006; Kasson et al., 2013b) with the tree being said to be “almost immune to attack by insects” (Swingle, 1916). A survey of Virginia’s *A. altissima* populations showed that current insect damage to the tree is insignificant (Kok et al., 2008). Insects that do feed on *A. altissima* foliage include the Ailanthus webworm (*Atteva punctella* Cramer), the cynthia moth (*S. cynthia*), and the Asiatic garden beetle (*Maladera castanea* Arrow) (Miller, 1990). *Euwallacea validus* Eichhoff, an ambrosia beetle introduced to North America from China, attacks the wood of stressed or dying *A. altissima*, but its impact on tree populations appears to be minimal (Kasson et al., 2013b; Kasson et al., 2015; Kok et al., 2008). Adding to the above, *Agrilus smaragdifrons* Ganglbauer was first observed in June 2011 in North America, and appears to be present and potentially established in parts of PA, NJ, NY, and CT (Hoebeke et al., 2017). Currently, *A. altissima* is the only known host of this beetle though its impact is currently unknown both in its native and introduced ranges (Hoebeke et al., 2017). Furthermore, the recent accidental introduction and spread of the spotted lanternfly (*Lycorma delicatula* White) to North America, which preferentially feeds on *A. altissima*, has become a significant management concern (Song et al., 2018). Overall, no insects in the tree’s invaded range currently cause enough damage to limit its success.

In *A. altissima's* native range, 16 fungal species have been associated with the tree (Ding et al., 2006). Outside of its native range, *A. altissima* is rarely impacted significantly by disease (Hepting, 1971; Miller, 1990), though 68 fungal species have been identified as being associated with the tree (Ding et al., 2006). For example, heart rot is common in *A. altissima*, and increases in prevalence with increasing tree DBH and age (Kasson et al., 2013a; Knüsel et al., 2015). Only *Verticillium* and *Fusarium* fungi have been recorded as causing a significant impact to the tree outside of its native range (Armel et al., 1997; Ding et al., 2006). *Fusarium oxysporum* has been listed as occurring on *A. altissima* in North America (Ding et al., 2006) and was isolated from *A. altissima* with typical wilt symptoms along the Blue Ridge Parkway in Virginia (Armel et al., 1997). This isolate of *F. oxysporum* was re-inoculated into healthy *A. altissima* seedlings and its pathogenicity confirmed (Armel et al., 1997), though no additional tests have since been conducted. Verticillium wilt fungi, which cause the most severe impact to *A. altissima*, are discussed in more detail in the following sections.

4.0 Verticillium wilt fungi

4.1 Taxonomy

Verticillium wilt is caused by ascomycete plant pathogens in the order *Glomerellales,* the family *Plectosphaerellaceae*, and the genus *Verticillium* (Giraldo and Crous, 2019; Pegg and Brady, 2002). Since *Verticillium*'s first description in 1817, identification of specific *Verticillium* species has been controversial and inaccurate with lots of debate regarding how to organize the genus into species or varieties (Inderbitzin and Subbarao, 2014; Pegg and Brady, 2002). In the 1970s a consensus was reached regarding species identification using morphology of resting structures and pigmented hyphae (Inderbitzin et al., 2011a; Inderbitzin and Subbarao, 2014), and by the 21st century, five species were commonly recognized in this genus (Pegg and Brady, 2002). As the genus became more defined, it was renamed to *Verticillium* sensu stricto with *V. dahliae* as the conserved type (Gams et al., 2005). However, recent molecular work by Inderbitzin et al. (2011a) has again redefined how *Verticillium* species are classified, splitting the genus into ten species, including one hybrid. The diploid hybrid, *V. longisporum,* is considered a unique species that evolved from three separate hybridization events between other *Verticillium* species, including *V. dahliae* (Inderbitzin et al., 2011b). With this new classification, there are now morphologically cryptic species (Inderbitzin et al., 2011a). Therefore, any research on Verticillium wilt not supported by molecular sequencing or voucher specimens may not be confirmed to the species level (Inderbitzin and Subbarao, 2014).

4.2 Host range

Verticillium species impact hundreds of different plant species including agricultural and horticultural crops such as herbaceous annuals, perennials, and woody plants (Dung and Weiland, 2015; Pegg and Brady, 2002). Because of this, this genus is considered to cause one of the most devastating fungal diseases in the world, Verticillium wilt disease, which impacts over 350 plant species, including many high value crops (Fradin and Thomma, 2006; Pegg and Brady, 2002).

Dicotyledonous trees from many genera are susceptible to *Verticillium* wilt, while monocotyledons and gymnophytes are not impacted (Hiemstra and Harris, 1998). Susceptible host trees include maples (*Acer* spp.), elms (*Ulmus* spp.), and European olive (*Olea europaea*) (Hiemstra and Harris, 1998). *Verticillium* wilt very rarely impacts forest trees, and is mostly found in ornamental plantings, in orchards, or on nursery trees (Hiemstra and Harris, 1998). Only three species of forest trees have ever been recorded as being impacted by Verticillium wilt (listed as *V. albo-atrum* at the time) in North America: *Liriodendron tulipifera* in Delaware (native), a *Ceanothus* species in California (native), and *A. altissima* (non-native) in eastern North America (Schall and Davis, 2009a).

Within a single species of *Verticillium*, isolates from a specific host tend to be the most aggressive on the host from which they were originally isolated, with varying degrees of pathogenicity when inoculated into other hosts (Bhat and Subbarao, 1999). A minimal degree of genetic variation has been observed between different isolates within a single species of *Verticillium,* but has yet to be connected to pathogenicity or host range (Bhat and Subbarao, 1999; Jelen et al., 2016; Kasson et al., 2014). Recent proteomic work found that *V. nonalfalfae* isolated from *A. altissima* clustered separately from *V. nonalfalfae* isolated from hops (*Humulus lupulus* L.) or Solanaceous species (*Solanum* spp.), indicating that some underlying variation in isolates exists, potentially accounting for the observed host differences Wickert (2019). Additionally, after laboratory work using the Austrian wild-type strain G1/5 resulted in strains with different levels of aggressiveness, comparative genomics work was completed, setting the stage to further understand the isolate's evolutionary potential (Berger et al., 2020).

4.3 Disease cycle

The life cycles of *Verticillium* species are all similar and have been described well by Fradin and Thomma (2006), Pegg and Brady (2002), and Hiemstra and Harris (1998). Overall, fungal resting structures (which vary by species) lay dormant in soil until exposed to root exudates (Fradin and Thomma, 2006; Inderbitzin et al., 2011a). These then germinate and grow towards the root, usually entering at the root tip or at damaged sites (Fradin and Thomma, 2006). The fungus then grows intra- and intercellularly to cross the endodermis into the xylem vessels (Fradin and Thomma, 2006). Once in the vascular tissue, *Verticillium* starts budding (producing asexual conidia) that are rapidly carried in the plant's sap stream until stopped by pit cavities or vessel ends (Fradin and Thomma, 2006; Hiemstra and Harris, 1998). At these sites, the conidia germinate, penetrate the plant tissue, and find another vessel element in which to bud, expanding the infection (Fradin and Thomma, 2006).

Symptoms develop when water transport in the plant is limited by fungal growth, degraded plant tissue, or plant defense responses (Hiemstra and Harris, 1998; Keykhasaber et al., 2017). During necrosis or plant senescence, *Verticillium* produces resting structures in the dying plant material, which will eventually be freed into the upper layers of soil during plant tissue decomposition (Fradin and Thomma, 2006; Hiemstra and Harris, 1998). These resting structures are the major inoculum source and the primary means of long-term survival for *Verticillium* (Fradin and Thomma, 2006). In soil, resting structures can survive for long periods of time, with microsclerotia lasting over 10 years (Pegg and Brady, 2002; Sewell and Wilson, 1966; Wilhelm, 1955).

Resting structures in the soil and infected plant material (including fruit, stems, petioles, and leaves) can be moved by wind, water, and animals (Fradin and Thomma, 2006; Hiemstra and Harris, 1998; Pegg and Brady, 2002). In addition, insects have the potential to transmit *Verticillium* between plants and tree root grafts can serve as a rapid intravascular means of transport (Fradin and Thomma, 2006; Hiemstra and Harris, 1998; Pegg and Brady, 2002).

Verticillium species reproduce only asexually, though both genetic recombination and gene flow can still occur within vegetative compatibility groups (Bhat and Subbarao, 1999; Hiemstra and Harris, 1998).

4.4 Typical symptoms

Susceptible plants infected by Verticillium wilt fungi produce a range of symptoms depending on host species, but which generally consist of leaf chlorosis, marginal and interveinal necrosis, wilting, defoliation, vascular discoloration, and premature senescence (Dung and Weiland, 2015). In woody species, symptoms can either progress rapidly or slowly (Hiemstra and Harris, 1998). Symptoms can be expressed asymmetrically or evenly, and can result in plant death, stunted growth, or complete recovery (Dung and Weiland, 2015; Fradin and Thomma, 2006).

4.5 Management options

Verticillium fungi are difficult to control due to their long-term survival in the soil, their broad host range, and the fact that fungicides are ineffective once the pathogen is inside a plant (Fradin and Thomma, 2006; Keykhasaber et al., 2017). Methods to help reduce inoculum in soil include solarization, soil fumigation, and crop rotation, but can only be used in certain areas and tend to be inefficient (Fradin and Thomma, 2006). Therefore, limiting disease spread should be the main focus of *Verticillium* management (Hiemstra and Harris, 1998). This can include reducing use of contaminated machinery, minimizing practices that damage roots, using drip irrigation to limit the spread of infected substances, destroying diseased material, and using clean planting stock (Hiemstra and Harris, 1998). Watering may reduce symptoms, as *Verticillium* appears more aggressively in *Liriodendron tulipifera* when stressed by periods of low moisture (Morehart and Melchior, 1982). Selecting for host resistance has been the most effective management technique, but is not a viable option in natural systems (Dung and Weiland, 2015).

5.0 Verticillium wilt outbreaks on *A. altissima*

5.1 Outbreaks in North America

Verticillium wilt has historically been observed on *A. altissima* throughout its invaded range (Hepting, 1971), though data are limited regarding *Verticillium* species identification. Typical symptoms observed include vascular discoloration, necrosis and chlorosis of leaves, premature defoliation, and mortality (Kasson et al., 2014). In 1915 the earliest report of Verticillium wilt on *A. altissima* was recorded by the Pennsylvania State University Disease Clinic (Kasson et al., 2014). In the late 1920s and early 1930s, *Verticillium* wilt was observed on *A. altissima* in Roanoke, VA; New York City, NY; and Philadelphia, PA (Gravatt and Clapper, 1932). In the 1990s *Verticillium* wilt was also observed on *A. altissima* trees within and bordering New York City (Emmerich et al., 1998). It is very possible that historic *Verticillium* outbreaks were mistaken for natural senescence or roadside damage since *A. altissima* tends to grow in low quality sites and is usually thought of as having a short life span (Kasson et al., 2014).

In 2002 and 2003, a high level of *A. altissima* mortality was identified in the Tuscarora State Forest in south-central Pennsylvania (Franklin and Perry Counties) and *V. nonalfalfae* was confirmed as the causal agent (Schall and Davis, 2009a). An isolate collected from one of these symptomatic *A. altissima* in 2005 (referred to as VnAa140, PSU140, or NRRL66861) has been the focus of the Pennsylvania research discussed later on (Kasson et al., 2019). *Verticillium dahliae* was also found in smaller and less severely diseased patches of *A. altissima* in the area (Schall and Davis, 2009a). In 2010, another Pennsylvanian *V. nonalfalfae* outbreak was confirmed over 200 km west of the previous infection site (Kasson et al., 2014).

Verticillium nonalfalfae was then confirmed killing 1,100 *A. altissima* at two locations in Virginia in July 2009 (Snyder et al., 2013). Following this confirmation, a windshield survey was conducted on over 26,500 km of roadway in VA, NC, and SC over the next few years to locate additional stands impacted by *Verticillium* (Snyder et al., 2014). Ninety potential sites were identified, and four more *V. nonalfalfae* infested sites were confirmed in the mountain region of Virginia (Snyder et al., 2014). Comparisons between 2011 and 2012 showed disease incidences increased at all six of these Virginian sites (Snyder et al., 2014). *Verticillium dahliae* was also tentatively isolated (identified only morphologically) from five Virginian sites during this survey (Snyder et al., 2014).

In June 2012, *A. altissima* mortality was also observed in southern Ohio (Pike County) and confirmed to be caused by *V. nonalfalfae* (Rebbeck et al., 2013).

5.2 Outbreaks in Europe

In 2011 in Austria, *A. altissima* mortality in two stands located in Styria and Lower Austria Province were confirmed as being caused by *V. nonalfalfae* (Maschek and Halmschlager, 2016). This work also identified *V. dahliae* as naturally widespread, causing low levels of disease throughout the geographic range of *A. altissima* in Austria (Maschek and Halmschlager, 2017). In 2017, on the southern side of the Alps in Italy, two stands of *A. altissima* displaying

Verticillium-wilt-like symptoms were confirmed to be declining due to *V. dahliae* (Longa et al., 2019).

5.3 Potential origin of *A. altissima* pathogenicity

It is impossible to know exactly when, how, or where the relationship between *Verticillium* fungi and *A. altissima* developed. Since the invasion of the tree occurred before the first description of *Verticillium*, the exact history will never be known. Ding et al. (2006) completed a thorough review of literature of natural enemies of *A. altissima* in its Chinese native range, and was unable to find any account of Verticillium wilt disease, supporting the idea that *Verticillium* did not coevolve with the tree in China. This severe susceptibility of *A. altissima* supports the lack of coevolution between the two species (Showalter et al., 2018). It is therefore plausible that Verticillium fungi host-jumped to *A. altissima* in the pathogen's native range (and *A. altissima*'s invaded range). A similar pathogen host range jump has been demonstrated in Switzerland, where two powdery mildews appear to have successfully jumped from their known tree hosts to *A. altissima,* supporting the idea that plant pathogens exposed to new hosts may be able to effectively colonize them (Beenken, 2017).

Despite observing differences in pathogenicity at the host level, multi-locus phylogenetic analyses have not been able to detect any genetic differences within *Verticillium* species (Kasson et al., 2014). However, a recent study looking at the expressed proteome of 32 *Verticillium* species from 12 different plant hosts resulted in unique protein profiles for both species and host origin (Wickert, 2019), indicating that some evolutionary differences between isolates of the same species do exist. In fact, they found that *V. nonalfalfae* isolated from *A. altissima* clustered closely with those isolated from the *Acer pensylvanicum* (Wickert, 2019)*,* a tree native to the US, potentially identifying the original host.

6.0 *Verticillium nonalfalfae* Inderb.

6.1 Background

V. nonalfalfae Inderb. (formerly *Verticillium albo-atrum* Reinke and Berthold) has currently been confirmed in over eleven countries (including Canada, China, Japan, Netherlands, Portugal, Slovenia, UK, and USA) and on over 10 host species (alfalfa, common hops, sweet orange, cotton, hop, petunia, potato, spinach, tomato, *A. altissima*, *Pelargonium grandiflorum*, snapdragon, and wild celery) (Garibaldi et al., 2016; Giraldo and Crous, 2019; Inderbitzin and Subbarao, 2014). *Verticillium nonalfalfae* forms melanized hyphae and is morphologically indistinguishable from *V. alfalfa* which causes disease mainly on lucerne or alfalfa hosts (Inderbitzin et al., 2011a).

6.2 On *A. altissima*

The life cycle of this pathogen on *A. altissima* was studied by Schall (2008) who determined that *V. nonalfalfae* can survive in *A. altissima*-infected leaves and infected xylem over winter. For general spread, infected leaves, petioles, and seeds have been confirmed to contain *V. nonalfalfae*, though movement of leaves and petioles is minimal compared to seeds (Schall, 2008). Inoculated seedlings indicate that *V. nonalfalfae* can spread rapidly throughout the tree's xylem (Schall, 2008).

Inoculation of *V. nonalfalfae* isolates from five plant hosts (*A. altissima*, deerbrush, Chinese fruit tree, eggplant, and potato) into healthy *A. altissima* seedlings revealed that *V. nonalfalfae* isolates from hosts other than *A. altissima* are not pathogenic to *A. altissima* (Kasson et al., 2014). This indicates that the isolates causing *A. altissima* mortality may be host specific (Kasson et al., 2014).

In Pennsylvania, 100 *A. altissima* trees were inoculated with *V. nonalfalfae* isolates (collected from *A. altissima*) in 12 stands between 2006 and 2009 (Kasson et al., 2014). These inoculated trees were dead within 12 months with symptoms appearing just weeks after inoculation (Kasson et al., 2014). By 2011, mortality had spread from the initial 100 *A. altissima* trees to over 14,000 canopy *A. altissima* trees and between 10 - 15,000 *A. altissima* sprouts (Kasson et al., 2014). This rapid spread of *V. nonalfalfae* to other *A. altissima* has been attributed to functional root grafts that are frequently found between *A. altissima* trees (O'Neal and Davis, 2015). These root grafts are common even in young stands and likely increase in number as the stand ages (O'Neal and Davis, 2015). This inoculation event left the stand free of *A. altissima*, except for a few lingering vegetative sprouts (Kasson et al., 2014). Potential insect vectors that may accelerate the distance of spread appear to include *Euwallacea validus* Eichhoff and *Xylosandrus germanus* Blandford, two non-native ambrosia beetles found abundantly in declining or dead *A. altissima* (Schall, 2008).

To confirm that *V. nonalfalfae* is pathogenic to *A. altissima* throughout its North American range, *A. altissima* seeds were collected from throughout North America, germinated, and inoculated with a *V. nonalfalfae* isolate from *A. altissima* (Kasson et al., 2015). All seed sources showed susceptibility to the pathogen, indicating that large-scale host-tolerance is unlikely (Kasson et al., 2015). However, *A. altissima* on a reclaimed surface mine in southeast Ohio appeared to become tolerant of *V. nonalfalfae* after absorbing iron from the soils, indicating that environmentally acquired tolerance may be possible (Wickert, 2019). Additionally, inoculation work in Austria has shown that high temperatures may impede *V. nonalfalfae* disease progression on *A. altissima* (Maschek and Halmschlager, 2017; O'Neal and Davis, 2015).

6.3 Host range

Host range testing of *V. nonalfalfae* isolates collected from *A. altissima has* been done using two different methods: by observing species surrounding infected *A. altissima* trees in the field (natural spread) or by directly inoculating the fungus to a species either in the greenhouse or in the field. These methods help give us a good idea of risk and hazard, respectively (Kasson, 2012).

A few studies have used direct inoculations to study *V. nonalfalfae* isolates from *A. altissima.* Schall and Davis (2009b) inoculated northern red oak (*Quercus rubra*), sugar maple (*Quercus saccharum*), and white ash (*Fraxinus americana*) seedlings and found them all to be resistant (no symptoms) to *V. nonalfalfae.* They also directly inoculated trees in the field and showed that chestnut oak (*Quercus montana*), northern red oak, red maple (*Acer rubrum*), sugar maple, white ash, and yellow-poplar (*Liriodendron tulipifera*) trees produced no symptoms and were considered resistant, while striped maple (*Acer pensylvanicum*) appeared susceptible (Schall and Davis, 2009b). Kasson et al. (2015) also tested 71 non-target woody species by direct inoculation in the greenhouse or in the field. Overall, nine of these species (striped maple, autumn olive (*Elaeagnus umbellata*), black locust (*Robinia pseudoacacia*), corkwood (*Leitneria floridana*), eastern redbud (*Cercis canadensis*), Japanese maple (*Acer palmatum*), northern catalpa (*Catalpa speciosa*), Norway maple (*Acer platanoides*), and sassafras (*Sassafras albidum*)) were classified as susceptible (symptoms were observed and the isolate was re-isolated), with eight species (staghorn sumac (*Rhus typhina*), amur corktree (*Phellodendron amurense*), angelica tree (*Aralia elata*), crossvine (*Bignonia caprolata*), northern spicebush

(*Lindera benzoin*), poison-ivy (*Toxicodendron radicans*), redbay, and red elderberry (*Sambucus racemosa*)) being classified as possibly susceptible (symptoms were observed but the pathogen could not be re-isolated) (Kasson et al., 2015).

The natural spread from *V. nonalfalfae*-infected *A. altissima* to non-target species appears limited. Schall and Davis (2009b) found that surrounding inoculated *A. altissima*, only 1.3% of striped maples were in decline due to *V. nonalfalfae* infections with no other wilt symptoms observed on other tree species (Schall and Davis, 2009b). Kasson et al. (2015) surveyed 38 non-target species within an infected *A. altissima* stand, and confirmed susceptibility in 17% of devil's walking sticks and 3% of striped maples. Additionally, they observed that 16% of staghorn sumacs displayed symptoms, though fungal isolates could not be recovered to confirm pathology (Kasson et al., 2015). The long-term monitoring completed by O'Neal and Davis (2015) at a site in Pennsylvania in which *A. altissima* canopy trees had been infected with *V. nonalfalfae* for over a decade, found the area dominated by native tree species including black birch (*Betula lenta*), black locust (*Robinia pseudoacacia*), red maple, and importantly striped maple with the highest stem density (O'Neal and Davis, 2015). The only observable wilt symptoms were found on the few remaining *A. altissima* seedlings (O'Neal and Davis, 2015). The contrast between the host-range testing that showed striped maple to be susceptible from both natural spread and direct inoculation compared to the actual long-term outcomes of an inoculated stand with striped maple is important to consider (O'Neal and Davis, 2015).

7.0 *Verticillium dahliae* Kleb.

7.1 Background

Verticillium dahliae Kleb. has been found in over 25 countries including the US and China, and found in over 70 hosts including many crops species, green ash (*Fraxinus pennsylvanica*), Japanese maple, tulip tree, and *A. altissima* (Giraldo and Crous, 2019; Inderbitzin and Subbarao, 2014). It is the *Verticillium* species reported as having the greatest economic impact and largest host range (Pegg and Brady, 2002). *Verticillium dahliae* produces microsclerotia, which morphologically resemble those of *V. longisporum,* but has smaller conidia (Inderbitzin et al., 2011a).

7.2 On *A. altissima*

Verticillium dahliae is usually found in small areas of diseased *A. altissima*, causing lower levels of disease than *V. nonalfalfae* (Maschek and Halmschlager, 2017; Schall and Davis, 2009a; Snyder et al., 2014), yet is more prevalent than *V. nonalfalfae* (Maschek and Halmschlager, 2017). Within the first growing season, disease severity on *A. altissima* increases slowly, with wilting symptoms appearing on small portions of the tree (Schall and Davis, 2009a). After a full year post inoculation, mortality remained low in seedlings (around 16%) and was rare in canopy trees (Schall and Davis, 2009a). Due to the higher virulence of *V. nonalfalfae* to *A. altissima,* most research has focused on *V. nonalfalfae* and not *V. dahliae*.

7.3 Host range

Besides *A. altissima, V. dahliae* has never been reported causing serious tree mortality in a forest setting (Schall and Davis, 2009b). *Verticillium dahliae* isolates from *A. altissima* have not been tested on other species, and no additional host range information is available.

8.0 Biological control potential

Given the virulence and host specificity of *V. nonalfalfae* on *A. altissima,* research has focused on the practical biological control application of *V. nonalfalfae*. To create a liquid inoculum, artificially infested soils or agar-grown colonies can be easily mixed with water (O'Neal and Davis, 2015). Application of liquid inoculum directly to artificially wounded *A. altissima* (using a "hack n squirt" type method) is both effective and the currently preferred method of inoculation (Kasson et al., 2015; O'Neal and Davis, 2015), though soil drenching (especially with artificial root wounding) may also cause disease (Schall, 2008). As an alternative inoculum source, lab-infested rye grain placed around *A. altissima* also resulted in symptom development and mortality (Wickert, 2019). In lieu of laboratory-prepared inoculum, natural inoculum sources have also been applied to artificial wounds. In this context, the application of discolored xylem has led to disease progression, while field collected soil and symptomatic leaves have not (O'Neal and Davis, 2015). Optimal inoculation timing in Pennsylvania and Austria occurs during late spring (Maschek and Halmschlager, 2017; O'Neal and Davis, 2015), likely corresponding to just after *A. altissima* bud break.

To date, all reports indicate that *V. nonalfalfae* has the potential to serve as a highly effective biological control agent of *A. altissima* in its invaded North American range. With the hope that this alternative management option could help control *A. altissima* over a long timescale and over a wide spatial scale, work towards registering *V. nonalfalfae* as a biopesticide is ongoing. Additional research on the distribution and impact of *V. dahliae* and *V. nonalfalfae* in both naturally infested and artificially inoculated *A. altissima* stands is explored in the following chapters.

References

Albright, T.P., Chen, H., Chen, L., Guo, Q., 2010. The ecological niche and reciprocal prediction of the disjunct distribution of an invasive species: the example of *Ailanthus altissima*. Biological Invasions. 12**,** 2413-2427. https://doi.org/10.1007/s10530-009-9652-8.

Armel, G.R., Richardson, R.J., Stipes, R.J., Hipkins, P.L., 1997. Biocontrol of the invasive *Ailanthus altissima* with fungal weaponry. Virginia Journal of Science. 48.

Asaro, C., Becker, C., Creighton, J., 2009. Control and utilization of tree-of-heaven: A guide for Virginia landowners. Virginia Department of Forestry Publication P00144, Charlottesville, VA.

Beenken, L., 2017. First records of the powdery mildews *Erysiphe platani* and *E. alphitoides* on *Ailanthus altissima* reveal host jumps independent of host phylogeny. Mycological Progress. 16**,** 135-143. https://doi.org/10.1007/s11557-016-1260-2.

Berger, H., Maschek, O., Halmschlager, E., 2020. Draft genome sequences of three strains of *Verticillium nonalfalfae* exhibiting different levels of aggressiveness on *Ailanthus altissima*. Microbiology Resource Announcements. 9. https://doi.org/10.1128/MRA.01384-19.

Bhat, R.G., Subbarao, K.V., 1999. Host range specificity in *Verticillium dahliae*. Phytopathology. 89**,** 1218-1225. https://doi.org/10.1094/PHYTO.1999.89.12.1218.

Burch, P.L., Zedaker, S.M., 2003. Removing the invasive tree *Ailanthus altissima* and restoring natural cover. Journal of Arboriculture. 29**,** 18-24.

Carter, W.K., Fredericksen, T.S., 2007. Tree seedling and sapling density and deer browsing incidence on recently logged and mature non-industrial private forestlands in Virginia, USA. Forest Ecology and Management. 242**,** 671-677. https://doi.org/10.1016/j.foreco.2007.01.086.

Chuanhui, Y., Qiuju, H., Lin, W., Kuang, R., 2010. The utilization of insect-resources in Chinese rural area. Journal of Agricultural Science. 2**,** 146.

Del Tredici, P., 2017. The Introduction of Japanese plants into North America. The Botanical Review. 1-38. https://doi.org/10.1007/s12229-017-9184-3.

Ding, J.Q., Wu, Y., Zheng, H., Fu, W.D., Reardon, R., Liu, M., 2006. Assessing potential biological control of the invasive plant, tree-of-heaven, *Ailanthus altissima*. Biocontrol Science and Technology. 16**,** 547-566. https://doi.org/10.1080/09583150500531909.

Dung, J.K.S., Weiland, J., 2015. Verticillium wilt in the Pacific Northwest. Pacific Northwest Plant Disease Management Handbook. Pacific Northwest Extension.

EDDmapS. 2019. Tree-of-heaven *Ailanthus altissima* (P. Mill.) Swingle. Early Detection & Distribution Mapping System. The University of Georgia, Center for Invasive Species and Ecosystem Health. https://www.eddmaps.org/distribution/uscounty.cfm?sub=3003 (accessed Nov 2019).

Emmerich, T., Birmingham, M., Daughtrey, M., 1998. Naturally-occurring pathogen is killing *Ailanthus* (New York). Restoration and Management Notes. 16**,** 223.

Feret, P.P., Bryant, R.L., Ramsey, J.A., 1974. Genetic variation among American seed sources of *Ailanthus altissima* (Mill.) Swingle. Scientia Horticulturae. 2**,** 405-411. https://doi.org/10.1016/0304-4238(74)90047-8.

Fotiadis, G., Kyriazopoulos, A., Fraggakis, I., 2011. The behaviour of *Ailanthus altissima* weed and its effects on natural ecosystems. Journal of Environmental Ecology. 32**,** 801.

Fradin, E.F., Thomma, B.P., 2006. Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology. 7**,** 71-86. https://doi.org/10.1111/j.1364-3703.2006.00323.x.

Gaertner, M., Den Breeyen, A., Hui, C., Richardson, D.M., 2009. Impacts of alien plant invasions on species richness in Mediterranean-type ecosystems: a meta-analysis. Progress in Physical Geography. 33**,** 319-338. https://doi.org/10.1177/0309133309341607.

Gams, W., Zare, R., Summerbell, R.C., 2005. (1654) Proposal to conserve the generic name *Verticillium* (Anamorphic Ascomycetes) with a conserved type. Taxon. 54**,** 179-179. https://doi.org/10.2307/25065318.

Garibaldi, A., Bertetti, D., Pensa, P., Ortega, S.F., Gullino, M., 2016. First report of Verticillium wilt caused by *Verticillium nonalfalfae* on *Pelargonium grandiflorum* in Italy. Plant Disease. 100**,** 2322-2322. https://doi.org/10.1094/PDIS-03-16-0285-PDN.

Giraldo, A., Crous, P., 2019. Inside Plectosphaerellaceae. Studies in Mycology. 92**,** 227-286. https://doi.org/10.1016/j.simyco.2018.10.005.

Gover, A., Johnson, J., Lloyd, K., Sellmer, J., 2013. Tree-of-heaven (*Ailanthus altissima*), Quicksheet 5. Wildland Weed Management, Penn State, College of Agricultural Sciences.

Gravatt, G.F., Clapper, R.B., 1932. Verticillium wilt of maple, *Ailanthus*, and elm. Plant Disease. 96**,** 96-98.

Greer, G.K., Dietrich, M.A., Lincoln, J.M., Nicotra, A., 2016. *Ailanthus altissima* stimulates legume nodulation in *Trifolium pratense* via root exudates: a novel mechanism facilitating invasion? International Journal of Plant Sciences. 177**,** 000-000. https://doi.org/10.1086/685659.

Heisey, R.M., 1996. Identification of an allelopathic compound from *Ailanthus altissima* (Simaroubaceae) and characterization of its herbicidal activity. American Journal of Botany. 83**,** 192-200. https://doi.org/10.1002/j.537-2197.1996.tb12697.x.

Heisey, R.M., Heisey, T.K., 2003. Herbicidal effects under field conditions of *Ailanthus altissima* bark extract, which contains ailanthone. Plant and Soil Journal. 256**,** 85-99. https://doi.org/10.1023/A:1026209614161.

Hepting, G., 1971. *Ailanthus altissima*. In: Diseases of forest and shade trees in the United States, Handbook 386. USDA Forest Service, Washington, D.C. pp. 63-64.

Herrick, N.J., McAvoy, T.J., Snyder, A.L., Salom, S.M., Kok, L.T., 2012. Host-range testing of *Eucryptorrhynchus brandti* (Coleoptera: Curculionidae), a candidate for biological control of tree-of-heaven, *Ailanthus altissima*. Environmental Entomology. 41**,** 118-124. https://doi.org/10.1603/EN11153.

Hiemstra, J., Harris, D., 1998. A compendium of Verticillium wilts in tree species. Ponsen & Looijen, Wageningen, The Netherlands. ISBN 9073771250.

Hoebeke, E.R., Jendek, E., Zablotny, J.E., Rieder, R., Yoo, R., Grebennikov, V.V., Ren, L., 2017. First North American records of the Eastasian metallic wood-boring beetle *Agrilus smaragdifrons* Ganglbauer (Coleoptera: Buprestidae: Agrilinae), a specialist on tree of heaven (*Ailanthus altissima*, Simaroubaceae). Procedings of the Entomological Society of Washington. 119**,** 408-422. https://doi.org/10.4289/0013-8797.119.3.408.
Hu, S.Y., 1979. *Ailanthus*. Arnoldia. 39**,** 29-50.
Hubner, C.D., Rebbeck, J., 2014. *Ailanthus*: A non-native urban weed is causing trouble in our forests. In: NRS Research Review. Northern Research Station, USDA Forest Service. pp. 1-5.
Inderbitzin, P., Bostock, R.M., Davis, R.M., Usami, T., Platt, H.W., Subbarao, K.V., 2011a. Phylogenetics and taxonomy of the fungal vascular wilt pathogen Verticillium, with the descriptions of five new species. PLoS One. 6**,** e28341. https://doi.org/10.1371/journal.pone.0028341.
Inderbitzin, P., Davis, R.M., Bostock, R.M., Subbarao, K.V., 2011b. The ascomycete V*erticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. PLoS One. 6**,** e18260. https://doi.org/10.1371/journal.pone.0018260.
Inderbitzin, P., Subbarao, K.V., 2014. *Verticillium* systematics and evolution: how confusion impedes Verticillium wilt management and how to resolve it. Phytopathology. 104**,** 564-574. https://doi.org/10.1094/PHYTO-11-13-0315-IA.
Jelen, V., de Jonge, R., Van de Peer, Y., Javornik, B., Jakse, J., 2016. Complete mitochondrial genome of the Verticillium-wilt causing plant pathogen *Verticillium nonalfalfae*. PLoS One. 11. https://doi.org/10.1371/journal.pone.0148525.
Kasson, M.T., 2012. *Verticillium nonalfalfae*: A potential biological control of the invasive *Ailanthus altissima* in Pennsylvania. PhD. The Pennsylvania State University. https://etda.libraries.psu.edu/catalog/16084.
Kasson, M.T., Davis, M.D., Davis, D.D., 2013a. The invasive *Ailanthus altissima* in Pennsylvania: A case study elucidating species introduction, migration, invasion, and growth patterns in the Northeastern US. Northeast Naturalist. 20**,** 1-60. https://doi.org/10.1656/045.020.m101.
Kasson, M.T., Kasson, L.R., Wickert, K.L., Davis, D.D., Stajich, J.E., 2019. Genome sequence of a lethal vascular wilt fungus, *Verticillium nonalfalfae*, a biological control used against the invasive *Ailanthus altissima*. Microbiology Resource Announcements. 8**,** e01619-01618. https://doi.org/10.1128/MRA.01619-18.
Kasson, M.T., O'Donnell, K., Rooney, A.P., Sink, S., Ploetz, R.C., Ploetz, J.N., Konkol, J.L., Carrillo, D., Freeman, S., Mendel, Z., 2013b. An inordinate fondness for Fusarium: phylogenetic diversity of fusaria cultivated by ambrosia beetles in the genus *Euwallacea* on avocado and other plant hosts. Fungal Genetics and Biology. 56**,** 147-157. https://doi.org/10.1016/j.fgb.2013.04.004.
Kasson, M.T., O'Neal, E.S., Davis, D.D., 2015. Expanded host range testing for *Verticillium nonalfalfae*: potential biocontrol agent against the invasive *Ailanthus altissima*. Plant Disease. 99**,** 823-835. https://doi.org/10.1094/PDIS-04-14-0391-RE.
Kasson, M.T., Short, D.P., O'Neal, E.S., Subbarao, K.V., Davis, D.D., 2014. Comparative pathogenicity, biocontrol efficacy, and multilocus sequence typing of *Verticillium nonalfalfae* from the invasive *Ailanthus altissima* and other hosts. Phytopathology. 104**,** 282-292. https://doi.org/10.1094/PHYTO-06-13-0148-R.

Keykhasaber, M., Thomma, B.P., Hiemstra, J.A., 2017. Verticillium wilt caused by *Verticillium dahliae* in woody plants with emphasis on olive and shade trees. Eur. J. Plant Pathology., 1-17. https://doi.org/10.1007/s10658-017-1273-y.

Knapp, L.B., Canham, C.D., 2000. Invasion of an old-growth forest in New York by *Ailanthus altissima*: sapling growth and recruitment in canopy gaps. Journal of the Torrey Botanical Society., 307-315. https://doi.org/10.2307/3088649.

Knüsel, S., Conedera, M., Rigling, A., Fonti, P., Wunder, J., 2015. A tree-ring perspective on the invasion of *Ailanthus altissima* in protection forests. Forest Ecology and Management. 354, 334-343. https://doi.org/10.1016/j.foreco.2015.05.010.

Knusel, S., De Boni, A., Conedera, M., Schleppi, P., Thormann, J.J., Frehner, M., Wunder, J., 2017. Shade tolerance of *Ailanthus altissima* revisited: novel insights from southern Switzerland. Biological Invasions. 19, 455-461. https://doi.org/10.1007/s10530-016-1301-4.

Kok, L.T., Salom, S.M., Yan, S., Herrick, N.J., Mcavoy, T.J., 2008. Quarantine evaluation of *Eucryptorrhynchus brandti* (Harold) (Coleoptera : Curculionidae), a potential biological control agent of tree ofheaven, *Ailanthus altissima*, in Virginia, USA. Proceedings of the XII International Symposium on Biological Control of Weeds. 292-300.

Kota, N.L., Landenberger, R.E., McGraw, J.B., 2007. Germination and early growth of *Ailanthus* and tulip poplar in three levels of forest disturbance. Biological Invasions. 9, 197-211. https://doi.org/10.1007/s10530-006-9026-4.

Kowarik, I., 1995. Clonal growth in *Ailanthus altissima* on a natural site in West Virginia. Journal of Vegetable Science. 6, 853-856.

Kowarik, I., Säumel, I., 2007. Biological flora of Central Europe: *Ailanthus altissima* (Mill.) Swingle. Perspectives in Plant Ecology, Evolution, and Systematics. 8, 207-237. https://doi.org/10.1016/j.ppees.2007.03.002.

Kowarik, I., Säumel, I., 2008. Water dispersal as an additional pathway to invasions by the primarily wind-dispersed tree *Ailanthus altissima*. Plant Ecology. 198, 241-252. https://doi.org/10.1007/s11258-008-9398-x.

Kowarik, I., Von der Lippe, M., 2011. Secondary wind dispersal enhances long-distance dispersal of an invasive species in urban road corridors. NeoBiota. 9, 49. https://doi.org/10.3897/neobiota.9.1469.

Landenberger, R.E., Kota, N.L., McGraw, J.B., 2007. Seed dispersal of the non-native invasive tree *Ailanthus altissima* into contrasting environments. Plant Ecology. 192, 55-70. https://doi.org/10.1007/s11258-006-9226-0.

Laskar, S., 2010. A brief resume on the genus *Ailanthus*: chemical and pharmacological aspects. Phytochemistry Reviews. 9, 379-412. https://doi.org/10.1007/s11101-009-9157-1.

Lawrence, J.G., Colwell, A., Sexton, O.J., 1991. The ecological impact of allelopathy in *Ailanthus altissima* (Simaroubaceae). American Journal of Botany. 78, 948-958. https://doi.org/10.2307/2445173.

Longa, C.M.O., Pietrogiovanna, M., Minerbi, S., Andriolo, A., Tolotti, G., Maresi, G., 2019. First observation of Verticillium wilt on *Ailanthus altissima* in the Eastern Italian Alps (Trentino-South Tyrol). Journal of Plant Pathology. 101, 757-757. https://doi.org/10.1007/s42161-018-00217-y.

Maschek, O., Halmschlager, E., 2016. First Report of Verticillium Wilt on *Ailanthus altissima* in Europe Caused by *Verticillium nonalfalfae*. Plant Disease. 100**,** 529. https://doi.org/10.1094/PDIS-07-15-0733-PDN.

Maschek, O., Halmschlager, E., 2017. Natural distribution of Verticillium wilt on invasive *Ailanthus altissima* in eastern Austria and its potential for biocontrol. Forest Pathology. 47**,** 1-11. https://doi.org/10.1111/efp.12356.

McAvoy, T.J., Snyder, A.L., Johnson, N., Salom, S.M., Kok, L.T., 2012. Road survey of the invasive tree-of-heaven (*Ailanthus altissima*) in Virginia. Invasive Plant Science and Management. 5**,** 506-512. https://doi.org/10.1614/Ipsm-D-12-00039.1.

Meloche, C., Murphy, S.D., 2006. Managing tree-of-heaven (*Ailanthus altissima*) in parks and protected areas: a case study of Rondeau Provincial Park (Ontario, Canada). Environmental Management. 37**,** 764-772. https://doi.org/10.1007/s00267-003-0151-x.

Mergen, F., 1959. A toxic principle in the leaves of Ailanthus. Botanical Gazette. 121**,** 32-36. https://doi.org/10.1086/336038.

Miller, J.H., 1990. *Ailanthus altissima* (Mill.) Swingle. In: Silvics of North America. pp. 101-104.

Morehart, A., Melchior, G., 1982. Influence of water stress on Verticillium wilt of yellow-poplar. Canadian Journal of Botany. 60**,** 201-209. https://doi.org/10.1139/b82-027.

Motard, E., Dusz, S., Geslin, B., Akpa-Vinceslas, M., Hignard, C., Babiar, O., Clair-Maczulajtys, D., Michel-Salzat, A., 2015. How invasion by *Ailanthus altissima* transforms soil and litter communities in a temperate forest ecosystem. Biological Invasions. 17**,** 1817-1832. https://doi.org/10.1007/s10530-014-0838-3.

Motard, E., Muratet, A., Clair-Maczulajtys, D., MacHon, N., 2011. Does the invasive species *Ailanthus altissima* threaten floristic diversity of temperate peri-urban forests? Comptes Rendus Biologies. 334**,** 872-879. https://doi.org/10.1016/j.crvi.2011.06.003.

Mousavi, F., Majd, A., Shahali, Y., Ghahremaninejad, F., Shokouhi Shoormasti, R., Pourpak, Z., 2017. Immunoproteomics of tree of heaven (*Ailanthus atltissima*) pollen allergens. Journal of Proteomics. 154**,** 94-101. http://dx.doi.org/10.1016/j.jprot.2016.12.013.

Nilsen, E.T., Huebner, C.D., Carr, D.E., Bao, Z., 2018. Interaction between *Ailanthus altissima* and native *Robinia pseudoacacia* in early succession: Implications for forest management. Forests. 9**,** 221. https://doi.org/10.3390/f9040221.

O'Neal, E.S., Davis, D.D., 2015. Biocontrol of *Ailanthus altissima*: Inoculation protocol and risk assessment for *Verticillium nonalfalfae* (Plectosphaerellaceae: Phyllachorales). Biocontrol Science and Technology. 25**,** 950-969. https://doi.org/10.1080/09583157.2015.1023258.

Pegg, G.F., Brady, B.L., 2002. Verticillium wilts. CABI Publishing, New York. ISBN 0–85199–529–2.

Petruzzellis, F., Peng, G., Tyree, M.T., Tonet, V., Savi, T., Torboli, V., Pallavicini, A., Bacaro, G., Nardini, A., 2018. Plasticity of functional traits of tree of heaven is higher in exotic than in native habitats. Trees. 1-10. https://doi.org/10.1007/s00468-018-1787-8.

Planchuelo, G., Catalan, P., Delgado, J.A., 2016. Gone with the wind and the stream: Dispersal in the invasive species *Ailanthus altissima*. Acta Oecologica. 73**,** 31-37. https://doi.org/10.1016/j.actao.2016.02.006.

Rebbeck, J., Jolliff, J., 2018. How long do seeds of the invasive tree, *Ailanthus altissima* remain viable? Forest Ecology and Management. 429**,** 175-179. https://doi.org/10.1016/j.foreco.2018.07.001.

Rebbeck, J., Malone, M.A., Short, D.P.G., Kasson, M.T., O'Neal, E.S., Davis, D.D., 2013. First report of Verticillium wilt caused by *Verticillium nonalfalfae* on tree-of-heaven (*Ailanthus altissima*) in Ohio. Plant Disease. 97**,** 999-999. https://doi.org/10.1094/pdis-01-13-0062-pdn.
Richardson, D.M., Rejmánek, M., 2011. Trees and shrubs as invasive alien species – a global review. Diversity and Distributions. 17**,** 788-809. https://doi.org/10.1111/j.1472-4642.2011.00782.x.
Rohe, R., 1984. Just scratching the surface: geographers and the mining west. The Geographical Bulletin. 26**,** 35.
Schall, M.J., 2008. Verticillium wilt of *Ailanthus altissima*. Dissertation, Ph.D. The Pennsylvania State University. https://etda.libraries.psu.edu/catalog/8954.
Schall, M.J., Davis, D.D., 2009a. *Ailanthus altissima w*ilt and mortality: etiology. Plant Disease. 93**,** 747-751. https://doi.org/10.1094/Pdis-93-7-0747.
Schall, M.J., Davis, D.D., 2009b. Verticillium wilt of *Ailanthus altissima*: susceptibility of associated tree species. Plant Disease. 93**,** 1158-1162. https://doi.org/10.1094/Pdis-93-11-1158.
Sewell, G.W.F., Wilson, J.F., 1966. Verticllium wilt of the hop: the survival of *V. albo-atrum* in soil. Annals of Applied Biology. 58**,** 241-249. https://doi.org/10.1111/j.1744-7348.1966.tb04383.x.
Showalter, D.N., Raffa, K.F., Sniezko, R.A., Herms, D.A., Liebhold, A.M., Smith, J.A., Bonello, P., 2018. Strategic development of tree resistance against forest pathogen and insect invasions in defense-free space. Frontiers in Ecology and Evolution. 6**,** 124. https://doi.org/10.3389/fevo.2018.00124.
Snyder, A.L., Kasson, M.T., Salom, S.M., Davis, D.D., Griffin, G.J., Kok, L.T., 2013. First report of Verticillium wilt of *Ailanthus altissimain* Virginia caused by *Verticillium nonalfalfae*. Plant Disease. 97**,** 837-837. https://doi.org/10.1094/pdis-05-12-0502-pdn.
Snyder, A.L., Salom, S.M., Kok, L.T., 2014. Survey of *Verticillium nonalfalfae* (Phyllachorales) on tree-of-heaven in the southeastern USA. Biocontrol Science and Technology. 24**,** 303-314. https://doi.org/10.1080/09583157.2013.860426.
Snyder, A.L., Salom, S.M., Kok, L.T., Griffin, G.J., Davis, D.D., 2012. Assessing *Eucryptorrhynchus brandti* (Coleoptera: Curculionidae) as a potential carrier for *Verticillium nonalfalfae* (Phyllachorales) from infected *Ailanthus altissima*. Biocontrol Science and Technology. 22**,** 1005-1019. https://doi.org/10.1080/09583157.2012.707639.
Song, S., Kim, S., Kwon, S.W., Lee, S.-I., Jablonski, P.G., 2018. Defense sequestration associated with narrowing of diet and ontogenetic change to aposematic colours in the spotted lanternfly. Scientific Reports. 8**,** 16831. https://doi.org/10.1038/s41598-018-34946-y.
Swingle, W.T., 1916. The early European history and the botanical name of the tree of heaven, Ailanthus altissima. Journal of the Washington Academy of Sciences. 6**,** 460-498.
Traveset, A., Brundu, G., Carta, L., Mprezetou, I., Lambdon, P., Manca, M., Médail, F., Moragues, E., Rodríguez-Pérez, J., Siamantziouras, A.-S.D., 2008. Consistent performance of invasive plant species within and among islands of the Mediterranean basin. Biological Invasions. 10**,** 847-858. https://doi.org/10.1007/s10530-008-9245-y.
Vilà, M., Tessier, M., Suehs, C.M., Brundu, G., Carta, L., Galanidis, A., Lambdon, P., Manca, M., Médail, F., Moragues, E., 2006. Local and regional assessments of the impacts of plant invaders on vegetation structure and soil properties of Mediterranean islands.

Journal of Biogeography. 33, 853-861. https://doi.org/10.1111/j.1365-2699.2005.01430.x.
Walker, G., Gaertner, M., Robertson, M., Richardson, D., 2017. The prognosis for *Ailanthus altissima* (Simaroubaceae; tree of heaven) as an invasive species in South Africa; insights from its performance elsewhere in the world. South African Journal of Botany. 112, 283-289.
Wickert, K.L., 2019. Elucidating disease dynamics in the biocontrol of *Ailanthus altissima* while confiriming the host specificity of the vascular wilt pathogen *Verticillium nonalfaflae*. Dissertation, Ph.D. West Virignia University. https://researchrepository.wvu.edu/etd/3854/.
Wickert, K.L., O'Neal, E.S., Davis, D.D., Kasson, M.T., 2017. Seed production, viability, and reproductive limits of the invasive *Ailanthus altissima* (tree-of-heaven) within invaded environments. Forests. 8. https://doi.org/10.3390/f8070226.
Wilhelm, S., 1955. Longevity of the Verticillium wilt fungus in the laboratory and field. Phytopathology. 45, 180-181. <Go to ISI>://WOS:A1955WJ01900013.
Wu, Z.Y., Raven, P.H., Hong, D.Y., 2008. Simaroubaceae. In: Flora of China, Vol 11: Oxalidaceae through Aceraceae. Missouri Botanical Garden Press, St. Louis, MO. ISBN 978-1-930723-73-3.

Chapter 2: First report of Verticillium wilt caused by *Verticillium dahliae* impacting *Ailanthus altissima* in Virginia, U.S.A.[1]

R. K. Brooks[1]

A. L. Snyder[2]

E. A. Bush[1]

S. M. Salom[2]

A. Baudoin[1]

[1]School of Plant and Environmental Sciences, Virginia Tech, Blacksburg, VA 24061

[2]Department of Entomology, Virginia Tech, Blacksburg, VA 24061

Author contribution statement:

Brooks: Conceptualization, Methodology, Analysis, Writing – original draft. **Snyder:** Methodology, Writing – review & editing. **Bush:** Methodology, Writing – review & editing. **Salom:** Conceptualization, Funding acquisition, Writing – review & editing. **Baudoin:** Conceptualization, Methodology, Funding acquisition, Writing – review & editing.

1.0 Note

The invasive tree of heaven, *Ailanthus altissima* (Miller) Swingle, was first confirmed dying from Verticillium wilt (caused by both *Verticillium nonalfalfae* Inderb. et al. formerly *V. albo-atrum* Reinke & Berthold [Inderbitzin et al. 2011] and *V. dahliae* Kleb.) in Pennsylvania, U.S.A., in 2002 (Schall et al. 2009). Following this, a Virginia, U.S.A., survey found and confirmed the reportedly more virulent *V. nonalfalfae* infecting *A. altissima* and also collected putative *V. dahliae* isolates, identified morphologically based on the production of brown-pigmented microsclerotia and verticillate whorls that amassed oval conidia (Snyder et al. 2013,

---

[1]Reprinted with permission from: Brooks, R.K., Snyder, A.L., Bush, E.A., Salom S.M., Baudoin A., 2020. First report of Verticillium wilt caused by Verticillium dahliae impacting Ailanthus altissima in Virginia, US. Plant Disease. https://doi.org/10.1094/PDIS-10-19-2064-PDN.

Reprinted with permission from: Brooks, R.K., Snyder, A.L., Bush, E.A., Salom S.M., Baudoin A., 2020. First report of Verticillium wilt caused by Verticillium dahliae impacting Ailanthus altissima in Virginia, US. Plant Disease. https://doi.org/10.1094/PDIS-10-19-2064-PDN.

[2]Reprinted with permission from: Brooks, R.K., Wickert, K.L., Baudoin, A., Kasson, M.T., Salom, S.M., 2020.

2014). Now, with increasing attention on the biological control potential of these pathogens, we have completed molecular identification and Koch's postulates for one of these *V. dahliae* isolates (VdAaVA2) collected from *A. altissima* displaying typical Verticillium wilt symptoms (chlorotic and necrotic leaflets, severe wilt, defoliation, canopy dieback, and vascular discoloration) in Nelson County, VA (37.842, –78.964) in 2009.

VdAaVA2 was confirmed as *V. dahliae* by amplification of a portion of the ITS gene region via a multiplex PCR for *V. dahliae-V. longisporum* lineages (Inderbitzin et al. 2013). This resulted in the expected amplification product (490 bp), which was sequenced bidirectionally. The consensus sequence had a 100% match and a 78% query coverage with the *V. dahliae* type PD322 sequence (GenBank NR_126124, Inderbitzin et al. 2013). In addition, conidia measuring 5.9 ± 0.9 μm (n = 100) distinguished the isolate from *V. longisporum*. The consensus sequence from VdAaVA2 was deposited into GenBank as MN496381, and voucher specimens of VdAaVA2 and VnAa200 were submitted to the ARS Culture Collection (NRRL 66917 and 66918; Peoria, IL).

VdAaVA2 was used for a comparative pathogenicity four-treatment greenhouse test in which 3-month-old *A. altissima* trees were syringe inoculated with 0.1 ml of a suspension of 107 conidia/ml of VdAaVA2, *V. nonalfalfae* isolate VnAa200 (Snyder et al. 2013), a 1:1 combination of the two isolates, or water, with 20 trees per treatment. The trees were placed into a stacked Latin square design and monitored weekly. Trees were destructively sampled when they died or at 15 weeks postinoculation. At the end of the experiment, nine VdAaVA2, 15 VnAa200, and 15 combination-inoculated trees had died after showing typical Verticillium wilt symptoms. An additional three VdAaVA2, two VnAa200, and four combination-inoculated trees were symptomatic. The remaining trees (n = 32), including all of the controls (n = 20), remained healthy. Treatment differences were significant (Fisher's exact test, P = 9.20e-08), and a posthoc pairwise comparison using a Bonferroni correction (R Core Team 2018) showed that all *Verticillium* spp. treatments were different from the control but not different from each other (α = 0.05). The morphology of isolates recovered from inoculated trees matched that of the initial inoculum (VdAaVA2 n = 17, VnAa200 n = 19) with combination treatment yielding only *V. nonalfalfae* (n = 18) and control yielding no *Verticillium* spp. Only *V. nonalfalfae* was reisolated from the combination treatment, indicating that VnAa200 outcompeted VdAaVA2, although tree survival compared with the other Verticillium treatments did not change.

Confirmation that *V. dahliae* causes disease on *A. altissima* in Virginia is important for understanding *Verticillium* spp. host range and for biopesticide registration efforts.

2.0 References

Inderbitzin, P., et al. 2011. PLoS ONE. 6:e28341. doi:/10.1371/journal.pone.0028341
Inderbitzin, P., et al. 2013. PLoS ONE. 8:e65990. doi:/10.1371/journal.pone.0065990
R Core Team. 2018. R: R Foundation for Statistical Computing. https://www.R-project.org/
Schall, M.J., et al. 2009. Plant Dis. 93:747. doi:/10.1094/PDIS-93-7-0747
Snyder, A.L., et al. 2013. Plant Dis. 97:837. doi:/10.1094/PDIS-05-12-0502-PDN
Snyder, A.L., et al. 2014. Biocontrol Sci Technol. 24:303. doi:/10.1080/09583157.2013.860426

3.0 Figures

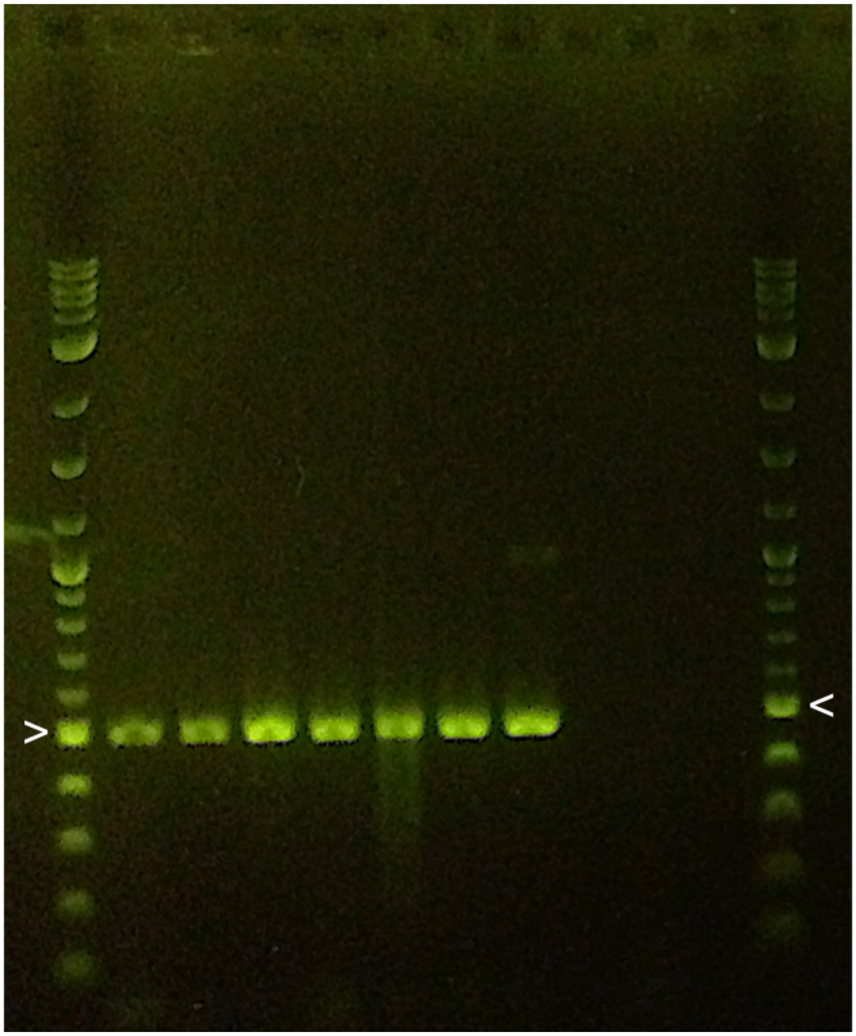


Figure S1. Multiplex PCR assays amplification results. Lanes 1 & 12: 2-log DNA ladder. Lane 2, 4, 5, 6, 7, & 8: other *Verticillium* spp. isolates. Lane 3: VdAaVA2 isolate used for Koch's postulate fulfillment. Lane 9 & 11: blank. Lane 10: nuclease free water & master mix control. Relevant size marker of 500bp indicated by ">" or "<".

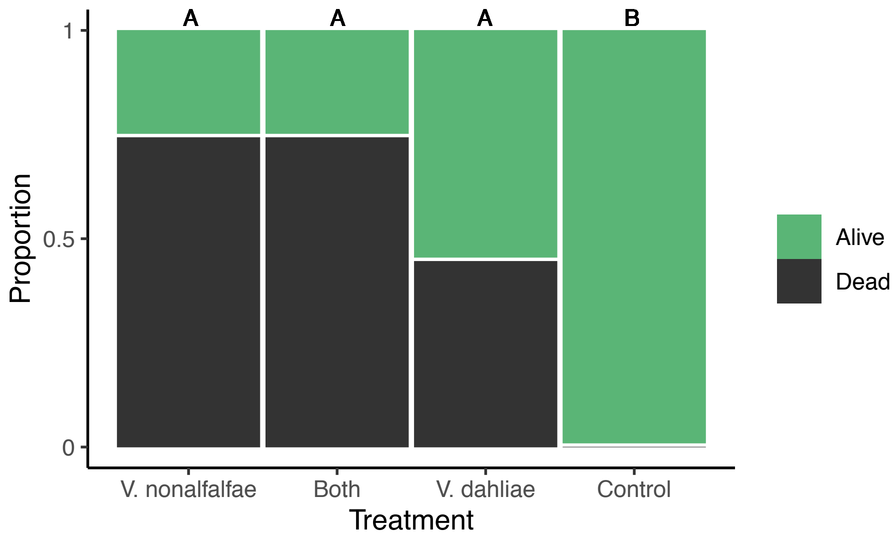


Figure S2. Mosaic plot displaying the proportion of *A. altissima* alive or dead for each inoculated treatment (*V. dahliae* isolate VdAaVA2, a 1:1 combination of the 2 species, *V. nonalfalfae* isolate VnAa200, or distilled water for a control) at 15 weeks post inoculation. Though trees were rated using an ordinal scale (1= non-symptomatic leaves, 2=wilting leaflets, 3=chlorotic/necrotic leaflets, 4=defoliating leaves, and 5=seedling mortality as detailed by Snyder et al. (2014)), analysis was completed at the end of the experiment on the number of alive and dead trees. Analysis using Fisher's Exact Test followed by a post-hoc pairwise comparison using a Bonferroni correction showed that all Verticillium treatments were significantly different than the control treatment, but not significantly different from each other, as indicated by the letters above each column (R Core Team 2018).

Chapter 3: Field-inoculated *Ailanthus altissima* stands reveal the biological control potential of *Verticillium nonalfalfae* in the mid-Atlantic region of the United States[2]

Rachel K. Brooks[a] (ORCID: 0000-0001-5172-7267)
Kristen L. Wickert[b]
Anton Baudoin[a]
Matt T. Kasson[b]
Scott Salom[c]

[a] Virginia Tech, School of Plant and Environmental Sciences, Blacksburg, VA, USA
[b] West Virginia University, Division of Plant and Soil Sciences, Morgantown, WV, USA
[c] Virginia Tech, Department of Entomology, Blacksburg, VA, USA

Authorship contribution statement

Brooks: Conceptualization, Data curation, Methodology, Formal analysis, Visualization, Writing – original draft. **Wickert:** Conceptualization, Data curation, Methodology, Writing – original draft. **Baudoin:** Methodology, Supervision, Writing – review & editing. **Kasson:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Salom:** Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Highlights

- Assessed regional biological control effectiveness against *Ailanthus altissima.*
- Used two naturally occurring fungi, *Verticillium nonalfalfae* and *V. dahliae.*
- *V. nonalfalfae* proved to be effective regardless of climate of stand differences.
- Inoculation of both *Verticillium* spp. did not change *V. nonalfalfae* effectiveness.

---

[2] Reprinted with permission from: Brooks, R.K., Wickert, K.L., Baudoin, E.B., Kasson, M.T., Salom S.M., 2020. Field-inoculated *Ailanthus altissima* stands reveal the biological control potential of *Verticillium nonalfalfae* in the mid-Atlantic region of the United States. Biological Control. https://doi.org/10.1016/j.biocontrol.2020.104298.

Abstract

Ailanthus altissima, perhaps the best-known example of an entrenched invasive weed tree in North America, was introduced to the Eastern U.S. roughly 240 years ago. The biological control of *A. altissima* has been a topic of interest since the discovery of a destructive naturally occurring Verticillium wilt disease of *A. altissima* in 2002. After nearly 20 years of research, an augmentative commercial release of this disease agent, *Verticillium nonalfalfae*, could be initiated in the near future. However, a few questions still remain: i) does the interaction of *V. nonalfalfae* with the less virulent *V. dahliae* inhibit the biological control effectiveness of *V. nonalfalfae*, and ii) do climate and *A. altissima* stand variables affect this biological control's efficacy? To help answer these questions, a three-year field inoculation study including 3,245 *A. altissima* trees in 13 sites across four hardiness zones of Pennsylvania and Virginia, U.S. was implemented. Disease progressed and spread at similar rates in *A. altissima* trees co-inoculated with *V. nonalfalfae* and *V. dahliae* as those inoculated with *V. nonalfalfae* alone, with no indication of disease progression changing in co-inoculated trees. *Verticillium dahliae* alone resulted in lower levels of disease, and no disease spread. Similar results were seen in a supplemental greenhouse inoculation study. Despite slight regional variation of disease progression and spread correlated to climate or stand variables, *V. nonalfalfae* always caused severe disease and spread rapidly to other *A. altissima* trees through the forested plots. Our results support the use of *V. nonalfalfae* as a biological control agent throughout the mid-Atlantic region of the U.S. regardless of stand and climate variables, and including sites where trees are already infected with *V. dahliae*.

Keywords

Tree of heaven, biopesticide, Verticillium wilt, invasive, nonnative, *Verticillium dahliae*

1.0 Introduction

1.1 The invasive *Ailanthus altissima*

Ailanthus altissima (Miller) Swingle, commonly known as the tree-of-heaven, is native to most regions of China (Wu et al., 2008). After being intentionally introduced to Pennsylvania, US in 1784 as a prized ornamental tree (Hu, 1979; Kasson et al., 2013a), it can now be found in over 40 US states with its highest densities around the mid-Atlantic region (EDDmapS, 2019). Similarly, *A. altissima* is now established on all continents except Antarctica (Kowarik and Säumel, 2007).

This tree's aggressive growth (Kasson et al., 2013a; Wu et al., 2008), vegetative reproduction (Hu, 1979), and prolific seed production (Wickert et al., 2017) can result in building and infrastructure damage (Hu, 1979; Kowarik and Säumel, 2007), overtaken agricultural lands (Hepting, 1971), and obstructed line-of-sights (Burch and Zedaker, 2003). Its brittle and weak wood (Hepting, 1971), allelochemical production (Heisey, 1996; Heisey and Heisey, 2003; Lawrence et al., 1991; Mergen, 1959), and ability to outcompete native plants, including the regeneration of oaks (*Quercus* spp.) (Huebner and Rebbeck, 2014), can lead to the reduction of wildlife habitat and timber resources. Its role as a preferred host for several invasive insects, including the spotted lanternfly (*Lycorma delicatula* (White)) (Song et al., 2018), the brown marmorated stink bug (*Halyomorpha halys* (Stål)) (Wallner et al., 2014), the Asiatic shot-hole borer (*Euwallacea validus* (Eichoff)) (Kasson et al., 2013b), and the East Asian buprestid (*Agrilus smaragdifrons* (Ganglbauer)) (Hoebeke et al. 2017) also makes it undesirable. Therefore, the control and management of this tree is highly desired, if not required, as *A. altissima* is currently listed on 14 US state noxious weed lists (nationalplantboard.org/laws-and-regulations/ accessed Dec 2019).

Current recommended control methods include a combination of both mechanical and chemical techniques (Asaro et al., 2009; Gover et al., 2013), which can only reasonably manage small-scale infestations. No biological control methods are available, and management over large spatial scales is currently impractical (Asaro et al., 2009; Gover et al., 2013).

1.2 A promising biological control agent

Within the past 20 years, both *Verticillium nonalfalfae* Inderb. (formerly "*V. albo-atrum*" Reinke & Berthold) and *V. dahliae* Kleb. have been found impacting A. altissima in three US states: Pennsylvania (Schall and Davis, 2009a); Virginia (Snyder et al., 2013); and Ohio (Rebbeck et al., 2013), in addition to Austria in Europe (Maschek and Halmschlager, 2017). No reports of *Verticillium* spp. impacting *A. altissima* in its native range of China have been documented.

In Pennsylvania, surveys of co-occurring plants in the field and artificial inoculation trials determined that *V. nonalfalfae* isolated from *A. altissima* is predominantly host-specific (Kasson et al., 2015; Kasson et al., 2014; O'Neal and Davis, 2015; Schall and Davis, 2009b). Additionally, it was determined that this *V. nonalfalfae* isolate is significantly more virulent to *A. altissima* than *V. dahliae* (Schall and Davis, 2009a), is easily and effectively inoculated into *A. altissima* (O'Neal and Davis, 2015), and spreads rapidly through functional root grafts (O'Neal and Davis, 2015). Notably, within just a few years, *V. nonalfalfae* with minimal maintenance or additional inputs appeared to effectively remove *A. altissima* from a forest system (Kasson et al., 2015; Kasson et al., 2014; Schall and Davis, 2009a). Due to this promising biological control potential, biopesticide registration efforts of the Pennsylvania *V. nonalfalfae* isolate PSU140/VnAa140/NRRL66861 have been initiated, including the recent sequencing of its genome (Kasson et al. 2019).

1.3 Regional effectiveness unknown

Despite the promising research from Pennsylvania, the large-scale regional effectiveness of this biological control still remains to be confirmed. For example, even though both *V. nonalfalfae* and *V. dahliae* have been found co-occurring in *A. altissima* stands, their interaction within the vascular system of a single plant is unknown. This interaction could potentially result in hybridization or an increase or decrease in disease progression. Hybridization is not unheard of among closely related plant pathogenic fungi (Stukenbrock, 2016). In fact, hybridization has been reported previously within the *Verticillium* genus when the haploid *V. dahliae* and another unknown *Verticillium* spp. hybridized to form the diploid *V. longisporum*, which has an expanded host range (Inderbitzin et al., 2011b). No other hybridization events within the genus *Verticillium* (either in vitro or in vivo) have been reported. As for co-occurring pathogens influencing disease progression, the available biological control agent *V. albo-atrum* WCS850 (sold as DutchTrig®) is used commercially to exclude the Dutch elm disease vascular wilt pathogens (*Ophiostoma ulmi* and *O. novo-ulmi*) from elm trees (*Ulmus* spp.; Postma and Goossen-van de Geijn, 2016). Similarly, co-inoculations of different *V. dahliae* isolates have been shown to affect disease expression and fungal colonization of potatoes (Wheeler and Johnson, 2019). Alternatively, an increase in control is possible, and appears relatively common when biological control agents are combined (Xu et al., 2011).

The regional effectiveness of *Verticillium* spp. as biological control agents are unknown throughout *A. altissima*'s range. In North America, where climates vary greatly, *A. altissima* can be found throughout most of the U.S. (EDDmapS, 2019). In contrast, *V. nonalfalfae* impacting *A. altissima* has only been confirmed in three mid-Atlantic states in locations ranging from plant hardiness zone 6a to 7a. Nevertheless, seed sources from across the U.S. have proven susceptible to *V. nonalfalfae* (Kasson et al. 2015). Regardless of host, research has shown that *V. dahliae* is usually found in warmer climates and can maintain growth at higher temperatures than "*V. albo-atrum*" (Fradin and Thomma, 2006; Inderbitzin et al., 2011a; Maschek and Halmschlager, 2017;

Pegg and Brady, 2002). As for moisture influencing disease progression, drought has been shown to exacerbate *Verticillium* wilt symptoms in *Liriodendron tulipifera* (L.) (Morehart and Melchior, 1982) while flooding may stress *A. altissima* directly allowing for increased symptomology (Marks and Van Driesche, 2016).

Local stand level characteristics of *A. altissima* may also influence disease progression. Within this study system, it is known that *V. nonalfalfae*-inoculated *A. altissima* seedlings die faster than inoculated *A. altissima* canopy trees, likely due to the pathogen's ability to faster colonize the smaller diameter and volume of the xylem tissue (Schall and Davis, 2009a). Similarly, younger almond orchards and small diameter maple trees tend to be more severely impacted by Verticillium wilt diseases than older almond orchards and larger diameter maple trees (Sinclair et al., 1981; Stapleton, 1997). Vessel arrangement and number of rings present in active sapwood also influence disease dynamics (Kasson et al. 2015).

1.4 Goal of research

To determine if the presence of *V. dahliae* inhibits the biological control effectiveness of *V. nonalfalfae* and to establish if climate and *A. altissima* stand variables affect this biological control's efficacy, we implemented a field inoculation study on *A. altissima*-dominated stands found in Virginia and Pennsylvania. We then studied the relationship between *V. nonalfalfae* and *V. dahliae* and disease severity through the expanded hardiness zone range of 5b to 7b. We hypothesized that *V. nonalfalfae* would be an effective biological control agent throughout the region, regardless of its interaction with *V. dahliae*.

2.0 Materials and methods

2.1 Site selection & mapping

We located 12 forested sites dominated by *A. altissima* canopy throughout Virginia and Pennsylvania on both private and public land (Fig. 1, Supplemental Table 1). These sites contained healthy *A. altissima* trees without symptoms of Verticillium wilt and had the appropriate permissions granted to allow access for three consecutive field seasons. At each site, four circular 0.04-hectare (0.1-acre) plots dominated by *A. altissima* (containing a minimum of 10 trees with a diameter at breast height (DBH) $\geq$ 2.5 cm) were established if possible. The exact number of plots located at each site depended on available *A. altissima* abundance and distribution (Supplemental Table 1). A distance of at least 20 m was maintained between plots.

In the spring of 2017, all plot centers were physically marked and their latitude and longitude recorded. Within a radius of 11.4 m, all living *A. altissima* trees with a DBH ≥ 2.5 cm were mapped (by recording the distance and azimuth from the center of the plot) and labeled with a unique ID number (Supplemental Fig. 1). Additionally, the DBH of each tree was recorded. A total of 2,661 trees in Virginia and 584 trees in Pennsylvania were utilized for this study. A list of all co-occurring woody plants was recorded at the final site visit. Site and plot information are detailed in Supplemental Tables 1 & 2.

2.2 Inoculation

Due to regulations limiting movement of plant pathogens across state borders, Virginia inoculum was prepared using isolates collected in Virginia: *V. nonalfalfae* VnAa200/NRRL66918 (Snyder et al., 2013) and *V. dahliae* VdAaVA2/NRRL66917 (Brooks et al., 2019), while Pennsylvania inoculum was prepared using isolates collected in Pennsylvania: *V. nonalfalfae* PSU140/VnAa140/NRRL66861 and *V. dahliae* PSU154 (Schall and Davis, 2009a). Pure colonies of these isolates were grown for one to three weeks on prune extract agar amended with streptomycin sulfate and neomycin sulfate (PEA + SN) to produce large amounts of conidia (Talboys, 1960). Sterile 0.1% peptone in water was then used to suspend and dilute the conidia to 10^7 conidia ml^{-1}. Inocula of *V. nonalfalfae* only, a 1:1 mixture of both *Verticillium* spp. (“combination”), *V. dahliae* only, and a control of sterile 0.1% peptone were created for each state. Inocula were kept at 4°C or on ice and used within three days. Viability of conidial suspensions was confirmed to be over 75% both before and after daily use in the field by plating on water or potato dextrose agar.

To create a randomized block design, plots at each site were randomly assigned to one of the four treatments. The *A. altissima* trees closest to the plot’s center that accounted for 20% of the plot’s total *A. altissima* basal area were inoculated with their corresponding inoculum (Supplemental Fig. 1). This clumped inoculation method ensured a distinct disease center and allowed us to monitor the spread from the center-inoculated trees to the surrounding non-inoculated trees. Inoculation was performed by wounding the base of the tree in two or three locations with a sterile straight gouge (05D04 - #5 Sweep Gouge 8 mm, Full Size or 05E07 - #7

Sweep Gouge 25 mm) and allowing the tree to absorb 1 or 3 ml of the treatment inoculum (trees with DBH ≥ 18 cm received the larger amount) (Maschek and Halmschlager, 2016). Since inoculations are most effective in April or May (O'Neal and Davis, 2015), selected *A. altissima* trees were inoculated in Virginia between 11 and 17 May 2017 and in Pennsylvania between 19 and 21 May 2017. Inoculations of all plots at each site occurred on the same day. In total, 520 trees in Virginia and 136 trees in Pennsylvania were inoculated.

2.3 Monitoring

Every mapped tree was monitored at 0, 0.5, 1, 2, 3, 13, 15, 25, and 27 months post inoculation (mpi) in Virginia and 0, 0.5, 1, 2, 3, 15, and 28 mpi in Pennsylvania, with the main gaps in monitoring corresponding to the months when *A. altissima* leaves are senescing, dormant, or not fully leafed out. Monitoring consisted of recording the health of every tree's crown, presence of epicormic sprouts, and presence of a secondary flush of canopy foliage. If present, these were then rated using the following scale: 1 = non-symptomatic, 2 = chlorotic leaves, 3 = wilting leaves, 4 = both wilting and chlorotic leaves, 5 = defoliated canopies, and 6 = dead. A photographic time series of the four plots found at a single site can be seen in Supplemental Fig. 2. Each monitoring series occurred over a period no longer than 11 days, with all plots within a site always monitored on the same day.

Local weather conditions (maximum temperature, average temperature, and total rainfall) during the entirety of this work (May 2017 – August 2019) were extrapolated from the PRISM Climate Group's Date Explorer (available online: http://www.prism.oregonstate.edu/explorer/) using the GPS point of each plot's center.

2.4 Re-isolation

Throughout the experiment, discolored xylem tissue or rachises from a subset of recently senesced *A. altissima* at each plot were collected using sterile tools. A portion of the collected xylem tissue that had not previously been exposed was removed, returned to the lab, and plated on PEA + SN and stored at room temperature. Any Verticillium-like growth (white mycelial colonies with verticillate whorls of oval conidia) was isolated to single-spore colonies and allowed to grow until resting structure morphology (melanized hyphae or microsclerotia) could be observed to distinguish between *V. nonalfalfae* and *V. dahliae* (Inderbitzin et al., 2011a). At the final sampling period, additional symptomatic trees were selected for additional re-isolation.

2.5 Molecular analysis

To explore the possibility that the two *Verticillium* spp. might be capable of hybridizing during co-colonization of host vascular tissues, at least one isolate from each of the combination treatment plots in Virginia and Pennsylvania was selected for molecular characterization. For each isolate, the recently developed multiplex PCR primer set for known *Verticillium* species (Inderbitzin et al., 2013) was used to confirm amplification of specific bands corresponding to either *V. nonalfalfae* and/or *V. dahliae*. Failure to detect either band or successful amplification of both bands would require sequence confirmation and characterization of additional loci.

For Virginia isolates, genomic DNA was extracted from single-conidium colonies grown on PEA + SN using Whatman® Indicating FTATM Classic Cards (Sigma-Aldrich Inc, St. Louis, MO). All PCR was performed on a Mastercycler® EP Gradient PCR Thermal Cycler (Eppendorf AG, Hamburg, Germany) using the custom DNA primers (Integrated DNA Technologies, Coralville, Iowa) to amplify either the GDP, EF, or ITS target locus (Inderbitzin et al., 2013). Reaction mixtures contained 2.5 µL of 0.5 µM working stock of the primers, 9 µL of nuclease-free water, and 12.5 µL of MyTaqTM Red Mix (Bioline USA Inc, Taunton, MA) for a total of 24 µL. For gel electrophoresis, 5 µL of PCR product was loaded into a gel comprising 0.5% Tris-Borate-EDTA buffer (Fisher Scientific, Waltham, MA), 1.5% w/v agarose (UltraPureTM Agarose, Invitrogen, Carlsbad, CA), and 5 µL/100 µL of SmartGlowTM Pre Stain (Accuris, Edison, NJ). Successful amplification was confirmed by electrophoresis at 90 V for 70 min and DNA bands were visualized on a Smart-DocTM Gel Imaging System (Accuris, Edison, NJ). A 2-log DNA ladder (Quick-Load® Purple, New England BioLabs, Ipswich, Massachusetts) was used for size comparison while VdAaVa2, VnAa200, and a nuclease-free water and master mix were used as controls.

For Pennsylvania isolates, genomic DNA was extracted from fungal mycelial plugs harvested from Difco potato dextrose broth (PDB; BD and Co., Franklin Lakes, NJ, USA) following procedures described by Short et al. (2015). All PCR was performed on a MJ Research PTC-200 Peltier Thermal Cycler (GMI, Ramsey, MN) using primers (Integrated DNA Technologies, Coralville, IA, USA) developed by Inderbitzin et al. (2013) for *V. dahliae* and *V. nonalfalfae* and Bioline PCR Kits (Bioline USA Inc, Taunton, MA) in 25.5 µL reactions containing: 1 µL of each of two primers, 1 µL genomic DNA, 10 µL nuclease-free water, and 12.5 µL Bioline PCR Mastermix. For gel electrophoresis, 4 µL of SYBR Gold (Invitrogen, Grand Island, NY, USA) and 4 µL of loading dye (5Prime, Gaithersburg, MD) were added to PCR products which were then loaded into a gel comprising 0.5% Tris-Borate-EDTA buffer (Amresco, Solon, OH, USA) and 1.5% w/v agarose (Amresco, Solon, OH, USA). Electrophoresis was performed at 90 V for 45 min and DNA bands were visualized on a UV transilluminator (Syngene, Frederick, MD, USA). A nuclease-free water and master mix control and a 100-bp molecular ladder (Omega Bio-tek, Norcross, GA, USA) for size comparison was included.

2.6 Pennsylvania greenhouse study

To accompany results from the Pennsylvania field study, remove potential outside variables, and match previous work done in Virginia (Brooks et al., 2019), an inoculation greenhouse study was completed in Pennsylvania. In total, 120 three-month old A. altissima seedlings (susceptible seed source HPA-62; Wickert et al., 2017) were root-dip inoculated in four solutions matching those used in the regional field study before being planted in sterile potting soil. This resulted in 30 plants per treatment. After inoculation, plants were allowed to grow for three weeks, and rated on a weekly basis using a 0 - 4 ordinal scale (0 = healthy, 1 = necrotic margins, 2 = chlorosis, 3 = severe wilt, and 4 = dead). At the conclusion of the three weeks, plants were destructively harvested for re-isolation. Re-isolated *Verticillium* spp. cultures were identified using the morphological and molecular methods detailed above.

2.7 Analysis

2.7.1 Regional considerations

Since *A. altissima* grows clonally and *V. nonalfalfae* has been shown to spread through functional root grafts (O'Neal and Davis, 2015), individual trees at a plot were not considered independent from each other. Therefore, all regional field analyses analyzed the percentage of trees that were symptomatic (including dead) at each plot, not individual tree ratings.

Though the attempt was made to keep the regional experimental design in both states identical, some important differences between the two states could not be avoided. Due to the requirement to use state-specific isolates, the differences in times that the sites could be revisited for rating, and the lack of *V. dahliae*-only inoculations in Pennsylvania, a separate analysis was performed for each state. Furthermore, the data from each state were split into two categories: i) the inoculated trees and ii) the non-inoculated trees. This additional split allowed us to make conclusions about the disease progression of directly inoculated trees and the disease spread to non-inoculated trees within a stand. This resulted in the analysis of four separate subsets of the regional data: Virginia inoculated trees, Pennsylvania inoculated trees, Virginia non-inoculated trees, and Pennsylvania non-inoculated trees.

For each of these four data subsets, the percentage of symptomatic trees (including dead trees) for each monitoring event was collapsed into a single disease rating using the Area Under the Disease Progress Curve (AUDPC) formula (Vanderplank, 1963) for each plot. Prior to analysis, all general linear model assumptions were confirmed to be met.

2.7.2 Regional treatment impacts

To compare overall treatment success accounting for the randomized block design, an additive general linear model of treatment and site (AUDPC ~ Treatment + Site) was applied to the four subsets of data. Since normality was met, the simplest appropriate model was chosen for this analysis, an additive general linear model. If the model was significant, a post-hoc pairwise comparison test (Tukey's HSD) was run (R Core Team, 2018). All significance tests were performed at the $P = 0.05$ level unless otherwise noted.

2.7.3 Regional climate and stand influences

To evaluate if climate or stand variation influenced regional disease progression, three of all the possible variables (elevation, total rainfall, average temperature, maximum temperature, average diameter at breast height, and total basal area) were selected based on their biological significance and lack of correlation (pair-wise correlation coefficients < 0.75). The selected terms were built into a series of 16 representative linear models in combination with treatment and site terms (Table 1). An additional intercept-only model was included as a null model. The model selection method using the Akaike Information Criterion corrected for small sample sizes (AICc) was then used to quantify evidence for these 17 models predicting disease rating (AUDPC) for each of the four subsets of data. The model(s) with the smallest AICc (ΔAICc < 2) was selected as the best fitting for each subset of data (R Core Team, 2018).

2.7.4 Greenhouse treatments

Statistical analysis of symptomatic versus healthy greenhouse *A. altissima* at the conclusion of the three weeks was performed using Fisher's Exact Test followed by a post-hoc pairwise comparison using a Bonferroni correction to determine treatment differences (R Core Team, 2018).

3.0 Results

3.1 Sites

Sites were selected throughout Pennsylvania and Virginia, with hardiness zones ranging from 5b to 7b (Fig. 1, Supplemental Fig. 1, Supplemental Table 1). In Virginia, six sites containing one plot of each treatment were included in the analysis with the exception of one control plot that was contaminated with *V. nonalfalfae* by some unknown source. In Pennsylvania, plot numbers were limited by lower *A. altissima* densities, the destruction of one entire site by the installation of a pipeline, and the accidental contamination of *V. dahliae*-only treatments with *V. nonalfalfae*. This resulted in seven sites containing a total of five *V. nonalfalfae*, six combination, no *V. dahliae*, and four control plots in Pennsylvania that were included in the analysis (Supplemental Fig. 1).

3.2 Re-isolation

Re-isolation results at *V. nonalfalfae* or *V. dahliae* inoculation plots matched their inoculum type (n = 35 and 19, respectively in Virginia and n = 20 at *V. nonalfalfae* sites in Pennsylvania). At combination plots, Virginia re-isolation (n = 43) resulted in *V. nonalfalfae* in all but one small-diameter (3.5 cm DBH) understory tree sampled during the first field season, which yielded *V. dahliae*. Pennsylvania combination sites resulted in only *V. nonalfalfae* (n = 15). Control sites included in the analysis yielded no *Verticillium* spp.

3.3 Molecular analysis

PCR results of selected isolates obtained in the second and third sampling year confirmed the *V. nonalfalfae* identifications for combination sites in Virginia (n = 10) and Pennsylvania (n = 7), as each isolate produced a single band consistent with *V. nonalfalfae* but not *V. dahliae*. Further molecular characterization was not pursued.

3.4 Treatment impact

Verticillium nonalfalfae alone or in combination caused significantly more disease than *V. dahliae* alone, or the control. No matter the state or the inoculation status, all of the treatment and site additive linear models (AUDPC ~ Treatment + Site) were significant (Fig. 2). Additionally, in all of these models the treatment variable was significant while the site variable was never significant. Post-hoc pairwise comparison tests (Tukey's HSD, $\alpha < 0.05$) were run on all these models (Fig. 2). For all four subsets of data, *V. nonalfalfae* alone or in combination caused significantly more disease than *V. dahliae* (when present) or the control. When present, the *V. dahliae* inoculation caused higher disease ratings than the control treatment, but less than the treatments containing *V. nonalfalfae* (Fig. 2a). By contrast, when present, the *V. dahliae* treatment in the non-inoculated trees was not significantly different from the control, but significantly different from the other two *Verticillium* spp. treatments (Fig. 2c). The proportion of trees symptomatic or dead at the final field visit is detailed in Supplemental Table 3.

3.5 Regional variation

Average temperature (°C), total rainfall (mm), and average diameter at breast height (cm) were selected as appropriate for model creation. The ranges for each variable split by state, inoculation status, and treatment are listed in Supplemental Table 4. None of these three variables were included in every model (or even the majority of models) selected on the basis of the smallest AICc.

All selected models were themselves significant, in addition to always including the treatment term, regardless of state or inoculation status. This treatment term was also significant in every selected model (Table 1).

For inoculated Virginia trees, the model with the best support contained both treatment and average DBH terms as interactive effects (AUDPC ~ treatment * DBH). This model, its predictor variables, and their interaction were all significant. The interaction between average DBH and treatment appears to be driven exclusively by the *V. dahliae*-only inoculation, with *V. dahliae* disease ratings decreasing as tree size increases (Table 1, Fig. 3a).

For non-inoculated Virginia trees, the data were best explained by two models. The first model contained just treatment as a response variable, while the second contained both treatment and average DBH as additive effects. Both of these models were significant themselves; however, only the treatment parameter in both models was significant, while the average DBH parameter in the second model was not (Table 1, Fig. 3d and e).

For inoculated Pennsylvania trees, again, the data were best explained by two models. The first model contained both treatment and average temperature as additive effects, while the second contained only treatment. Both models were significant along with their treatment terms, while the average temperature term included in the first was borderline insignificant ($P = 0.0506$, Table 1, Fig. 3b and c).

For non-inoculated Pennsylvania trees, two models were best supported by the data. They both contained treatment and another term in an additive model, with the first model including average temperature and the second total rainfall. In both cases, the model and all of the parameters were significant, with increasing average temperature and total rainfall correlated with decreasing levels of disease (Table 1, Fig. 3f and g).

3.6 Greenhouse

By the conclusion of the three-week greenhouse experiment, of the 30 *V. nonalfalfae*-inoculated seedlings, 21 were dead and nine were severely wilted, with symptoms appearing during the second week. Similarly, of the 30 combination-inoculated seedlings, 19 were dead and 11 were severely wilted, also with symptoms appearing during the second week. In contrast, of the 30 *V. dahliae*-inoculated seedlings, one was severely wilted, 13 were chlorotic, and 16 were asymptomatic, with symptoms appearing during the third week. Lastly, of the 30 control seedlings, 29 were asymptomatic and one was dead, with the single mortality not being attributed to *Verticillium* fungi (confirmed by unsuccessful re-isolation attempts).

Analysis of the number of symptomatic and healthy seedlings at three weeks post inoculation indicated significant differences between treatments did exist (Fisher's Exact Test, $P = 2.2e\text{-}16$). A post-hoc pairwise comparison using a Bonferroni correction ($\alpha < 0.00625$) showed that *V. nonalfalfae* inoculations in combination or alone caused a higher number of symptomatic seedlings than *V. dahliae*, which in turn caused higher levels of symptomatic seedlings than the control (R Core Team, 2018).

Re-isolated cultures matched initial treatments for *V. nonalfalfae* ($n = 30$) and *V. dahliae* ($n = 30$), with combination seedlings resulting in only *V. nonalfalfae* isolations ($n = 30$) and no *Verticillium* spp. were isolated from controls. PCR results validated findings based on culture morphology.

4.0 Discussion

4.1 Treatment impacts

4.1.1 *Verticillium nonalfalfae*, regardless of other variables, is highly effective and spreads quickly

We found that in all analyses (the regional treatment analyses, regional model selection, and the greenhouse analysis), *V. nonalfalfae* inoculations alone or in combination with *V. dahliae* had higher disease ratings than *V. dahliae* alone or the control. This difference was consistent regardless of location, average temperature, total rainfall, average DBH, or inoculation type, solidifying the overarching effectiveness of *V. nonalfalfae* against *A. altissima*. For example, when considering climate and stand variation, the AICc-selected models always included a significant treatment term, while no other term was included in all or even a majority of the selected models. This was true for both directly inoculated trees and its spread to the surrounding non-inoculated trees.

The model selection analysis incorporating climate and stand variables was utilized to help make predictions outside of this study's geographic range. And though this model selection is limited by the ranges of the average temperature, total rainfall, and average DBH variables at these sites (Supplemental Table 4), the disease ratings of *V. nonalfalfae*, either in combination or alone, were never diminished by any of the included predictor terms (average DBH, average temperature, or total rainfall) to control levels (Fig. 3). Therefore, the effectiveness of *V. nonalfalfae* as a biological control agent in and likely adjacent to Virginia and Pennsylvania was confirmed by this study, as all plots inoculated with *V. nonalfalfae* showed severe disease progression and rapid spread within a stand. Like any model, these analyses can only accurately make predictions within, or closely around, the range of the variables included in the analysis, and therefore these results may not be accurate nationally or globally. However, since both of these states represent the densest portion of *A. altissima*'s range (EDDmapS, 2019) they can reasonably project how *V. nonalfalfae* can control a substantial portion of *A. altissima* in North America.

It is also important to note that by the conclusion of this work, it was obvious to us that disease in the *V. nonalfalfae* and combination treatment plots had spread to adjacent *A. altissima* not included in this experiment. Therefore, at the final visit, *A. altissima* adjacent to the mapped plots (if present) were surveyed for symptoms of Verticillium wilt, confirming that *V. nonalfalfae*, with or without *V. dahliae* present, had spread to adjacent *A. altissima* not included in the study from 20 of 22 plots (Supplemental Table 5). Therefore, this analysis may actually underestimate the effectiveness of *V. nonalfalfae* within a stand during these three field seasons.

4.1.2 Impact of *V. nonalfalfae* is not limited by presence of *V. dahliae*

When considering the combination treatments, it is likely that *V. nonalfalfae* is causing mortality alone. First, all but one of the 48 field re-isolations resulted in *V. nonalfalfae*, with the only *V. dahliae* isolate collected from a small-diameter tree which rapidly succumbed to the disease after inoculation. All re-isolated cultures from the Pennsylvania greenhouse combination treatment resulted in only *V. nonalfalfae*, matching results seen by Brooks et al. (2019) using the Virginia isolates. Second, there was no significant difference between the disease ratings between the *V. nonalfalfae* alone or combination treatments in any of the field inoculation analyses or in the greenhouse study. This lack of difference indicates the absence of additive, subtractive, or interactive pathogenicity interaction between these two fungal species. This is not surprising, as true synergistic or antagonistic relationships in plant disease biological control agents are rare (Xu et al., 2011). Therefore, the potential impact of *V. dahliae* on *V. nonalfalfae* disease progression is negligible, allowing for the use of *V. nonalfalfae* biological control on all *A. altissima* stands regardless of the presence of *V. dahliae*.

It is important to note that for the combination treatment, the inoculation of *V. nonalfalfae* and *V. dahliae* in both field and greenhouse studies occurred simultaneously by inoculating a mixed suspension of conidia. Although simultaneous inoculations have been shown to impact disease severity within the *Verticillium* genus in other studies (Price and Sackston, 1989; Wheeler and Johnson, 2019), it is possible that if our inoculations had been separated by time, a different interaction may have been observed. For example, the staggered inoculation of different *Verticillium* spp. has been shown to impact disease rating on tomato and sunflower (Matta and Garibaldi, 1977; Wheeler and Johnson, 2019) and induced resistance in *Ulmus* species to *Ophiostoma* spp. is triggered by *V. dahliae* inoculations prior to exposure of the Dutch elm disease pathogen (Postma and Goossen-van de Geijn, 2016).

4.1.3 *Verticillium dahliae* causes low levels of disease but does not effectively spread

Both the regional study and the greenhouse inoculations indicate that *V. dahliae* can cause low levels of disease in *A. altissima*, as *V. dahliae* alone caused higher disease ratings than the control treatment, but less than any of the treatments with *V. nonalfalfae*. This matches work done previously in Pennsylvania identifying *V. nonalfalfae* as the more effective biological control agent (Schall and Davis, 2009a). Additionally, there was no indication of *V. dahliae* effectively spreading during the 3-year regional study from the inoculated to the non-inoculated trees. Although much less is known about the host range of *V. dahliae* isolates from *A. altissima*, it had been suggested that these isolates may potentially provide a warm-region alternative to *V. nonalfalfae* for biological control of *A. altissima*, but our data do not support this as a promising option.

4.1.4 Mortality in control plots attributed to competition

Any decline in the regional control plots included in this analysis was attributed to competition and overtopping by other *A. altissima*, not Verticillium wilt. This is expected, as in any forest stand, you should expect a background level of dying trees (Stephenson et al., 2011). In fact, as *V. nonalfalfae* removes *A. altissima* from the canopy and other woody species start filling in the canopy gap (Supplemental Fig. 2), additional suppression of *A. altissima* through intraspecies competition may also be seen.

4.2 Regional climate and stand variables

4.2.1 Temperature not a limiting factor

The average plot temperature ("temperature") was only included in the Pennsylvania selected models. In this state's inoculated trees, the model with the lower AICc score contained temperature as a term, but in the model itself, the temperature term was not significant (Table 1). This becomes obvious when the model is plotted (Fig. 3b), since the disease rating only slightly decreases with increased temperatures and is therefore unlikely to substantively impact disease progression in *A. altissima* stands. In the state's non-inoculated trees, the temperature term was also included in the model with the lowest AICc value as an additive term with treatment. In this model the temperature term was a significant factor, indicating that as average temperatures increase, disease spread may slow (Fig. 3f). However, the other selected model did not include the temperature parameter, signifying that it was not a main driving factor.

Despite not being a huge driver of disease, it is reasonable to consider temperature accounting for some of the variation seen, as it has previously been shown that geographic ranges and growth rates of *Verticillium* spp. vary in different temperatures (Fradin and Thomma, 2006; Pegg and Brady, 2002). For example, microsclerotia numbers of *V. dahliae* in broccoli are reduced at higher temperatures (Subbarao and Hubbard, 1996) and *V. nonalfalfae* appeared limited by high temperatures in Austria (Maschek and Halmschlager, 2017). However, the successful disease progression and spread seen of *V. nonalfalfae* in the Virginia Piedmont, a region warmer than any other location where *V. nonalfalfae* had previously been found on *A. altissima* (Snyder et al., 2014) and during years that were relatively warm (https://www.ncdc.noaa.gov/temp-and-precip/), highlights this pathogen's ability to thrive in warmer regions than shown previously.

4.2.2 Rainfall not a limiting factor

Total rainfall was only included in one of the two models selected from Pennsylvania non-inoculated trees (Table 1). The rainfall term was significant in the model; as rainfall increased, disease ratings declined (Fig. 3g). However, since rainfall is not selected in both models and disease levels at the highest rainfall were still far above those of the controls, it does not appear to be an important predictor of disease. This negative relationship, though not very substantial since the disease killed *A. altissima* even when rainfall was plentiful, does seem plausible. This is because vascular wilts have a direct effect on water transport and storage in trees and can accelerate drought-induced mortality (Oliva et al., 2014). This matches research on Dutch elm disease, in which the combination of the vascular wilt pathogen and drought conditions caused increased disease symptoms (Solla and Gil, 2002) and with Verticillium wilt on *Liriodendron tulipifera* in which drought stress aggravated the disease (Morehart and Melchior, 1982).

4.2.3 Tree size influences *V. dahliae* disease progression

Tree size (average DBH) was only included in the Virginia selected models (Table 1). The inclusion of tree size in this state's inoculated model was driven by the *V. dahliae* treatment alone, because only *V. dahliae* disease ratings varied based on average tree diameter (Fig. 3a). Similarly, the Virginia non-inoculated trees included a model where tree size is additive with treatment, though the average DBH term itself was not significant and did not appear to do more than cause a slight decrease in disease ratings (Fig. 3e). This relationship, where an increase of tree size may decrease disease ratings, might have also been selected for in Pennsylvania if the *V. dahliae* treatment had been included in that state as well.

The indication that disease progression and potential spread of *V. dahliae* is influenced by average DBH is not surprising. When considering a single tree, a larger tree may either have more time to form barrier zones before its xylem becomes completely occluded or the percentage of xylem affected may be less (Beier et al., 2017). This was found previously with *V. nonalfalfae* (formerly *V. albo-atrum*) in which *A. altissima*-inoculated seedlings died faster than canopy trees, likely due to the pathogen's ability to faster colonize the smaller xylem volume (Schall and Davis, 2009a). However, when comparing our greenhouse results to our field results, where trees vary drastically in size and age, symptoms appeared at a similar rate in the greenhouse (within 2–3 weeks) and in the field (within half a month).

4.3 Conclusions

This study further supports the use of *V. nonalfalfae* as an effective biological control agent against *A. altissima* throughout the mid-Atlantic region of the United States, regardless of the presence of *V. dahliae* and despite slight variations of disease progression and spread correlated to climate and stand variables. Ongoing work towards registration will ensure that the risk regarding environment (especially host range and persistence) and human health will be sufficiently addressed prior to wide-scale use. With other species filling in the canopy gaps left after the removal of *A. altissima* (Supplemental Fig. 2) and the presence of numerous other woody species (Supplemental Table 2), the restoration of our forest lands and management of our urban areas at a large scale may become more manageable now that the aggressive *A. altissima* can be successfully removed.

Acknowledgements

We thank Tom McAvoy, Laney Metz, Abby Biggs, Caleb Gore, Ryan Mays, Jamie Buttler, Andrew Dechaine, Jeremiah Foley, Holly Wantuch, Max Ragozzino, Allison Sylvester, Toby Grapener, Russel Gibbs, Ben Gamble, and Andrew Rohrbach for their help. Thank you to the Virginia State Parks & Wildlife Management Areas, the Shenandoah Valley AREC, the Radford Army Ammunition Plant, the Pennsylvania Department of Conservation and Natural Resources, Anita Simmons, Ernie Jenkins, and Kirk Hanlon for allowing research to occur on their property. This work was supported by the US Forest Service [15-CA-11420004-161] and the USDA Animal and Plant Health Inspection Services [agreement number

AP18PPQFO000C529]. Final thanks to the editor and the two anonymous reviewers whose comments helped improve this manuscript.

References

Asaro, C., Becker, C., Creighton, J., 2009. Control and utilization of tree-of-heaven: A guide for Virginia landowners. Virginia Department of Forestry Publication P00144, Charlottesville, VA.

Beier, G., Held, B.W., Giblin, C.P., Cavender-Bares, J., Blanchette, R.A., 2017. American elm cultivars: Variation in compartmentalization of infection by *Ophiostoma novo-ulmi* and its effects on hydraulic conductivity. For. Path. 47, e12369. https://doi.org/10.1111/efp.12369.

Brooks, R.K., Bush, E.E., Salom, S.M., Baudoin, A., 2019. First report of Verticillium wilt caused by *Verticillium dahliae* impacting *Ailanthus altissima* (tree of heaven) in Virginia, USA. Plant Dis. https://doi.org/10.1094/PDIS-10-19-2064-PDN.

Burch, P.L., Zedaker, S.M., 2003. Removing the invasive tree *Ailanthus altissima* and restoring natural cover. J. Arboric. 29, 18-24.

EDDmapS. 2019. Tree-of-heaven *Ailanthus altissima* (P. Mill.) Swingle. Early Detection & Distribution Mapping System. The University of Georgia, Center for Invasive Species and Ecosystem Health. https://www.eddmaps.org/distribution/uscounty.cfm?sub=3003 (accessed Nov 2019).

Fradin, E.F., Thomma, B.P., 2006. Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Mol. Plant Pathol. 7, 71-86. https://doi.org/10.1111/j.1364-3703.2006.00323.x.

Gover, A., Johnson, J., Lloyd, K., Sellmer, J., 2013. Tree-of-heaven (*Ailanthus altissima*), Quicksheet 5. In: Invasive Plant Species Management. Wildland Weed Management, Penn State, College of Agricultural Sciences

Heisey, R.M., 1996. Identification of an allelopathic compound from *Ailanthus altissima* (Simaroubaceae) and characterization of its herbicidal activity. Am. J. Bot. 83, 192-200. https://doi.org/10.1002/j.537-2197.1996.tb12697.x.

Heisey, R.M., Heisey, T.K., 2003. Herbicidal effects under field conditions of *Ailanthus altissima* bark extract, which contains ailanthone. Plant Soil. 256, 85-99. https://doi.org/10.1023/A:1026209614161.

Hepting, G., 1971. Ailanthus *Ailanthus altissima*. In: Diseases of forest and shade trees in the United States, Handbook 386. USDA Forest Service, Washington, D.C., pp. 63-64.

Hoebeke, E.R., Jendek, E., Zablotny, J.E., Rieder, R., Yoo, R., Grebennikov, V.V., Ren, L., 2017. First North American records of the Eastasian metallic wood-boring beetle *Agrilus smaragdifrons* Ganglbauer (Coleoptera: Buprestidae: Agrilinae), a specialist on tree of heaven (*Ailanthus altissima*, Simaroubaceae). Proc. Entomol. Soc. Wash. 119, 408-422. https://doi.org/10.4289/0013-8797.119.3.408.

Hu, S.Y., 1979. *Ailanthus*. Arnoldia. 39, 29-50.

Huebner, C.D., Rebbeck, J., 2014. *Ailanthus*: A non-native urban weed is causing trouble in our forests. In: NRS Research Review. Northern Research Station, USDA Forest Service, pp. 1-5.

Inderbitzin, P., Bostock, R.M., Davis, R.M., Usami, T., Platt, H.W., Subbarao, K.V., 2011a. Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. PLoS One. 6**,** e28341. https://doi.org/10.1371/journal.pone.0028341.
Inderbitzin, P., Davis, R.M., Bostock, R.M., Subbarao, K.V., 2011b. The ascomycete V*erticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. PLoS One. 6**,** e18260. https://doi.org/10.1371/journal.pone.0018260.
Inderbitzin, P., Davis, R.M., Bostock, R.M., Subbarao, K.V., 2013. Identification and differentiation of *Verticillium* species and *V. longisporum* lineages by simplex and multiplex PCR assays. PLoS One. 8**,** e65990. https://doi.org/10.1371/journal.pone.0065990.
Kahle, D., Wickham, H., 2013. ggmap: Spatial visualization with ggplot2. R J. 5**,** 144-161. https://doi.org/10.32614/RJ-2013-014.
Kasson, M.T., Davis, M.D., Davis, D.D., 2013a. The invasive *Ailanthus altissima* in Pennsylvania: A case study elucidating species introduction, migration, invasion, and growth patterns in the Northeastern US. Northeast Nat. 20**,** 1-60. https://doi.org/10.1656/045.020.m101.
Kasson, M.T., Kasson, L.R., Wickert, K.L., Davis, D.D., Stajich, J.E., 2019. Genome sequence of a lethal vascular wilt fungus, Verticillium nonalfalfae, a biological control used against the invasive Ailanthus altissima. Microbiol. Resour. Announc. 8, e01619-01618. https://doi.org/10.1128/MRA.01619-18.
Kasson, M.T., O'Donnell, K., Rooney, A.P., Sink, S., Ploetz, R.C., Ploetz, J.N., Konkol, J.L., Carrillo, D., Freeman, S., Mendel, Z., 2013b. An inordinate fondness for Fusarium: phylogenetic diversity of fusaria cultivated by ambrosia beetles in the genus *Euwallacea* on avocado and other plant hosts. Fungal Genet. Biol. 56**,** 147-157. https://doi.org/10.1016/j.fgb.2013.04.004.
Kasson, M.T., O'Neal, E.S., Davis, D.D., 2015. Expanded host range testing for *Verticillium nonalfalfae*: potential biocontrol agent against the invasive *Ailanthus altissima*. Plant Dis. 99**,** 823-835. https://doi.org/10.1094/PDIS-04-14-0391-RE.
Kasson, M.T., Short, D.P., O'Neal, E.S., Subbarao, K.V., Davis, D.D., 2014. Comparative pathogenicity, biocontrol efficacy, and multilocus sequence typing of *Verticillium nonalfalfae* from the invasive *Ailanthus altissima* and other hosts. Phytopathology. 104**,** 282-292. https://doi.org/10.1094/PHYTO-06-13-0148-R.
Kowarik, I., Säumel, I., 2007. Biological flora of Central Europe: *Ailanthus altissima* (Mill.) Swingle. Perspect. Plant Evol. Syst. 8**,** 207-237. https://doi.org/10.1016/j.ppees.2007.03.002.
Lawrence, J.G., Colwell, A., Sexton, O.J., 1991. The ecological impact of allelopathy in *Ailanthus altissima* (Simaroubaceae). Am. J. Bot. 78**,** 948-958. https://doi.org/10.2307/2445173.
Marks, C.O., Van Driesche, R.G., 2016. Designing restoration programs based on understanding the drivers of ecological change. In: Integrating Biological Control into Conservation Practice. John Wiley & Sons, Ltd., West Sussex, UK, pp. 4-21. https://doi.org/10.1002/9781118392553.ch2.
Maschek, O., Halmschlager, E., 2016. A rapid, reliable and less-destructive method for stem inoculations on trees. For. Path. 46**,** 171-173. https://doi.org/10.1111/efp.12266.

Maschek, O., Halmschlager, E., 2017. Natural distribution of Verticillium wilt on invasive *Ailanthus altissima* in eastern Austria and its potential for biocontrol. For. Path. 47, 1-11. https://doi.org/10.1111/efp.12356.

Matta, A., Garibaldi, A., 1977. Control of Verticillium wilt of tomato by preinoculation with avirulent fungi. Neth. J. Plant Pathol. 83, 457-462. https://doi.org/10.1007/BF03041463.

Mergen, F., 1959. A toxic principle in the leaves of Ailanthus. Botanical Gaz. 121, 32-36. https://doi.org/10.1086/336038.

Morehart, A., Melchior, G., 1982. Influence of water stress on Verticillium wilt of yellow-poplar. Can. J. Bot. 60, 201-209. https://doi.org/10.1139/b82-027.

O'Neal, E.S., Davis, D.D., 2015. Biocontrol of *Ailanthus altissima*: inoculation protocol and risk assessment for *Verticillium nonalfalfae* (Plectosphaerellaceae: Phyllachorales). Biocontrol Sci. Techn. 25, 950-969. https://doi.org/10.1080/09583157.2015.1023258.

O'Neal, E.S., Davis, D.D., 2015. Intraspecific root grafts and clonal growth within *Ailanthus altissima* stands influence *Verticillium nonalfalfae* transmission. Plant Dis. 99, 1070-1077. https://doi.org/10.1094/pdis-07-14-0722-re.

Oliva, J., Stenlid, J., Martínez‐Vilalta, J., 2014. The effect of fungal pathogens on the water and carbon economy of trees: Implications for drought‐induced mortality. New Phytol. 203, 1028-1035. https://doi.org/10.1111/nph.12857.

Pegg, G.F., Brady, B.L., 2002. Verticillium wilts. CABI Publishing, New York. ISBN 0–85199–529–2.

Postma, J., Goossen-van de Geijn, H., 2016. Twenty-four years of Dutch Trig® application to control Dutch elm disease. Biocontrol. 61, 305-312. https://doi.org/10.1007/s10526-016-9731-6.

Price, D., Sackston, W., 1989. Cross protection among strains of *Verticillium dahliae* on sunflower. In: Vascular Wilt Diseases of Plants. Springer, pp. 229-235. ISBN 9783642731662.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Rebbeck, J., Malone, M.A., Short, D.P.G., Kasson, M.T., O'Neal, E.S., Davis, D.D., 2013. First report of Verticillium wilt caused by *Verticillium nonalfalfae* on tree-of-heaven (*Ailanthus altissima*) in Ohio. Plant Dis. 97, 999-999. https://doi.org/10.1094/pdis-01-13-0062-pdn.

Schall, M.J., Davis, D.D., 2009a. *Ailanthus altissima w*ilt and mortality: etiology. Plant Dis. 93, 747-751. https://doi.org/10.1094/Pdis-93-7-0747.

Schall, M.J., Davis, D.D., 2009b. Verticillium wilt of *Ailanthus altissima*: susceptibility of associated tree species. Plant Dis. 93, 1158-1162. https://doi.org/10.1094/Pdis-93-11-1158.

Short, D.P., Double, M., Nuss, D.L., Stauder, C.M., MacDonald, W., Kasson, M.T., 2015. Multilocus PCR assays elucidate vegetative incompatibility gene profiles of *Cryphonectria parasitica* in the United States. Appl. Environ. Microbiol. 81, 5736-5742.

Sinclair, W., Smith, K., Larsen, A., 1981. Verticillium wilt of maples: symptoms related to movement of the pathogen in stems. Phytopathology. 71, 340-345. https://doi.org/10.1094/Phyto-71-340.

Snyder, A.L., Kasson, M.T., Salom, S.M., Davis, D.D., Griffin, G.J., Kok, L.T., 2013. First report of Verticillium wilt of *Ailanthus altissima in* Virginia caused by *Verticillium nonalfalfae*. Plant Dis. 97, 837-837. https://doi.org/10.1094/pdis-05-12-0502-pdn.

Snyder, A.L., Salom, S.M., Kok, L.T., 2014. Survey of *Verticillium nonalfalfae* (Phyllachorales) on tree-of-heaven in the southeastern USA. Biocontrol Sci. Techn. 24, 303-314. https://doi.org/10.1080/09583157.2013.860426.

Solla, A., Gil, L., 2002. Influence of water stress on Dutch elm disease symptoms in *Ulmus minor*. Can. J. Bot. 80, 810-817. https://doi.org/10.1139/b02-067.

Song, S., Kim, S., Kwon, S.W., Lee, S.-I., Jablonski, P.G., 2018. Defense sequestration associated with narrowing of diet and ontogenetic change to aposematic colours in the spotted lanternfly. Sci. Rep. 8, 16831. https://doi.org/10.1038/s41598-018-34946-y.

Stapleton, J., 1997. Verticillium wilt of almond in California. In: EPPO Bulletin. European and Mediterranean Plant Protection Organization, pp. 489-492. https://doi.org/10.1111/j.1365-2338.1997.tb00671.x.

Stephenson, N.L., Van Mantgem, P.J., Bunn, A.G., Bruner, H., Harmon, M.E., O'Connell, K.B., Urban, D.L., Franklin, J.F., 2011. Causes and implications of the correlation between forest productivity and tree mortality rates. Ecol. Monogr. 81, 527-555. https://doi.org/10.1890/10-1077.1.

Stukenbrock, E.H., 2016. The role of hybridization in the evolution and emergence of new fungal plant pathogens. Phytopathology. 106, 104-112. https://doi.org/10.1094/PHYTO-08-15-0184-RVW.

Subbarao, K., Hubbard, J., 1996. Interactive effects of broccoli residue and temperature on *Verticillium dahliae* microsclerotia in soil and on wilt in cauliflower. Phytopathology. 86, 1303-1310. https://doi.org/10.1094/Phyto-86-1303.

Talboys, P.W., 1960. A culture-medium aiding the identification of *Verticillium albo-atrum* and *V. dahliae*. Plant Path. 9, 57-58. https://doi.org/10.1111/j.1365-3059.1960.tb01147.x.

Vanderplank, J.E., 1963. Plant diseases: Epidemics and control. Academic Press, New York. ISBN 9781483262130.

Wallner, A.M., Hamilton, G.C., Nielsen, A.L., Hahn, N., Green, E.J., Rodriguez-Saona, C.R., 2014. Landscape factors facilitating the invasive dynamics and distribution of the brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae), after arrival in the United States. PLoS One. 9, e95691. https://doi.org/10.1371/journal.pone.0095691.

Wheeler, D.L., Johnson, D.A., 2019. Does coinoculation with different *Verticillium dahliae* genotypes affect the host or fungus? Phytopathology. 109, 780-786. https://doi.org/10.1094/PHYTO-11-18-0430-R.

Wickert, K.L., O'Neal, E.S., Davis, D.D., Kasson, M.T., 2017. Seed production, viability, and reproductive limits of the invasive *Ailanthus altissima* (tree-of-heaven) within invaded environments. Forests. 8. https://doi.org/10.3390/f8070226.

Wickham, H. 2016. ggplot2: elegant graphics for data analysis. Springer-Verlag New York. https://ggplot2.tidyverse.org.

Wu, Z.Y., Raven, P.H., Hong, D.Y., 2008. Simaroubaceae. In: Flora of China, Vol 11: Oxalidaceae through Aceraceae. Missouri Botanical Garden Press, St. Louis, MO. ISBN 978-1-930723-73-3.

Xu, X.M., Jeffries, P., Pautasso, M., Jeger, M.J., 2011. Combined use of biocontrol agents to manage plant diseases in theory and practice. Phytopathology. 101, 1024-1031. https://doi.org/10.1094/PHYTO-08-10-0216.

Figures

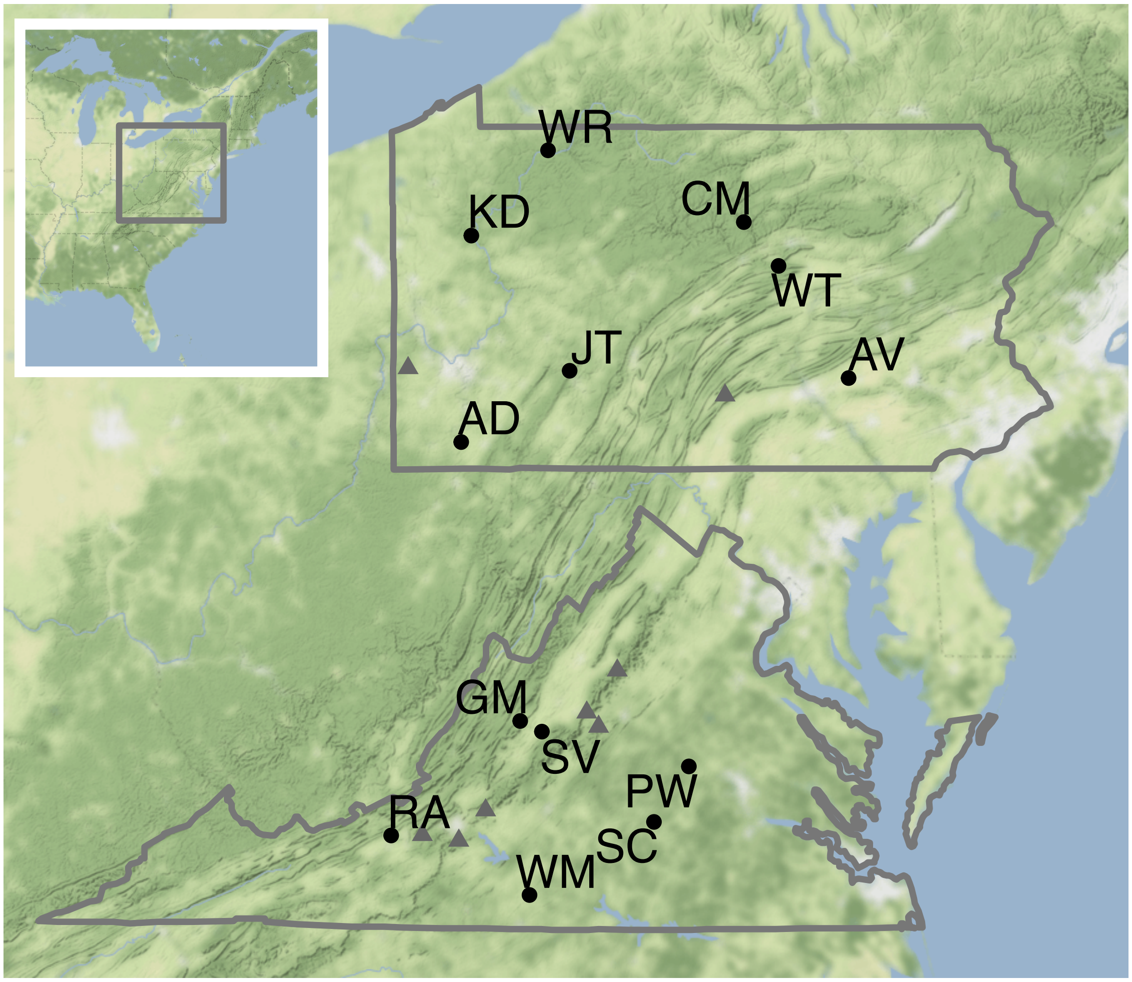


Figure 1: Location and site code of all sites in Pennsylvania (top) and Virginia (bottom) included in the regional field analysis marked by circular points. Previously located areas with natural *V. nonalfalfae* impacting *A. altissima* within these two states are indicated by gray triangles (Kahle and Wickham, 2013; R Core Team, 2018). Hardiness zones defined by USDA Agricultural Research Service's 2012 Plant Hardiness Zone Map (http://planthardiness.ars.usda.gov) for each site are as follows: 5b: KD, WR; 6a: CM, JT, WT; 6b: GM, RA, SV, AD, AV; 7a: PW, SC, WM.

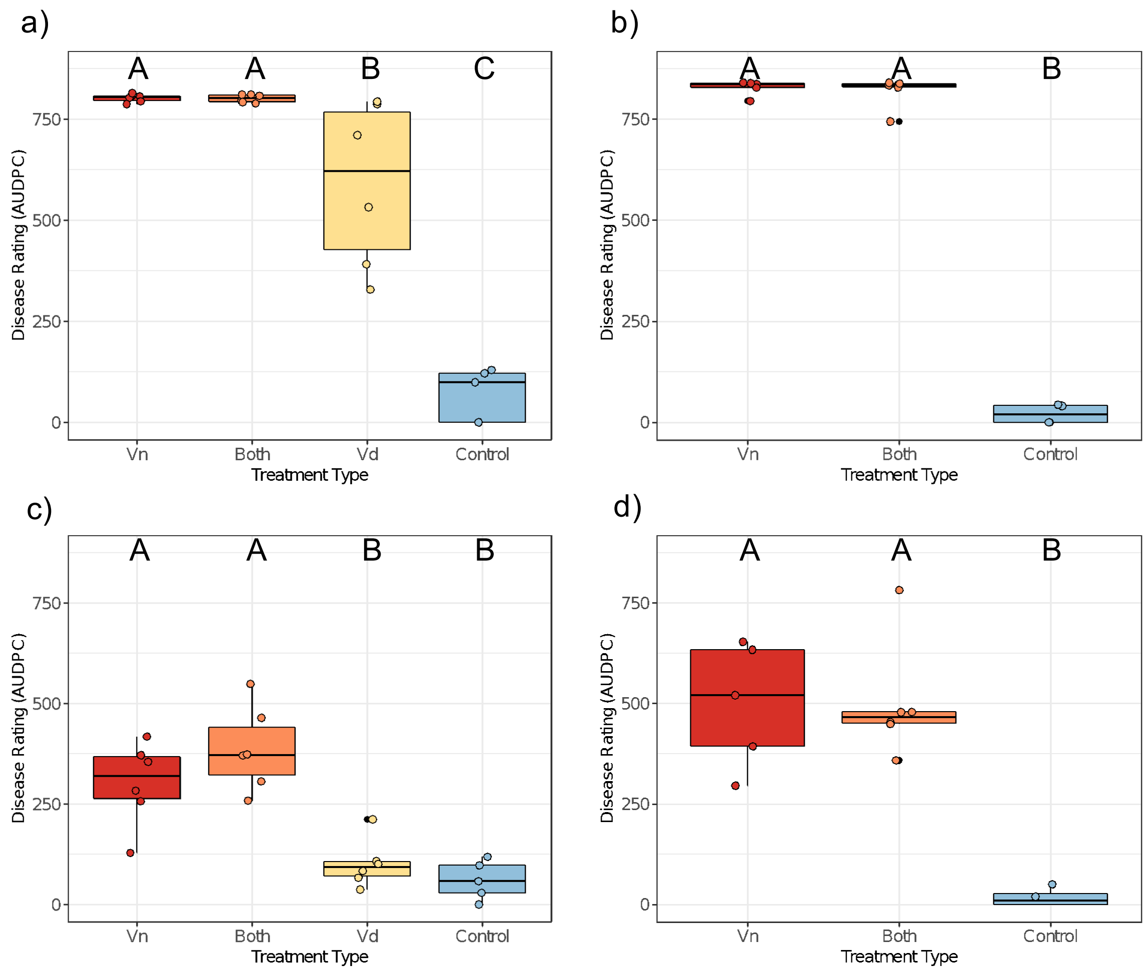


Figure 2: Boxplot of the area under the disease progress curve (AUDPC) values for each treatment (Vn = *Verticillium nonalfalfae*, Vd = *V. dahliae*) split by state and by inoculation status. Model and predictor significance for the additive linear model (Treatment + Site) is reported for each. Significant differences in AUDPC values are indicated by different letters displayed above the boxplots as determined by Tukey's HSD, $\alpha < 0.05$. Raw data are shown with jittered points (R Core Team, 2018; Wickham, 2016). a) Virginia inoculated results, Model AUDPC ~ Treatment + Site is significant: ($F(8, 14) = 25.29$, $p = 4.79e-7$), with parameter "Treatment" significant ($p = 1.95e-8$) and parameter "Site" not significant ($p = 0.182$). b) Pennsylvania inoculated results, Model AUDPC ~ Treatment + Site is significant: ($F(8, 6) = 288$, $p = 3.48e-7$), with parameter "Treatment" significant ($p = 1.77e-8$) and parameter "Site" not significant ($p = 0.468$). c) Virginia non-inoculated results: Model AUDPC ~ Treatment + Site is significant ($F(8 , 14) = 8.699$, $p = 0.000276$), with parameter "Treatment" significant (1.88e-5) and parameter "Site" not significant ($p = 0.317$). d). Pennsylvania non-inoculated results: Model AUDPC ~ Treatment + Site is significant ($F(8, 6) = 8.228$, $p = 0.00961$), with parameter "Treatment" significant (0.000933) and parameter "Site" not significant ($p = 0.259$).

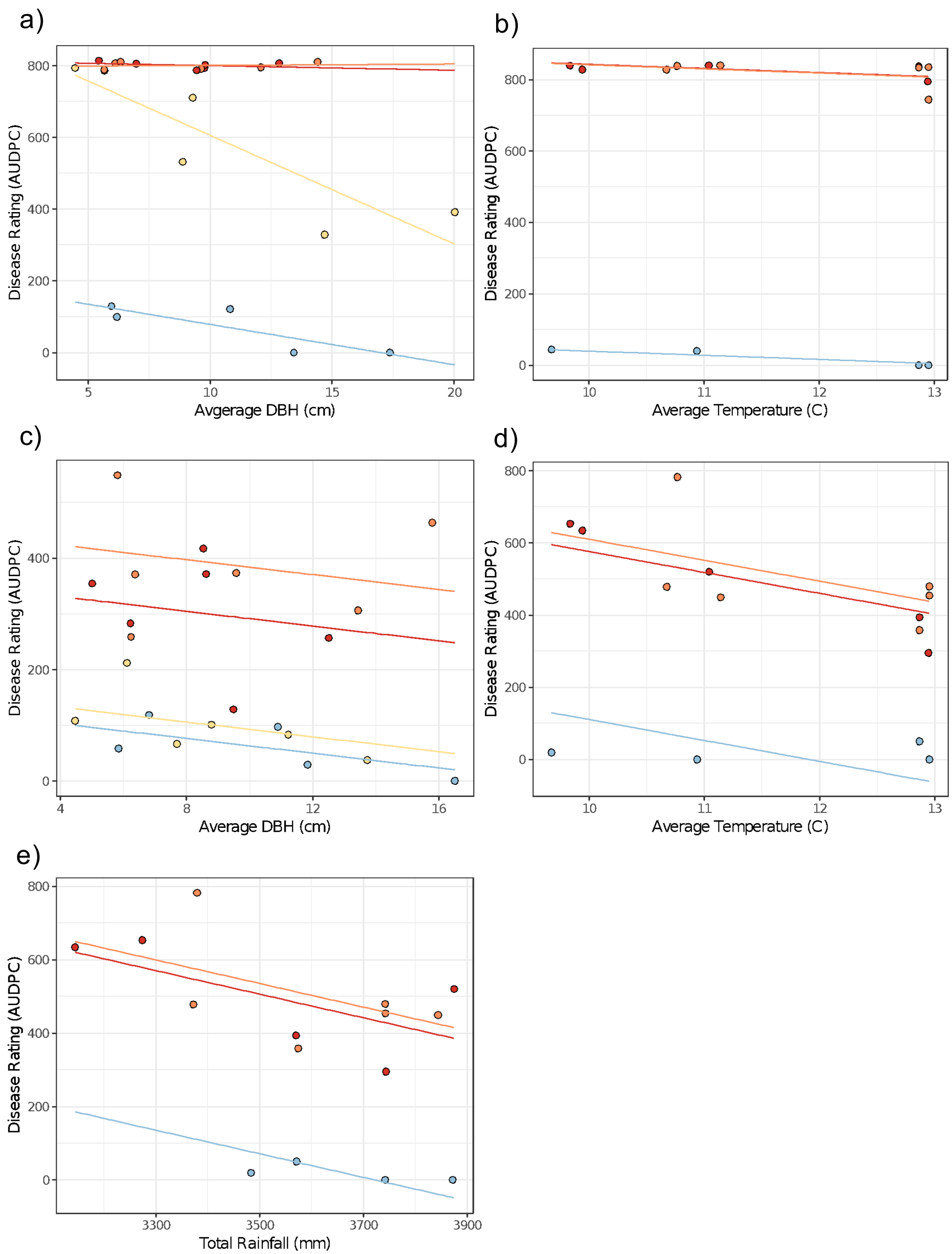


Figure 3: The best fitting selected linear regression models determined by AICc shown as lines and actual data displayed as points for Pennsylvania or Virginia data split by inoculation status. Treatment type indicated by color: *V. nonalfalfae* (red), combination (orange), *V. dahliae* (yellow), or the water control (blue; R Core Team, 2018; Wickham, 2016). The two selected models that included only treatment (from Pennsylvania-inoculated trees and Virginia non-

inoculated trees) are not shown, as they look identical to the results shown in Fig. 2. (a) Virginia-inoculated trees, area under the disease progress curve (AUDPC) ~ treatment * average diameter at breast height (DBH), (b) Pennsylvania-inoculated trees, AUDPC ~ treatment + average temperature, (c) Virginia-non-inoculated trees, treatment + average DBH, (d) Pennsylvania-non-inoculated trees, AUDPC ~ treatment + average temperature, (e) Pennsylvania-non-inoculated trees, AUDPC ~ treatment + total rainfall.

Tables

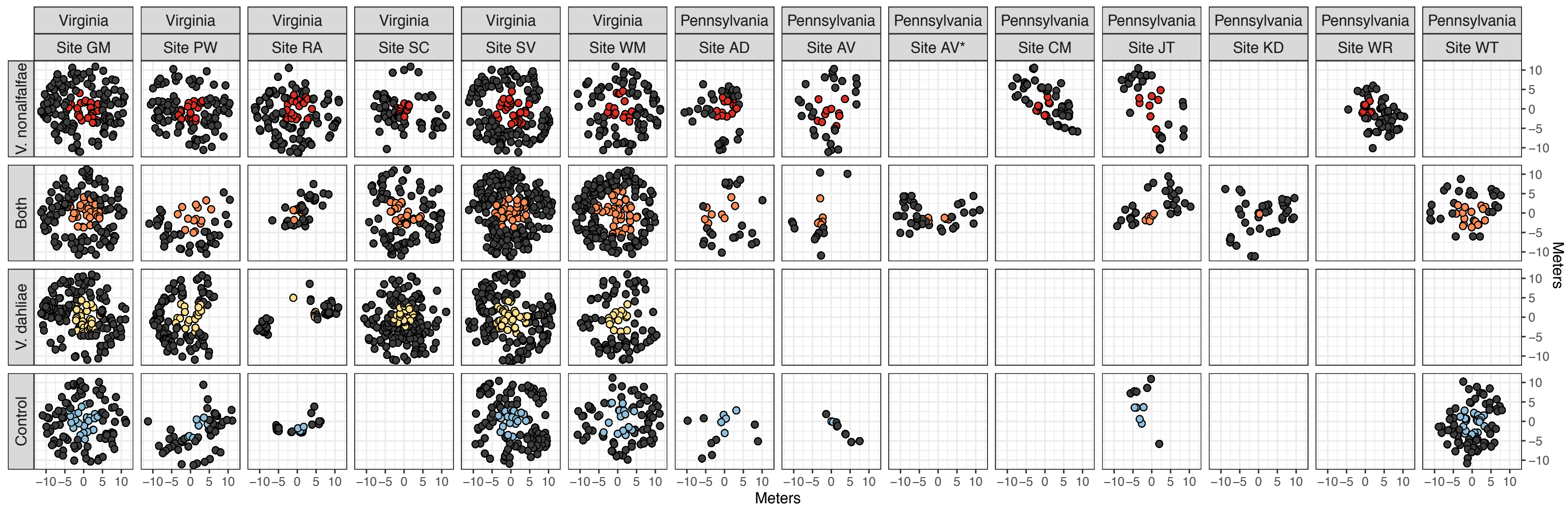
Table 1: Model selection statistics for i = 17 models predicting disease rating as a function of inoculation treatment ("Treatment"), average temperature ("AvgTemp"), total rainfall in mm ("Rainfall"), location ("Site"), or average diameter at breast height in cm (AvgDBH) for each state and inoculation status. "Intercept only" represents the null model. AICc is the Akaike Information Criterion corrected for small sample sizes, ΔAICc is the change in AICc values, "Wt" is the weight of the model, and "k" is the number of parameters. Selected models (ΔAICc < 2) are bolded and additional model information for each selected model is included in the footers (R Core Team, 2018).

State	Model information					
Type	*i*	Model	*k*	AICc	ΔAICc	Weight
Virginia						
Inoculated						
	16	**Treatment*AvgDBH[1]**	**9**	**275.38**	**0.00**	**0.78**
	9	Treatment+AvgDBH	6	278.40	3.02	0.17
	11	Treatment+AvgDBH+AvgTemp	7	282.24	6.86	0.03
	13	Treatment+AvgDBH+Rainfall	7	282.62	7.24	0.02
	14	Treatment+AvgTemp+Rainfall+AvgDBH	8	286.98	11.60	0.00
	17	Treatment*Rainfall	9	288.04	12.66	0.00
	10	Treatment+Rainfall	6	288.06	12.68	0.00
	12	Treatment+AvgTemp+Rainfall	7	289.01	13.63	0.00
	3	Treatment	5	289.97	14.59	0.00
	8	Treatment+AvgTemp	6	292.62	17.24	0.00
	7	Treatment+Site	10	303.46	28.08	0.00
	15	Treatment*AvgTemp	9	305.78	30.40	0.00
	5	AvgDBH	3	331.81	56.43	0.00
	1	Intercept Only	2	332.69	57.31	0.00
	6	Rainfall	3	335.04	59.66	0.00
	4	AvgTemp	3	335.33	59.95	0.00
	2	Site	7	348.03	72.65	0.00
Noninoculated						
	3	**Treatment[2]**	**5**	**278.60**	**0.00**	**0.45**
	9	**Treatment+AvgDBH[3]**	**6**	**280.35**	**1.75**	**0.19**
	8	Treatment+AvgTemp	6	281.10	2.50	0.13
	10	Treatment+Rainfall	6	281.23	2.63	0.12
	12	Treatment+AvgTemp+Rainfall	7	283.47	4.86	0.04
	11	Treatment+AvgDBH+AvgTemp	7	283.56	4.96	0.04
	13	Treatment+AvgDBH+Rainfall	7	284.57	5.96	0.02
	14	Treatment+AvgTemp+Rainfall+AvgDBH	8	288.07	9.47	0.00
	15	Treatment*AvgTemp	9	291.94	13.33	0.00
	17	Treatment*Rainfall	9	291.98	13.38	0.00
	16	Treatment*AvgDBH	9	293.50	14.90	0.00
	7	Treatment+Site	10	294.61	16.01	0.00
	1	Intercept Only	2	301.98	23.37	0.00
	5	AvgDBH	3	303.80	25.20	0.00
	6	Rainfall	3	304.47	25.87	0.00
	4	AvgTemp	3	304.58	25.98	0.00
	2	Site	7	316.36	37.76	0.00
Pennsylvania						
Inoculated						
	8	**Treatment+AvgTemp[4]**	**5**	**151.63**	**0.00**	**0.51**
	3	**Treatment[5]**	**4**	**152.41**	**0.78**	**0.34**
	10	Treatment+Rainfall	5	156.56	4.93	0.04
	9	Treatment+AvgDBH	5	156.71	5.07	0.04
	12	Treatment+AvgTemp+Rainfall	6	157.16	5.53	0.03
	11	Treatment+AvgDBH+AvgTemp	6	157.40	5.77	0.03

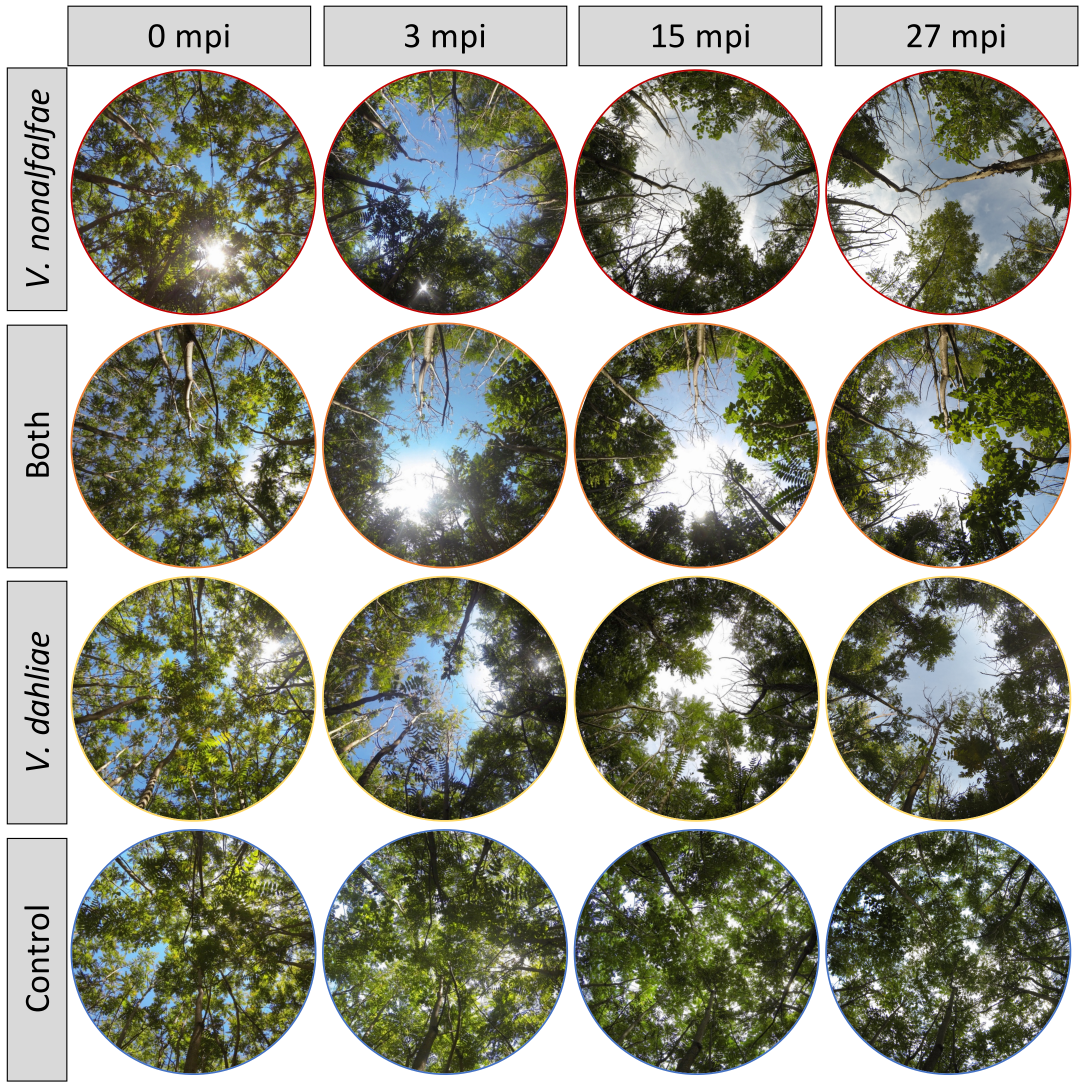
	13	Treatment+AvgDBH+Rainfall	6	162.13	10.49	0.00
	15	Treatment*AvgTemp	7	164.14	12.50	0.00
	14	Treatment+AvgTemp+Rainfall+AvgDBH	7	164.58	12.95	0.00
	16	Treatment*AvgDBH	7	168.95	17.32	0.00
	17	Treatment*Rainfall	7	169.54	17.90	0.00
	7	Treatment+Site	10	204.49	52.86	0.00
	1	Intercept Only	2	223.80	72.16	0.00
	6	Rainfall	3	226.29	74.66	0.00
	4	AvgTemp	3	226.96	75.33	0.00
	5	AvgDBH	3	226.97	75.34	0.00
	2	Site	8	256.96	105.33	0.00
Noninoculated						
	8	**Treatment+AvgTemp[6]**	**5**	**194.53**	**0.00**	**0.44**
	10	**Treatment+Rainfall[7]**	**5**	**195.57**	**1.04**	**0.26**
	3	Treatment	4	197.21	2.68	0.12
	12	Treatment+AvgTemp+Rainfall	6	197.82	3.29	0.08
	9	Treatment+AvgDBH	5	199.71	5.18	0.03
	11	Treatment+AvgDBH+AvgTemp	6	200.06	5.54	0.03
	15	Treatment*AvgTemp	7	201.00	6.47	0.02
	13	Treatment+AvgDBH+Rainfall	6	201.38	6.85	0.01
	14	Treatment+AvgTemp+Rainfall+AvgDBH	7	205.27	10.74	0.00
	16	Treatment*AvgDBH	7	205.87	11.34	0.00
	17	Treatment*Rainfall	7	208.25	13.72	0.00
	5	AvgDBH	3	212.03	17.50	0.00
	6	Rainfall	3	212.23	17.70	0.00
	1	Intercept Only	2	212.34	17.81	0.00
	4	AvgTemp	3	214.26	19.74	0.00
	2	Site	8	243.11	48.58	0.00
	7	Treatment+Site	10	245.10	50.57	0.00

[1] Model: F(7,15) = 82.5,p = 7.80e-11, treatment (p = 6.37e-12), DBH (p = 5.90e-05), interaction (p = 0.00796)
[2] Model: F(3,19) = 19.47,p = 5.11e-6, treatment (p = 5.11e-06)
[3] Model: F(4,18) = 15.47,p = 1.19e-5, treatment (p = 5.65e-6) average DBH (p = 0.221).
[4] Model: F(3,11) = 977,p = 1.26e-13, treatment (p = 4.52e-14) temperature (p = 0.0506).
[5] Model: F(2,12) = 1,110,p = 2.42e-14, treatment (p = 2.42e-14).
[6] Model: F(3,11) = 22.5,p = 5.36e-5, treatment (p = 3.37e-5) average temperature (p = 0.0232).
[7] Model: F(3,11) = 20.75,p = 7.80e-5, treatment (p = 4.64e-05) total rainfall (p = 0.0354).

Supplementary data



Supplemental Figure 1: All plot maps included in the final analysis showing *A. altissima* tree distribution as if seen from above. Rows separate plots by sites, while columns separate plots by treatment type. Inoculated *A. altissima* trees are shown in color (based on treatment), and non-inoculated *A. altissima* trees are shown in black (R Core Team, 2018; Wickham, 2016). *Site AV contained two plots with the same treatment, and therefore is shown in two separate columns.



Supplemental Figure 2: Representative canopy photograph series from the “GM” Virginia s
taken with a GoPro HERO3 Black camera (GoPro, Inc. San Mateo, CA) at the center of eac
plot looking up. Images organized by time taken in columns (0, 3, 15, 27 months post inocu
(mpi)) and by the four treatments in rows. Note that by 15 & 27 mpi the majority of green i
V. nonalfalfae and combination (“both”) treatment is mostly non-*A. altissima* vegetation gr
into the canopy gap. This dominance of non-*A. altissima* foliage is not the case for any of tl
other images.

Supplemental Table 1: Site and plot details for plots included in analysis: state, town, code, name, hardiness zone (zone), access information, and a brief description for each site and the latitude and longitude of each plot center. Treatments applied to each plot are indicated in the footer.

Location	Code	Site name	Zone*	Access/Permit	Plot 1	Plot 2	Plot 3	Plot 4	Site description
Goshen, VA	GM	Little North Mountain Wildlife Management Area	6b	Special Use Permit obtained	38.000222, -79.403194[D]	38.000889, -79.404083[N]	38.000306, -79.404000[B]	38.000444, -79.404583[C]	Clearcut regeneration
Powhatan County, VA	PW	Powhatan State Park	7a	Permit PWSC-RCP-022717	37.684639, -77.917917[D]	37.686278, -77.917778[B]	37.684611, -77.927472[C]	37.685278, -77.918556[N]	Field and forest edge
Radford, VA	RA	Radford Army Ammunition Plant	6b	Written permission received	37.202291, -80.539624[N]	37.201730, -80.541643[B]	37.203357, -80.535552[D]	37.201860, -80.540988[C]	Fence line in field
Rice, VA	SC	Sailors Creek Battlefield State Park	7a	Permit PWSC-RCP-022717	37.297111, -78.225167[D]	37.298222, -78.227278[N]	37.297917, -78.224833[B]		Abandoned field
Raphine, VA	SV	Shenandoah Valley AREC (Virginia Tech property)	6b	Written permission received	37.926500, -79.212073[B]	37.925417, -79.214472[N]	37.923278, -79.214194[C]	37.922694, -79.214167[D]	Clearcut regeneration
Pittsylvania County, VA	WM	White Oak Mountain Wildlife Management Area	7a	Special Use Permit obtained	36.783889, -79.321222[B]	36.791500, -79.324806[D]	36.785694, -79.334333[C]	36.799972, -79.317917[N]	Field, forest, and road edge
Adah, PA	AD	Private property	6b	Written permission received	39.908565, -79.922764[N]	39.908144, -79.923957[C]	39.905821, -79.925817[B]		Forest road or field edge
Anneville, PA	AV	Lebanon Valley College	6b	Written permission received	40.339109, -76.511169[B]	40.340534, -76.509585[N]	40.340870, -76.507294[C]	40.339824, -76.509531[B]	Field and forest edge
Cammal, PA	CM	Tiadaghton State Forest	6a	Written permission received	41.376946, -77.433758[N]				Trail and forest edge
Johnstown, PA	JT	Gallitzin State Forest	6a	Written permission received	40.388163, -78.967800[C]	40.373510, -78.961407[N]	40.363720, -78.948835[B]		River and road edge
Kennerdell, PA	KD	Clear Creek State Forest, Kennerdell Tract	5b	Written permission received	41.287920, -79.833510[B]				Forest gap
Warren, PA	WR	Washington Park	5b	Written permission received	41.849189, -79.158027[N]				Area cleared for view
Washington Township, PA	WT	Tiadaghton State Forest	6a	Written permission received	41.087169, -77.127885[C]				Forest gap

[N] assigned the *V. nonalfalfae* treatment
[D] assigned the *V. dahliae* treatment
[B] assigned the combination treatment (*V. nonalfalfae* and *V. dahliae*) treatment
[C] assigned the water control
*Hardiness zones defined by USDA Agricultural Research Service's 2012 Plant Hardiness Zone Map (https://planthardiness.ars.usda.gov). These zones are based on the average annual extreme minimum temperature during a 30-year period, where zone 5b is between -26.1 and 23.3°C, zone 6a is between -23.3 and -20.6°C, zone 6b is between -20.6 and -17.8°C, and zone 7a is between -17.8 and -15°C.

Supplemental Table 2: Co-occurring woody species found naturally in plots at the final site visit, separated by state and treatment type. None of these species displayed Verticillium wilt-like symptoms in the field.

	Treatment			
State	***V. nonalfalfae***	**Both**	***V. dahliae*[1]**	**Control**
Virginia	*Acer negundo*	*Acer negundo*	*Acer negundo*	*Acer negundo*
	Acer rubrum	*Asimina triloba*	*Acer rubrum*	*Acer rubrum*
	Asimina triloba	*Campsis radicans*	*Asimina triloba*	*Asimina triloba*
	Celtis occidentalis	*Elaeagnus umbellata*	*Berberis thunbergii*	*Berberis thunbergii*
	Elaeagnus umbellata	*Juniperus virginiana*	*Campsis radicans*	*Campsis radicans*
	Fraxinus pennsylvanica	*Lespedeza bicolor*	*Carya tomentosa*	*Elaeagnus umbellata*
	Juglans nigra	*Lindera benzoin*	*Celtis occidentalis*	*Lonicera spp.*
	Lindera benzoin	*Liquidambar styraciflua*	*Cercis canadensis*	*Liriodendron tulipifera*
	Liriodendron tulipifera	*Liriodendron tulipifera*	*Elaeagnus umbellata*	*Lonicera japonica*
	Lonicera spp.	*Lonicera* spp.	*Fraxinus americana*	*Parthenocissus quinquefolia*
	Parthenocissus quinquefolia	*Parthenocissus quinquefolia*	*Juniperus virginiana*	*Paulownia tomentosa*
	Paulownia tomentosa	*Paulownia tomentosa*	*Lespedeza bicolor*	*Platanus occidentalis*
	Prunus serotina	*Platanus occidentalis*	*Lindera benzoin*	*Quercus rubra*
	Quercus rubra	*Prunus serotina*	*Liriodendron tulipifera*	*Robinia pseudoacacia*
	Robinia pseudoacacia	*Quercus rubra*	*Lonicera* spp.	*Rosa multiflora*
	Toxicodendron radicans	*Robinia pseudoacacia*	*Parthenocissus quinquefolia*	*Toxicodendron radicans*
	Vitis spp.	*Rosa multiflora*	*Paulownia tomentosa*	
		Rubus phoenicolasius	*Prunus serotina*	
		Toxicodendron radicans	*Quercus marilandica*	
		Vitis spp.	*Robinia pseudoacacia*	
			Rubus allegheniensis	
			Toxicodendron radicans	
Pennsylvania	*Berberis thunbergii*	*Berberis thunbergii*		*Acer saccharum*
	Catalpa speciosa	*Celtis occidentalis*		*Betula lenta*
	Celtis occidentalis	*Juglans nigra*		*Celtis occidentalis*
	Hamamelis virginiana var. virginiana	*Liriodendron tulipifera*		*Lindera benzoin*
	Juglans nigra	*Lonicera* spp.		*Lonicera* spp.
	Lindera benzoin	*Robinia pseudoacacia*		
	Lonicera spp.	*Rubus phoenicolasius*		
	Platanus occidentalis	*Toxicodendron radicans*		
	Prunus serotina			
	Robinia pseudoacacia			
	Vitis spp.			

[1] Since no Pennsylvania plots contained exclusively *V. dahliae*, no co-occurring species are listed.

Supplemental Table 3: Proportion of trees symptomatic (including dead) at final field visit recorded by state, inoculation status, and treatment type. Sample size (n), mean, standard deviance (SD), maximum proportion (max), and minimum proportion (min) recorded for each category.

State	Type	Treatment	n	Mean	SD	Max	Min
VA	Inoculated	*V. nonalfalfae*	6	1.000	0.000	1.000	1.000
		Both	6	1.000	0.000	1.000	1.000
		V. dahliae	6	0.728	0.233	1.000	0.333
		Control	5	0.208	0.215	0.500	0.000
	Noninoc.	*V. nonalfalfae*	6	0.726	0.233	1.000	0.304
		Both	6	0.902	0.106	1.000	0.723
		V. dahliae	6	0.238	0.111	0.421	0.105
		Control	5	0.118	0.111	0.280	0.000
PA	Inoculated	*V. nonalfalfae*	5	1.000	0.000	1.000	1.000
		Both	6	1.000	0.000	1.000	1.000
		Control	4	0.104	0.121	0.217	0.000
	Noninoc.	*V. nonalfalfae*	5	0.993	0.017	1.000	0.963
		Both	6	0.945	0.090	1.000	0.769
		Control	4	0.086	0.118	0.250	0.000

Supplemental Table 4: Range of selected parameters included in the AICc model selection detailed by state, inoculation status, and treatment type. Maximum temperature and total rainfall were measured at a site level, and therefore do not vary as much as diameter at breast height, which was measured at a plot level.

				Maximum temperature (°C)				Total rainfall (mm)				Diameter at breast height (cm)			
State	**Type**	**Treatment**	**n**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**
VA	Inoculated	*V. nonalfalfae*	6	30.7	1.4	32.2	29.1	3180	320	3630	2700	9.0	2.6	12.8	5.4
		Both	6	30.7	1.4	32.2	29.1	3180	325	3650	2700	9.0	3.6	14.4	5.7
		V. dahliae	6	30.7	1.4	32.2	29.1	3180	323	3650	2700	10.5	5.9	20.0	4.5
		Control	5	30.3	1.3	32.0	29.1	3200	353	3640	2700	10.8	4.9	17.4	6.0
	Noninoc.	*V. nonalfalfae*	6	30.7	1.4	32.2	29.1	3180	320	3630	2700	8.4	2.6	12.5	5.0
		Both	6	30.7	1.4	32.2	29.1	3180	325	3650	2700	9.5	4.2	15.8	5.8
		V. dahliae	6	30.7	1.4	32.2	29.1	3180	323	3650	2700	8.7	3.4	13.7	4.5
		Control	5	30.3	1.3	32.0	29.1	3200	353	3640	2700	10.4	4.3	16.5	5.9
PA	Inoculated	*V. nonalfalfae*	5	28.8	1.5	30.9	27.2	3520	309	3880	3140	10.6	4.3	16.4	5.6
		Both	6	29.4	1.4	30.9	27.4	3610	200	3840	3370	16.3	11.1	38.1	6.4
		Control	4	29.1	1.6	30.9	27.0	3670	174	3870	3480	13.1	7.3	19.6	3.2
	Noninoc.	*V. nonalfalfae*	5	28.8	1.5	30.9	27.2	3520	309	3880	3140	11.1	4.6	16.1	5.1
		Both	6	29.4	1.4	30.9	27.4	3610	200	3840	3370	11.6	3.6	18.1	9.1
		Control	4	29.1	1.6	30.9	27.0	3670	174	3870	3480	16.0	11.2	30.6	3.7

Supplemental Table 5: Lists the number of plots within each treatment that have adjacent *A. altissima* trees, and of those, how many displayed Verticillium wilt symptoms.

Treatment	**Total plots**	**Plots with adjacent *A. altissima***	**Verticillium wilt observed in adjacent *A. altissima***
V. nonalfalfae	11	11	10
Both	11	11	10
V. dahliae	6	6	0
Control	9	8	0

Chapter 4: The natural persistence and distribution of the proposed biological control agent *Verticillium nonalfalfae* on *Ailanthus altissima* in Virginia, USA

Abstract

Reports of *Ailanthus altissima* stand declines in south-central Pennsylvania resulted in the identification of the causal agent, a vascular wilt fungus, *Verticillium nonalfalfae*. Additional surveys throughout North America and Europe found that this disease outbreak was not an isolated event. One of these surveys included identifying and then monitoring six naturally diseased *A. altissima* stands in Virginia, USA between 2009 and 2012. To help predict the long-term outcome of infested stands, these naturally infested sites were monitored again from 2017 to 2018. While *A. altissima* were still present at all sites, their densities and volumes were consistently reduced at all sites to just 13% of their original numbers. At all but one of these sites, *V. nonalfalfae* was still causing disease on the remaining *A. altissima*. This reduction of *A. altissima* and persistence of *V. nonalfalfae* highlights this pathogen's effectiveness at controlling this tree over long periods of time. A walking survey looking closely at all *A. altissima* stands within 1 km of these sites found additional *V. nonalfalfae* infections around four sites. Since no correlation existed between tree infection status and distance from the known site, this patchy disease occurrences may indicate this pathogen is able to spread over longer distances or is more abundant than the previous survey detected. Soil collected at these sites did not infect any *A. altissima* seedlings in a greenhouse study, suggesting that soil inoculum may not play an important role in *V. nonalfalfae* persistence. This research further supports the use of *V. nonalfalfae* as an effective biopesticide that can reduce *A. altissima* numbers consistently over many years.

Keywords

Tree of heaven, biocontrol, biopesticide, vascular wilt, non-native

1.0 Introduction

1.1 The invasive *Ailanthus altissima*

Ailanthus altissima (Miller) Swingle, commonly called the tree-of-heaven, is an abundant invasive Chinese tree found throughout all continents except Antarctica (Kowarik and Säumel, 2007). Its aggressive growth, prolific seed production, clonality, tolerance to poor growing conditions, and ability to produce root and stump sprouts make this tree difficult, laborious, and expensive to control. Consequently, eradication is only possible in small isolated areas using a combination of both mechanical and chemical tactics applied over numerous years (Asaro et al., 2009; Gover et al., 2013; Kowarik and Säumel, 2007; Meloche and Murphy, 2006). No other control methods, including biological controls, are currently available that would make the large-scale management of this tree feasible.

1.2 A naturally occurring Verticillium wilt pathogen

In 2002, a stand of diseased *A. altissima* in south-central Pennsylvania, USA was discovered, causing much excitement and interest. With the identification of the causal agent, *Verticillium nonalfalfae* Inderb. (formerly *Verticillium albo-atrum* Reinke and Berthold), by Schall and Davis (2009a), additional Pennsylvania research supporting its use as a biological control agent quickly followed (Kasson et al., 2013; Kasson et al., 2019; Kasson et al., 2015; Kasson et al., 2014; O'Neal and Davis, 2015; O'Neal and Davis, 2015; Schall and Davis, 2009b). With such promising local results, field surveys looking for other areas of decline found additional outbreaks in the mountains of Virginia in 2009 (Snyder et al., 2013; Snyder et al., 2014), in western Pennsylvania in 2010 (Kasson et al., 2014), in Austria, Europe in 2011 (Maschek and Halmschlager, 2017), and in southern Ohio in 2012 (Rebbeck et al., 2013).

Since the first diseased *A. altissima* site was found in Virginia, the number of known sites within the state has increased to six, with formal surveys of each site being conducted in 2011 and 2012 (Snyder et al., 2013; Snyder et al., 2014). These initial surveys found that all sites contained a variety of healthy, symptomatic, and dead *A. altissima*, with disease incidence increasing from one year to the next (Snyder et al., 2014).

1.3 Long-term site outcomes unknown

Research in Pennsylvania has shown that within five years, artificially inoculated sites can be left practically free of *A. altissima*, except for a few lingering vegetative sprouts or seedlings (Kasson et al., 2014; O'Neal and Davis, 2015). Determining if a similar disease progression is occurring in Virginia's naturally infested stands five years after their previous survey could prove helpful in predicting the long-term efficacy of using *V. nonalfalfae* as a biological control agent.

Besides determining the number of healthy, symptomatic, and dead *A. altissima* at these sites, the incidence of *V. nonalfalfae* in adjacent *A. altissima* stands would also help determine the pathogen's overall distribution in these areas. If locally present, its distribution might provide insight into the pathogen's prevalence or indicate how *V. nonalfalfae* spreads in addition to functional root grafts (O'Neal and Davis, 2015).

Regardless of *A. altissima* presence, *V. nonalfalfae* may still exist at these sites. For example, most *Verticillium* species develop resting structures in symptomatic host tissue in order to be reincorporated into the soil as the plant material decomposes (Fradin and Thomma, 2006; Hiemstra and Harris, 1998). These resting structures can allow Verticillium species to survive in soil for long periods of time without any hosts present (Pegg and Brady, 2002). In controlled lab conditions, *V. nonalfalfae* can produce effective inoculum for at least four to eight years after being stored in soil (O'Neal and Davis, 2015; Wickert, 2019). However, soil collected from around a single recently killed *A. altissima* tree applied directly to wounded *A. altissima* stems did not result in disease progression (O'Neal, 2014). Therefore, understanding if *V. nonalfalfae* persists in field soil would help us better understand the pathogen's disease cycle and predict if areas where *A. altissima* has been eradicated are protected from reinvasion.

1.4 Research goals

The goal of this research was to evaluate Virginia's six naturally occurring *V. nonalfalfae* infected *A. altissima* stands previously identified by Snyder et al. (2014) and (1) assess healthy, symptomatic, and dead *A. altissima* presence within the stand, (2) survey surrounding *A. altissima* stands to determine local disease distribution, and (3) establish if soil collected from those sites has the ability to infect healthy *A. altissima* seedlings. We hypothesized that (1) if any *A. altissima* remained at these sites *V. nonalfalfae* would still be found infecting them, (2) that symptomatic *A. altissima* stands could be located adjacent to these sites, (3) and that soil collected from these sites would quickly infect healthy *A. altissima* seedlings.

2.0 Materials and methods

2.1 Within-stand persistence of *A. altissima* and *V. nonalfalfae*

By 2011, Snyder et al. (2014) had confirmed the presence of six natural *V. nonalfalfae* infected *A. altissima* stands in Virginia (site 81, mv, 32, 30, 48, and 72; Table 1) and monitored them for two consecutive years (2011 and 2012). This monitoring including recording the number of alive, symptomatic, and dead *A. altissima* with a diameter at breast height (DBH) ≥ 2.5 cm (Table 2; Snyder et al., 2014). Therefore, to better understand the long-term persistence and distribution of *A. altissima* and *V. nonalfalfae*, we revisited each of these sites in 2017, five years after their previous survey. Access permission was obtained from the landowner or by acquiring necessary permits (VA Department of Transportation Land Use Permit 018-6614 and USDA NPS Scientific Research & Collecting Permit SHEN-2017-SCI-002 & SHEN-2018-SCI-0010).

At each site, the previously surveyed boundaries were determined based on all available GPS points, published site descriptions, and any remaining markings found in the field. Every *A. altissima* tree with a DBH ≥ 2.5 cm within these boundaries were then mapped using the Survey123 for ArcGIS smartphone app (ERSI®, http://survey123.arcgis.com/) in which the latitude and longitude (± 5 - 10 m), status (asymptomatic, displaying Verticillium wilt symptoms, or dead), and DBH (cm) of each tree was recorded. The area of each surveyed stand was then calculated using the convex hull of all mapped *A. altissima* datapoints (Hijmans, 2019; R Core Team, 2018).

Any symptomatic trees were subsampled at each site to confirm the presence of *V. nonalfalfae* by removing sections of discolored xylem with sterile tools. A portion of this tissue not previously exposed was then plated on prune extract agar amended with streptomycin sulfate and neomycin sulfate (PEA+SN) and monitored every other day for Verticillium-like growth (Talboys, 1960). Single-conidia cultures of any resulting colonies were allowed to produce resting structures to confirm *V. nonalfalfae* identification (Inderbitzin et al., 2011).

A direct comparison between the previous survey reported in Snyder et al. (2014) and these findings was made using only density-depended measurements (live trees per hectare and average basal area (m^2) per hectare) to mitigate any possible differences in the exact area surveyed.

2.2 Local *V. nonalfalfae* distribution in *A. altissima* stands

Infected *A. altissima* around the sites identified by Snyder et al. (2014) were revisited in 2018 to better understand the local prevalence of *V. nonalfalfae*. In addition, two control sites known by the authors to have never shown any symptoms of Verticillium wilt (site c1 and c2; Figure 1 and Table 1) were also visited. At each site, all adjacent public rights-of-way were walked for 1 km in every available direction. During this radial foot survey, continuous 10-m sections were paced out, and the presence of either healthy or symptomatic *A. altissima* within each section was recorded. Any symptomatic trees found were subsampled to confirm *V. nonalfalfae* presence using the methods described above.

The collected data were then analyzed using a logistic regression to determine if the probability of finding *V. nonalfalfae*-infected *A. altissima* varied as distance from known infection sites changed (R Core Team, 2018). The model that was analyzed, a generalized linear model function with a logit link, contained two additive predictive variables: the distance of an observation from the known infection site (m) and the site code. Site code was included to account for the lack of independence between adjacent 10-m section within a single site, as neighboring sections may be connected by functional root grafts or clonal growth. The significance ($\alpha = 0.05$) of the distance variable was used to assess this relationship.

2.3 Presence of *V. nonalfalfae* soil inoculum

A soil baiting method was used to detect if *V. nonalfalfae* capable of infecting *A. altissima* was present in site soils. This method was modified from Wilhelm (1955), who was able to determine *V. dahliae* inoculum load in agricultural lands by planting tomatoes into fields and monitoring them for symptoms. Accordingly, all *V. nonalfalfae*-positive sites (81, mv, 32, 48, and 72) and the two previously used control sites (c1 and c2) were revisited during the summer of 2018. At each site, 1 L of soil was collected using a spade sanitized with 70% EtOH from the base of 10 representative *A. altissima*. Symptomatic or dead *A. altissima* were selected at infested sites and healthy *A. altissima* were selected at control sites. Each sample included the top 10 cm of soil pooled from three evenly distributed locations 15 cm away from the tree base. These 70 samples were placed into sterile Ziplock bags and stored at 4°C.

Healthy *A. altissima* seedlings from a previously confirmed susceptible seed source (Brooks et al., 2019) were germinated in potting soil (Miracle-Gro® Potting Mix 0.21-0.11-0.16) and allowed to grow for two months. On 14 April, 2019, the field soil samples were moved to the greenhouse and individually mixed 1:2 with potting soil using a 70% EtOH cleaned spade in 3.8-L plastic pots. The potting soil was added to ensure that appropriate drainage and nutrient needs of the seedlings were met regardless of the characteristics of the collected soil sample. Then, three healthy *A. altissima* seedlings were randomly selected, transplanted into each pot, and monitored weekly for disease symptoms (Figure 4). These pots were randomly arranged and watered as needed. Any seedling that became symptomatic in the first 48 hrs was determined to be killed by transplant stress and replaced.

As the three seedlings outgrew their shared pot, the smallest seedling was removed and destructively sampled for *V. nonalfalfae* isolation at 43 (27 May), 81 (4 July), and 145 (6 September) days post-transplanting. This staggered sampling allowed for both continued monitoring for disease within the xylem and encouraged aggressive root growth within each pot. If seedlings died prior to the planned removal date, they were also immediately destructively sampled.

3.0 Results

3.1 Within-stand persistence of *A. altissima* and *V. nonalfalfae*

The center of all six previously identified sites were successfully located and all adjacent *A. altissima* were surveyed. Due to the limited number of original GPS points (ranging from one to eight for each site; Figure 2) and the speed at which *A. altissima* can both decay and/or grow into new areas, the initial site boundaries were difficult to determine with a high level of precision. This inability to identify exact boundaries resulted in a mismatch between previous and current stand areas between the two survey periods (Table 2). However, any potential *A. altissima* at each site that may have possibly been counted in the earlier monitoring events were included in 2017 to ensure that no *A. altissima* were missed.

In total, 1,509 *A. altissima* were measured, mapped, and rated in 2017 compared to the 9,383 and 9,437 counted in 2011 and 2012 by Snyder et al. (2014; Table 2). Of these mapped trees, the living *A. altissima* (healthy or symptomatic) were reduced to just 13% of their original number (960/7,440). Only five living *A. altissima* were relocated at all sites in 2017, as no sites were completely eradicated.

Out of these six sites, five sites contained symptomatic *A. altissima* that resulted in *V. nonalfalfae* reisolation (Table 2). Site 30, the one site that did not contain any symptomatic trees, was treated (along with many kilometers of roadway in either direction) with a hack-n-squirt herbicide targeting *A. altissima* in 2015. This treatment application was confirmed by observing hack-n-squirt wounds on all dead *A. altissima* along that section of roadway. This herbicide treatment greatly reduced the *A. altissima* number, and likely removed any symptomatic trees from the vicinity. No other sites had signs of herbicide treatments.

The number of living *A. altissima* per hectare (trees/ha) and the average basal area per hectare (m^2/ha) were compared between the two survey periods (Table 2). At all five sites not compromised by herbicide treatment, the number of living *A. altissima* per hectare was reduced (decreasing the overall average from 602 to 66 trees/ha in five years) as was the average basal area per hectare (decreasing the overall average from 16.1 to 2.9 m^2/ha in five years).

3.2 Local *V. nonalfalfae* distribution in *A. altissima* stands

In total, 43.64 km (4,364 10-m sections) of unique roadside was surveyed around sites 81, mv, 32, 48, 72, c1, and c2. Site 30 was not included in this survey since all surrounding roadsides had been treated with herbicide targeting *A. altissima*. *Ailanthus altissima* was present within 1 km of every site and overall was present in 12.8% (557) of the 10-m sections (Table 3).

Four of the five *V. nonalfalfae*-positive sites visited had *V. nonalfalfae* isolated from at least one symptomatic *A. altissima* stand found within the 1-km surrounding area, with symptomatic trees found in 17.3% of the 10-m sections that contained *A. altissima*. The last site, mv, and all the control sites did not contain any adjacent symptomatic *A. altissima* despite the presence of healthy *A. altissima* (Table 3).

A logarithmic regression was run on the data from all sites with surrounding *V. nonalfalfae* infections (site 32, 48, 72, and 81) to determine if the probability of there being symptomatic *A. altissima* within a 10-m section changed as distance from the site varied. The model's distance term was not significant (P = 0.598), with the model indicating there was between a 5 – 30% probability of finding symptomatic *A. altissima* in a section regardless of the distance from the site (Figure 3; R Core Team, 2018).

3.3 Presence of *V. nonalfalfae* soil inoculum

No Verticillium wilt symptoms were observed on any of the 210 seedlings and no destructive sampling resulted in any *V. nonalfalfae* isolation throughout this entire experiment. After the final destructive sampling event, prolific and dense root growth was confirmed in all pots when the soil was removed for disposal (Figure 4).

4.0 Discussion

4.1 *Verticillium nonalfalfae* persists, reducing, but not eradicating, *A. altissima*

We found that over the past five years, healthy *A. altissima* were able to persist at all Virginia sites infected with *V. nonalfalfae* disease, and in no cases was *A. altissima* completely eradicated. However, *A. altissima* densities (living trees per hectare) and volumes (BA per hectare) were considerably reduced over this time period (Table 2). This reduction, but lack of eradication, of *A. altissima* matches the disease progression seen at artificially inoculated stands in Pennsylvania two to five years post-inoculation (Kasson et al., 2014; O'Neal and Davis, 2015).

Due to the adjustments made to account for the difficulty in locating precise stand boundaries, the decrease of *A. altissima* at these Virginia sites may actually be more extreme than reported. Specifically, to ensure that our results did not exaggerate any *A. altissima* reductions, we generously estimated stand boundaries searching for any remaining *A. altissima* and then minimized our area calculations by using the convex hull of all mapped data points. This practice likely resulted in increased density and volume measurements in 2017. Nevertheless, the reduction of *A. altissima* at all sites was substantial, even using this conservative method.

In addition to *A. altissima*, we also showed that *V. nonalfalfae* was persisting and actively causing disease at all but one of these naturally infested sites. This long-term survival and activity of this pathogen demonstrates its ability to persist and continue to control *A. altissima* for many years. Site 30, the one site where *V. nonalfalfae* was no longer found causing disease, was located along a roadway in which an herbicide treatment targeting A. altissima had been previously applied. This herbicide application appears to have dramatically reduced *A. altissima* to just 6% of their original numbers. With this rapid reduction in host numbers, it is not surprising that there were no symptomatic *A. altissima* remaining.

Within all sites, tree status (healthy, diseased, or dead) was unevenly distributed spatially (Figure 2). This clumping makes sense, as *V. nonalfalfae* spreads rapidly to adjacent trees through functional root grafts but may be limited in its ability to spread over longer distances (O'Neal and Davis, 2015). Therefore, a well-distributed biological control application of *V. nonalfalfae* would likely increase the rate at which *V. nonalfalfae* could remove *A. altissima* in a stand by thoroughly spreading out disease. A finer-scale understanding of how disease distribution spreads within a stand would provide additional insights into how to determine which subset of trees should receive a biological control application, and we encourage someone to repeat this work in the future using our spatial information.

We would also like to note that five of these six stands were initially found during a roadside survey specifically looking for large areas of declining *A. altissima* (Snyder et al., 2014). However, when revisiting these sites, it was often difficult or even impossible to see any *A. altissima* from the roadway due to the overall *A. altissima* reduction. The inconspicuousness of sites with high levels of disease may limit our ability to survey for *V. nonalfalfae* infected *A. altissima* thoroughly in other areas, resulting in underestimates of *V. nonalfalfae* disease distribution.

Since this work was completed five years after the previous survey and eight years since the identification of the first Virginian site, *V. nonalfalfae* appears able to persist at naturally infested sites, reducing, but not eradicating, *A. altissima*. Because biological controls are used to manage pests, not eradicate them, these results support the long-term biological control effectiveness of *V. nonalfalfae* (DeBach, 1964).

4.2 Additional *V. nonalfalfae* infected *A. altissima* found locally

Not surprisingly for an invasive species, we found scattered *A. altissima* populations within 1 km of all seven surveyed sites (Table 3). This pervasiveness of *A. altissima* along roadsides throughout the mountain region of Virginia is well known, and does not make these specific sites unique (McAvoy et al., 2012). In contrast to *A. altissima*'s abundance, Snyder et al. (2014) had to survey 26,500 km of roadside in Virginia, North Carolina, and South Carolina, to locate half-a-dozen *V. nonalfalfae*-infected *A. altissima* sites. So, it was unexpected that *V. nonalfalfae*-infected *A. altissima* stands were found within 1 km of four of these infested sites. Since careful walking surveys of these areas had previously not been completed, it is impossible to know if these other stands were previously diseased or had become diseased within the past five years.

Within the 1 km surveyed area of these four sites, the probability of finding *V. nonalfalfae* infected *A. altissima* did not vary as the distance from the known surveyed site changed (Figure 3). Since no diseased trees were found around control sites, it is probable that these larger diseased areas are in themselves patchy, and if we had surveyed past the 1 km mark, we would eventually expect a reduction in this probability. Regardless, within this 1 km range it appears that diseased *A. altissima* are randomly distributed among healthy *A. altissima*. This may indicate that the local dispersal of *V. nonalfalfae* between *A. altissima* stands is driven by something fairly mobile, such as the ambrosia beetle, *Euwallacea validus* (Ploetz et al., 2013; Schall, 2008), not by something that would likely cause an even gradient, such as the dispersal of inoculum through the spread of plant material or functional root grafts (O'Neal and Davis, 2015).

This scattered distribution of diseased trees among local *A. altissima* stands shows that within six to eight years, *V. nonalfalfae* has not thoroughly dispersed to all *A. altissima* within 1 km. Therefore, a formulated biopesticide that could be dispersed throughout many stands would likely help increase the efficiency of this naturally occurring pathogen in areas where *A. altissima* are not especially dense.

4.3 Field collected soil did not infect *A. altissima* seedlings

None of the 210 *A. altissima* seedlings became symptomatic when planted in field-collected soil originating from the bases of symptomatic or healthy trees. This matched previous work in which *A. altissima* was not infected when soil collected around a single infected tree was applied to artificial stem wounds (O'Neal and Davis, 2015), and in which *V. nonalfalfae* was not successfully cultured or molecularly confirmed from soil collected at infested sites (Schall, 2008). Since most research regarding *Verticillium* species survival in soil has focused on *V. dahliae* and its production of microsclerotia, it is unclear if *V. nonalfalfae*'s lack of microsclerotia production would minimize its survival in soil. It is probable that compared to *V. dahliae*'s microsclerotia, *V. nonalfalfae*'s melanized hyphae might not survive as long (Sewell and Wilson, 1966; Pegg and Brady, 2002).

It is interesting to note that O'Neal and Davis (2015) were able to get disease to develop when symptomatic wood was taken from a living *A. altissima* and inserted into healthy *A. altissima* artificial stem wounds. They were also able to get disease development when applying symptomatic leaf tissue in the same manner. This could indicate that *V. nonalfalfae* survives within dead plant material at a site. However, since we did not exclude the top organic layer from these soil samples, it is likely that decomposing wood and dead leaves from infected trees were included in this baiting attempt. Therefore, it may be that only living or recently dead tissue contains viable inoculum, not long-dead tissue. Though other reservoirs of *V. nonalfalfae* could exist at these sites, such as survival in other symptomless carriers (Thanassoulopoulos et al., 1981), it is currently unknown how, or if, *V. nonalfalfae* survives at a site when no *A. altissima* remain.

Another explanation for this finding is that field collected soils may not favor *V. nonalfalfae,* limiting its ability to persist or germinate in the soils. Disease-suppressive soils have been shown to exist in other systems (Kinkel et al., 2011), and therefore could help explain the inability of field-collected soil to infect healthy *A. altissima* while greenhouse inoculated potting soil has been shown to successfully infect *A. altissima* seedlings (Kasson et al., 2015). If this is the case, transmission in the field could be limited to functional root grafts, not soil transmission. This would reduce the practical host-range of *V. nonalfalfae* to species able to form inter-specific functional root grafts with *A. altissima*, and could therefore explain the low infection rates of other species observed in the field (Kasson et al., 2015). Inter-specific root grafts between *A. altissima* and other species have yet to be investigated.

If *V. nonalfalfae* is unable to remain at these sites once *A. altissima* have been removed, these areas may not be protected from an *A. altissima* reinvasion. This is especially true because *A. altissima* is considered an early-successional gap-obligate species which could easily take advantage of the disturbance caused by the initial *A. altissima* removal (Carter and Fredericksen, 2007; Kasson et al., 2013; Knapp and Canham, 2000). *Ailanthus altissima* recolonization could occur through the reintroduction of seeds which can travel far (Kowarik and Von der Lippe, 2011; Landenberger et al., 2007) or by the germination of seedbank seeds which remain viable in soil for at least five years (Rebbeck and Jolliff, 2018). Therefore, if *V. nonalfalfae* is used as a biopesticide, just like traditional herbicides, repeat applications may be necessary for the long-term control of *A. altissima* in a single location. This lack of survival in soil, which may limit *V. nonalfalfae*'s long-term site protection, may actually help in biopesticide registration efforts, as the USDA Environmental Protection Agency often discourages pesticide products that have long-lasting site residuals.

We do acknowledge that this baiting isolation method may be limited in its conclusions, as it was only tested in one set of environmental and temporal conditions and did not result in any positive infections. For example, the storage, greenhouse, or watering conditions may have limited the viability of *V. nonalfalfae* in the soil samples or the volume of field soil used may not have been large enough to contain the inoculum load needed for infection. If resources were not a limiting factor, we suggest planting seedlings at these field sites and monitoring their survival and disease progression there to minimize these variables. Despite these weaknesses, we attempted to maximize the likelihood that this method would result in disease development by using a susceptible seed source, confirming aggressive root growth had occurred by the end of the experiment (Figure 4), and destructively sampling seedlings at three separate time periods. We also let the experiment run for 21 weeks, well past the typical two to four weeks needed between infection and symptom development (Schall and Davis, 2009a). Lastly, when we transplanted seedlings, we likely caused a low level of root damage creating additional infection points for *V. nonalfalfae* as Verticillium species tend to infect plants through root tips or damaged areas (Fradin and Thomma, 2006).

We believe this baiting method is practical because it not only can confirm the presence of *V. nonalfalfae* in soil, but can also confirm it is able to infect a specific host (Wilhelm, 1955). This host specificity information cannot be currently obtained using molecular tests or isolating directly from soil.

4.4 Conclusions

This work confirmed that naturally occurring *V. nonalfalfae* persists within *A. altissima* stands, reducing, but not eradicating *A. altissima* in Virginia over the course of six to eight years. Additionally, *V. nonalfalfae*-infected *A. altissima* was found more often than not in surrounding *A. altissima* stands, indicating that the infection sites are either more common than initially assumed or the disease is spreading between stands. Lastly, field collected soil was unable to infect *A. altissima* seedlings suggesting that these areas may not be protected from *A. altissima* recolonization. These findings support continuing biopesticide registration efforts of *V. nonalfalfae*.

Longer-term surveys like this are beneficial to help predict site outcome and biological control potential of plant pathogens.

Acknowledgements

We thank Tom McAvoy, Laney Metz, Caleb Gore, Amy Snyder, the Shenandoah National Park, the Virginia Department of Transportation, and Miller School of Albemarle. This work was supported by the US Forest Service [15-CA-11420004-161].

References

Asaro, C., Becker, C., & Creighton, J. (2009). Control and utilization of tree-of-heaven: A guide for Virginia landowners. Charlottesville, VA: Virginia Department of Forestry Publication P00144.

Brooks, R. K., Bush, E. E., Salom, S. M., & Baudoin, A. (2019). First report of Verticillium wilt caused by *Verticillium dahliae* impacting *Ailanthus altissima* (tree of heaven) in Virginia, USA. Plant Disease. doi:10.1094/PDIS-10-19-2064-PDN

Carter, W. K., & Fredericksen, T. S. (2007). Tree seedling and sapling density and deer browsing incidence on recently logged and mature non-industrial private forestlands in Virginia, USA. Forest Ecology and Management, 242(2), 671-677. doi:10.1016/j.foreco.2007.01.086

DeBach, P. (1964). Biological Control of Insect Pests and Weeds. London: Chapman and Hall Ltd.

Fradin, E. F., & Thomma, B. P. (2006). Physiology and molecular aspects of Verticillium wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology, 7(2), 71-86. doi:10.1111/j.1364-3703.2006.00323.x

Gover, A., Johnson, J., Lloyd, K., & Sellmer, J. (2013). Tree-of-heaven (*Ailanthus altissima*), Quicksheet 5: Wildland Weed Management, Penn State, College of Agricultural Sciences.

Hiemstra, J., & Harris, D. (1998). A compendium of Verticillium wilts in tree species. Wageningen, The Netherlands: Ponsen & Looijen.

Hijmans, R. J. (2019). geosphere: Spherical Trigonometry. R package version 1.5-10. Retrieved from https://CRAN.R-project.org/package=geosphere.

Inderbitzin, P., Bostock, R. M., Davis, R. M., Usami, T., Platt, H. W., & Subbarao, K. V. (2011). Phylogenetics and taxonomy of the fungal vascular wilt pathogen Verticillium, with the descriptions of five new species. PLoS One, 6(12), e28341. doi:10.1371/journal.pone.0028341

Kahle, D., & Wickham, H. (2013). ggmap: Spatial visualization with ggplot2. The R Journal, 5(1), 144-161. doi:10.32614/RJ-2013-014

Kasson, M. T., Davis, M. D., & Davis, D. D. (2013). The invasive *Ailanthus altissima* in Pennsylvania: A case study elucidating species introduction, migration, invasion, and growth patterns in the Northeastern US. Northeastern Naturalist, 20, 1-60. doi:10.1656/045.020.m101

Kasson, M. T., Kasson, L. R., Wickert, K. L., Davis, D. D., & Stajich, J. E. (2019). Genome sequence of a lethal vascular wilt fungus, *Verticillium nonalfalfae*, a biological control used against the invasive *Ailanthus altissima*. Microbiology Resource Announcements, 8(4), e01619-01618. doi:10.1128/MRA.01619-18

Kasson, M. T., O'Neal, E. S., & Davis, D. D. (2015). Expanded host range testing for *Verticillium nonalfalfae*: potential biocontrol agent against the invasive *Ailanthus altissima*. Plant Disease, 99(6), 823-835. doi:10.1094/PDIS-04-14-0391-RE

Kasson, M. T., Short, D. P., O'Neal, E. S., Subbarao, K. V., & Davis, D. D. (2014). Comparative pathogenicity, biocontrol efficacy, and multilocus sequence typing of *Verticillium nonalfalfae* from the invasive *Ailanthus altissima* and other hosts. Phytopathology, 104(3), 282-292. doi:10.1094/PHYTO-06-13-0148-R

Knapp, L. B., & Canham, C. D. (2000). Invasion of an old-growth forest in New York by *Ailanthus altissima*: sapling growth and recruitment in canopy gaps. Journal of the Torrey Botanical Society, 307-315. doi:10.2307/3088649

Kowarik, I., & Säumel, I. (2007). Biological flora of Central Europe: *Ailanthus altissima* (Mill.) Swingle. Perspectives in Plant Ecology, Evolution, and Systematics, 8, 207-237. doi:10.1016/j.ppees.2007.03.002

Kowarik, I., & Von der Lippe, M. (2011). Secondary wind dispersal enhances long-distance dispersal of an invasive species in urban road corridors. NeoBiota, 9, 49. doi:10.3897/neobiota.9.1469

Landenberger, R. E., Kota, N. L., & McGraw, J. B. (2007). Seed dispersal of the non-native invasive tree *Ailanthus altissima* into contrasting environments. Plant Ecology, 192(1), 55-70. doi:10.1007/s11258-006-9226-0

Maschek, O., & Halmschlager, E. (2017). Natural distribution of Verticillium wilt on invasive *Ailanthus altissima* in eastern Austria and its potential for biocontrol. Forest Pathology, 47(5), 1-11. doi:10.1111/efp.12356

McAvoy, T. J., Snyder, A. L., Johnson, N., Salom, S. M., & Kok, L. T. (2012). Road survey of the invasive tree-of-heaven (*Ailanthus altissima*) in Virginia. Invasive Plant Science and Management, 5(4), 506-512. doi:10.1614/Ipsm-D-12-00039.1

Meloche, C., & Murphy, S. D. (2006). Managing tree-of-heaven (*Ailanthus altissima*) in parks and protected areas: a case study of Rondeau Provincial Park (Ontario, Canada). Environmental Management, 37(6), 764-772. doi:10.1007/s00267-003-0151-x

O'Neal, E. S. (2014). Biological control of *Ailanthus altissima*: transmission, formulation, and risk assessment of *Verticillium nonalfalfae*. (M.S. Thesis). The Pennsylvania State University, The Pennsylvania State University.

O'Neal, E. S., & Davis, D. D. (2015). Biocontrol of *Ailanthus altissima*: Inoculation protocol and risk assessment for *Verticillium nonalfalfae* (Plectosphaerellaceae: Phyllachorales). Biocontrol Science and Technology, 25(8), 950-969. doi:10.1080/09583157.2015.1023258

O'Neal, E. S., & Davis, D. D. (2015). Intraspecific root grafts and clonal growth within *Ailanthus altissima* stands influence *Verticillium nonalfalfae* transmission. Plant Disease, 99(8), 1070-1077. doi:10.1094/pdis-07-14-0722-re

Pegg, G. F., & Brady, B. L. (2002). Verticillium wilts. New York: CABI Publishing.

Ploetz, R. C., Hulcr, J., Wingfield, M. J., & de Beer, Z. W. (2013). Destructive tree diseases associated with ambrosia and bark beetles: black swan events in tree pathology? Plant Disease, 97(7), 856-872. doi:10.1094/PDIS-01-13-0056-FE

R Core Team. (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Rebbeck, J., & Jolliff, J. (2018). How long do seeds of the invasive tree, *Ailanthus altissima* remain viable? Forest Ecology and Management, 429, 175-179. doi:10.1016/j.foreco.2018.07.001

Rebbeck, J., Malone, M. A., Short, D. P. G., Kasson, M. T., O'Neal, E. S., & Davis, D. D. (2013). First report of Verticillium wilt caused by *Verticillium nonalfalfae* on tree-of-heaven (*Ailanthus altissima*) in Ohio. Plant Disease, 97(7), 999-999. doi:10.1094/pdis-01-13-0062-pdn

Schall, M. J. (2008). Verticillium wilt of *Ailanthus altissima*. (Ph.D. Dissertation). The Pennsylvania State University,

Schall, M. J., & Davis, D. D. (2009a). *Ailanthus altissima* wilt and mortality: etiology. Plant Disease, 93(7), 747-751. doi:10.1094/Pdis-93-7-0747

Schall, M. J., & Davis, D. D. (2009b). Verticillium wilt of *Ailanthus altissima*: susceptibility of associated tree species. Plant Disease, 93(11), 1158-1162. doi:10.1094/Pdis-93-11-1158

Sewell, G. W. F., Wilson, J. F. (1966). Verticllium wilt of the hop: the survival of *V. albo-atrum* in soil. Annals of Applied Biology, 58, 241-249. doi:10.1111/j.1744-7348.1966.tb04383.x.

Snyder, A. L., Kasson, M. T., Salom, S. M., Davis, D. D., Griffin, G. J., & Kok, L. T. (2013). First report of Verticillium wilt of *Ailanthus altissimain* Virginia caused by *Verticillium nonalfalfae*. Plant Disease, 97(6), 837-837. doi:10.1094/pdis-05-12-0502-pdn

Snyder, A. L., Salom, S. M., & Kok, L. T. (2014). Survey of *Verticillium nonalfalfae* (Phyllachorales) on tree-of-heaven in the southeastern USA. Biocontrol Science and Technology, 24(3), 303-314. doi:10.1080/09583157.2013.860426

Talboys, P. W. (1960). A culture-medium aiding the identification of *Verticillium albo-atrum* and *V. dahliae*. Plant Pathology, 9(2), 57-58. doi:10.1111/j.1365-3059.1960.tb01147.x

Thanassoulopoulos, C., Biris, D., & Tjamos, E. (1981). Weed hosts as inoculum source of Verticillium in olive orchards. Phytopathologia Mediterranea, 20, 164-168. Retrieved from https://www.jstor.org/stable/42684562?seq=1

Wickert, K. L. (2019). Elucidating disease dynamics in the biocontrol of *Ailanthus altissima* while confiriming the host specificity of the vascular wilt pathogen *Verticillium nonalfaflae*. (Ph.D. Dissertation). West Virignia University, Morgantown, West Virginia.

Wickham, H. (2016). ggplot2: elegant graphics for data analysis: Springer-Verlag New York. Retrieved from https://ggplot2.tidyverse.org

Wilhelm, S. (1955). Longevity of the Verticillium wilt fungus in the laboratory and field. Phytopathology, 45(3), 180-181.

Figures

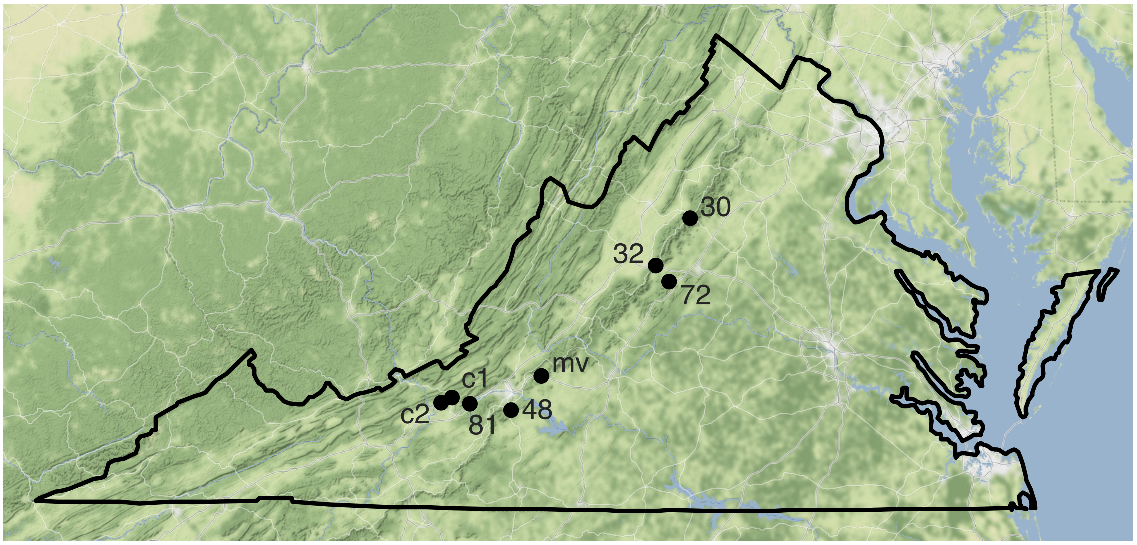


Figure 1: Location of the six *V. nonalfalfae* infected *A. altissima* sites (30, 32, 72, mv, 48, and 81) identified by Snyder et al. (2014) and two control sites (c1 and c2) in Virginia, USA (Kahle and Wickham, 2013; R Core Team, 2018).

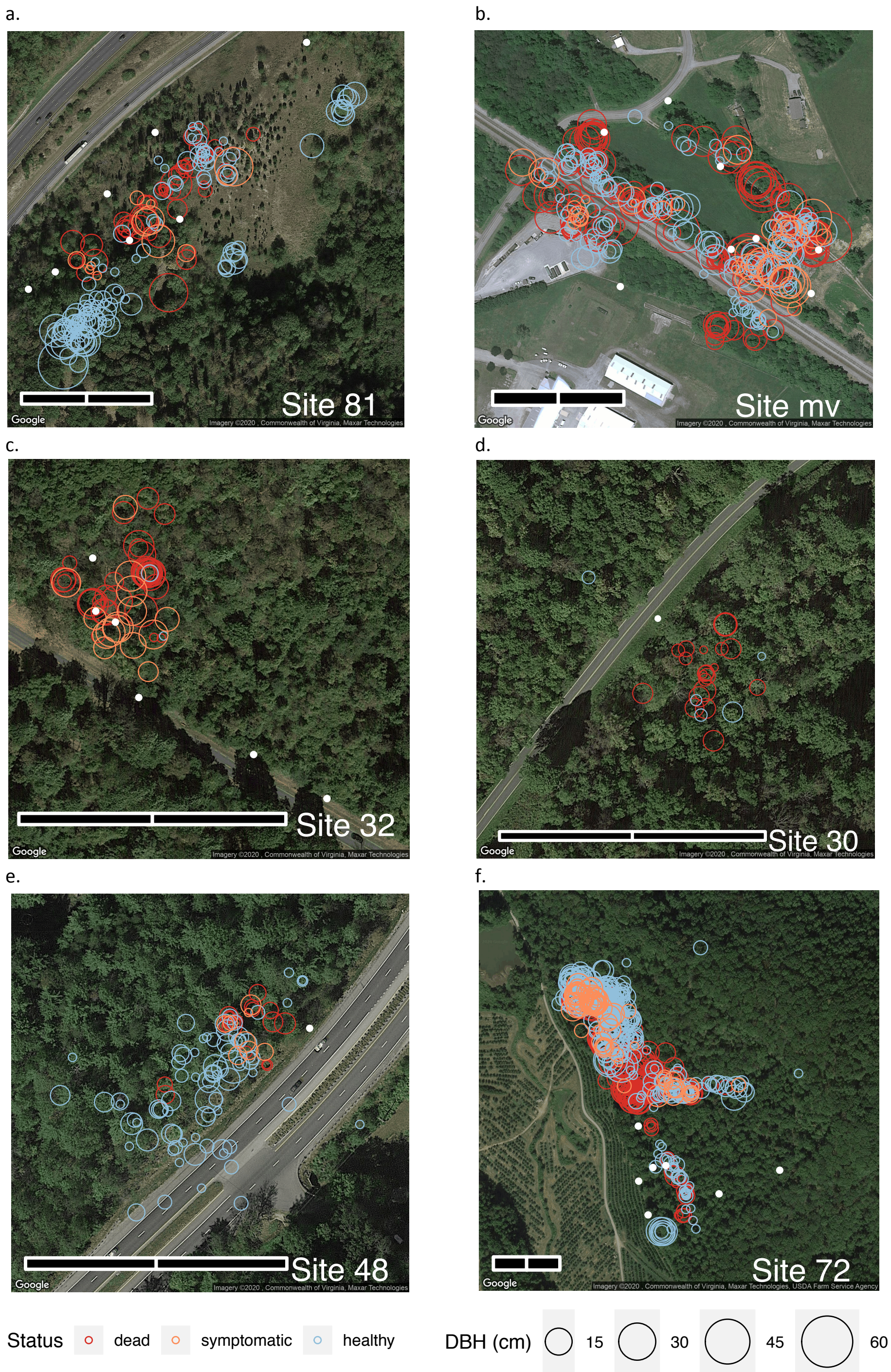


Figure 2: Maps of all *V. nonalfalfae* infested sites with the location of all standing *A. altissima* ≥ 2.5 cm indicated. Color represents tree health status (blue = healthy, orange = symptomatic, red = dead) and size represents diameter at breast height (DBH, cm). Datapoints were collected using Survey123 for ArcGIS smartphone app (ERSI®, http://survey123.arcgis.com/). White points represent Snyder et al (2014) GPS data available from original survey. (a) Site 81, (b) site mv, (c) site 32, (d) site 30, (e) site 48, and (f) site 72. Total scalebar length = 100 m. Note that all points are ± 5 – 10 m accurate, with the most apparently inaccurate site (site 48) likely being

influenced by the steepness of the stand's terrain. Maps created using the ggmap package in R (Kahle and Wickham, 2013; R Core Team, 2018).

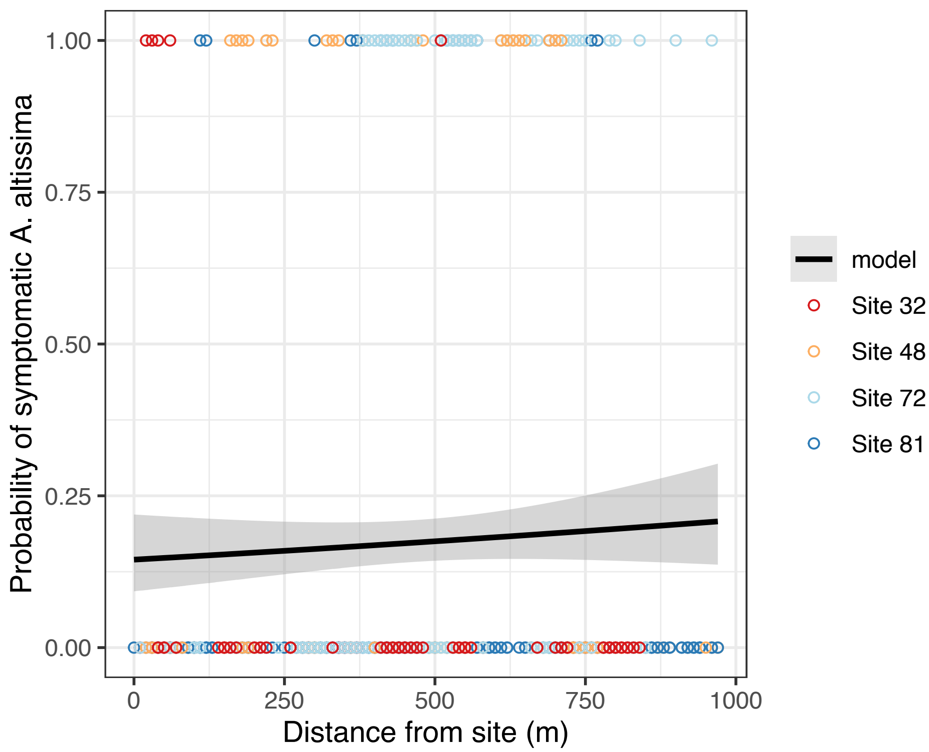


Figure 3: Relationship between the probability of finding symptomatic *A. altissima* and the distance from known *V. nonalfalfae* infection at the Virginia sites 81, 32, 48, and 72. Sites that had no *V. nonalfalfae* adjacent to the plot were excluded. Each point represents the presence of *A. altissima* in a 10-m stretch along a right-of-way as either symptomatic (1) or asymptomatic (0) with color indicating site. An additive logistic regression (status ~ distance + site) is shown in black, with its 95% confidence interval in gray. Distance from site is not significant (P = 0.598), suggesting that the probability of finding *V. nonalfalfae* infected *A. altissima* within 1 km of the site is randomly distributed (R Core Team, 2018; Wickham, 2016).

Figure 4: Experimental layout the day the 210 *A. altissima* seedlings were planted in field collected soil (left and middle) and an example of aggressive root growth observed the final day of the experiment (right).

Tables

Table 1: Name, type (*V. nonalfalfae* or control), town, location (latitude and longitude of center point), and owner information for all sites referenced throughout this study.

Site	Type	Town	Location	Owner
81	*V. nonalfalfae*	Shawsville	37.20592, -80.26466	VA Dept of Transportation
mv	*V. nonalfalfae*	Montvale	37.37880, -79.70790	Private
32	*V. nonalfalfae*	Dooms	38.05969, -78.81700	Shenandoah National Park
30	*V. nonalfalfae*	Elkton	38.35020, -78.54865	Shenandoah National Park
48	*V. nonalfalfae*	Roanoke	37.16628, -79.94394	VA Dept of Transportation
72	*V. nonalfalfae*	Samuel Miller	37.96107, -78.71275	Private
C1	Control	Blacksburg	37.24443, -80.40469	Town of Blacksburg
C2	Control	Blacksburg	37.21235, -80.48891	Virginia Tech

Table 2: Site information for each year sites 81, mv, 32, 30, 48, and 72 were visited. Area (m^2) of stand surveyed, count of healthy, symptomatic, and dead *A. altissima* ≥ 2.5 cm in diameter at breast height, the number of live *A. altissima* per hectare, *A. altissima* basal area per hectare (BA; m^2/ha), and the 2017 *V. nonalfalfae* isolation results are shown. 2011 and 2012 data obtained from Snyder et al. 2014. 2017 area based on the convex hull of each set of points (Hijmans, 2019; R Core Team, 2018).

	2011						2012[1]				2017						
Site	Area	Healthy	Sympt.	Dead	Live trees/ha	BA	Healthy	Sympt.	Dead	Live trees/ha	Area	Healthy	Sympt.	Dead	Live trees/ha	BA	Isolation
81	2,617	72	51	295	470.0	14.9	69	45	304	435.6	20,138	118	12	49	64.6	1.13	Positive
mv	24,519	395	286	501	277.7	14.0	392	280	510	274.1	25,130	160	41	195	80.0	3.90	Positive
32	3,219	23	39	45	192.6	12.1	14	42	51	174.0	1,657	2	15	39	108.6	8.04	Positive
30[2]	1,252	13	14	23	215.7	14.9	9	14	27	183.7	1,970	5	0	21	25.4	0.94	Negative
48	2,103	16	31	79	223.5	20.1	10	36	84	218.7	6,975	81	8	20	133.3	0.76	Positive
72	90,206	4,000	2,500	1,000	720.6	19.5	3,850	2,650	1,050	720.6	90,338	444	69	230	56.8	0.52	Positive

[1]Area and BA not included for 2012, but assumed the same as 2011, based on information published by Snyder et al. (2014).

[2]Site 30 was aggressively treated with a hack-n-squirt herbicide in 2015.

Table 3: Results of the 1 km radial survey surrounding sites 81, mv, 32, 48, 72, c1, and c2. The site name, type, and count of 10-m sections, and *V. nonalfalfae* isolation results are listed. Count of 10-m sections is divided into those without *A. altissima*, those with healthy *A. altissima*, and those with symptomatic *A. altissima*.

Site[1]	Type	Sections w/o *A. altissima*	Sections w/ healthy *A. altissima*	Sections w/ symptomatic *A. altissima*	*V. nonalfalfae* isolated
81	*V. nonalfalfae*	204	176	20	Yes
mv	*V. nonalfalfae*	697	63	0	n/a
32	*V. nonalfalfae*	727	68	5	Yes
48	*V. nonalfalfae*	509	82	21	Yes
72	*V. nonalfalfae*	241	60	35	Yes
C1	Control	1022	6	0	No
C2	Control	407	21	0	No

[1] Site 30 was excluded from this analysis due to the herbicide treatment that removed the majority of *A. altissima* along all adjacent rights-of-way.

Chapter 5: The invasive tree, *Ailanthus altissima,* impacts understory nativity, not seedbank nativity

Highlights

- *Ailanthus altissima* invasion decreases the proportion of the understory that is native
- Seedbank nativity or diversity is not influenced by *A. altissima* invasion
- *A. altissima* reduces woody understory nativity over time
- The proportion of *A. altissima* seeds in the seedbank increases with stand age
- Manage *A. altissima* early to reduce its impact

Abstract

Ailanthus altissima, the invasive forest tree commonly known as the tree-of-heaven, has been associated with decreased levels of plant species richness and native species diversity. However, this relationship with resident plants has been inconsistently found and the tree's influence on the seedbank has yet to be studied. To further understand this tree's long-term impact, ten paired invaded-uninvaded sites were identified in Virginia, USA in a variety of different-aged stands. The herbaceous and woody understories for each plot were inventoried and soil samples were collected and grown out for 34 weeks in a greenhouse. All plants were identified to the most detailed taxonomic level possible. In total, 35 woody understory species, 62 herbaceous understory taxa, and 77 seedbank taxa were identified. The relationship between *A. altissima* presence and i) the proportion of individual plants that are native, ii) the proportion of species that are native, iii) the native diversity, and iv) the nonnative diversity were analyzed. In addition, a model including the invasion age was also considered. We show that *A. altissima* invasions were associated with a decrease in the proportion of native plants and species in the understory, but not in the seedbank. Nonnative woody diversity also increased with *A. altissima* presence. Additionally, the impact on the woody understory became more extreme over time. We end by discussing the benefits of both managing *A. altissima* invasions early to limit its overall impact and including other nonnative plants in *A. altissima* restoration plans to account for the expected higher numbers of nonnatives established in the understory.

Keywords

Management practices, nonnative plants, invasion biology, uninvaded-invaded

Declaration of Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

1.0 Introduction

Invasive species are an ever-increasing global problem, with ecological and economic consequences that we are just starting to understand (Pyšek and Richardson, 2010). In the United States alone, it is estimated that 50,000 nonnative species are present and causing damages of at least 120 billion USD per year (Pimentel et al., 2005). Of these, 5,000 are plants, representing about one of every five plant species found in the country (Pimentel et al., 2005). With such a large incidence and impact comes the need for management (Pyšek and Richardson, 2010). However, to make matters more challenging, when an invasive plant is successfully removed from an area it often leaves behind a "weed shaped hole" ready to be filled by other nonnative plants (Buckley et al., 2007). This secondary invasion of other nonnative species is a global problem for any restoration effort (Pearson et al., 2016). Therefore, understanding how major invasive species impact local flora can help land managers better predict post-removal restoration needs.

One such major invader is the invasive forest tree, *Ailanthus altissima* (Mill.) Swingle (Sapindales: Simaroubaceae), commonly known as the tree-of-heaven. This tree has spread from its native Chinese range to the temperate and subtropical zones of all continents except Antarctica (Kowarik and Säumel, 2007). Since its first introduction to the United States in 1784 as a prized ornamental tree (Hu, 1979; Kasson et al., 2013), it has spread to 44 of the 50 states and is the dominant tree in many localized areas (EDDmapS, 2019). With this expansive presence, many corresponding impacts have been documented. These impacts include overtaking agricultural fields (Hepting, 1971), causing infrastructure damage (Hu, 1979; Kowarik and Säumel, 2007), and supporting invasive insects (Hoebeke et al., 2017; Song et al., 2018; Wallner et al., 2014). However, this tree's impact on the surrounding vegetation, including wildlife habitat and timber resources, may be the most concerning for land managers focused on long-term site restoration.

A variety of work comparing invaded areas to adjacent uninvaded areas has established that *A. altissima* can impact the resident vegetation. In the Mediterranean, areas invaded with *A. altissima* have decreased levels of native plant species diversity (Vilà et al., 2006), but do not differ in overall plant species richness (Traveset et al., 2008). In a Paris forest, it was found that understory vegetation was less diverse and made up of more common species under *A. altissima* trees, and that *A. altissima* sprout densities correlated negatively with floristic richness (Motard et al., 2011). In contrast in Greece, the overall floristic diversity was higher in *A. altissima* stands (Fotiadis et al., 2011). Though nativity of the resident vegetation was included in the first of these studies, it was not included in the latter two. Plant nativity is an important consideration since it is not only relevant to land managers who are often trying to increase the number of native species present, but may help explain some of the inconsistent impacts observed. For example, a meta-analysis in Mediterranean-type ecosystems found invasive tree species contributed to a decline in native plant richness, but did not to look at their impacts to nonnative species (Gaertner et al., 2009). Since the majority of ecosystems include invasions by multiple nonnative plant species, the inclusion of nonnatives is also important to consider (Kuebbing et al., 2013).

The corresponding studies looking at vegetation after *A. altissima* control have always considered nativity in their assessments. For example, when *A. altissima* was removed in Virginia using chemical herbicides, the number of native dominant herbaceous species increased (Burch and Zedaker, 2003). Similarly, when *A. altissima* was removed in Pennsylvania using the proposed biopesticide *Verticillium nonalfalfae,* stands became dominated by native tree species (O'Neal and Davis, 2015). However, they also noted that in some areas nonnative herbaceous plants became dominant (O'Neal and Davis, 2015). In two other studies in Pennsylvania, it was found that *A. altissima* removal caused very little impact on nonnative or native understory plants (Harris et al., 2013), and that there was no change in the mean number of native woody plants (Kasson et al., 2014). Therefore, to fully understand *A. altissima*'s impacts and to account for these inconsistent findings, additional research studying how *A. altissima* impacts the woody and herbaceous understory is still needed.

One potential difference between invaded-uninvaded and managed-unmanaged studies, is the importance of the seedbank (Brown, 1992). It is reasonable to consider that post-management findings may be exacerbated or minimized by the inputs from a seedbank. No previous studies have investigated how *A. altissima* impacts the seedbank. In a meta-analysis, Gioria et al. (2014) showed that the seedbank in areas invaded by invasive plants had lower native species richness and density and higher nonnative species richness and abundances. This diverging impact on the seedbank based on the nativity of the species within it could help explain and predict the impact *A. altissima* has on the surrounding plant composition.

Even once *A. altissima's* impact on the understory and the seedbank is understood, these impacts may become more severe as the invasion increases in age (Strayer et al., 2006). Though this trend has been seen in other systems, it is unknown how *A. altissima*'s impact will change. For example, an Australian study comparing the time since invasion (ranging between 8 - 25 years) of the woody shrub *Cytisus scoparius* (scotch broom) found that native species richness and cover declined over the length of the invasion (Wearne and Morgan, 2004). Therefore, knowledge of how *A. altissima's* impact on the understory and the seedbank changes over time may help shape our understanding of this highly impactful invasive tree and its post-management restoration needs.

The objective of this research was to study the relationship between *A. altissima* invasion and the nativity of the woody understory, herbaceous understory, and the seedbank by looking at paired invaded-uninvaded plots in Virginia, USA. The age of the invasion was also studied to determine if it accounted for any differences in impact observed. We hypothesized that *A. altissima*'s invasion will decrease the native vegetation in both the understory and the seedbank, and that this decrease will become more severe over time.

2.0 Materials and Methods

2.1 Site selection

Ten paired *A. altissima* invaded-uninvaded field plots were identified in Montgomery, Giles, and Pulaski Counties in Virginia, USA during the winter of 2017 to 2018 (Fig. 1). This paired invaded-uninvaded experimental design, commonly used to assess an invader's impact, is based on the assumption that the uninvaded area is reflective of what the invaded area would have been like if never invaded (Barney et al., 2015; Gioria et al., 2014; Levine et al., 2003; Walker and Smith, 1997). Therefore, sites were selected to minimize any differences besides the invasion status of *A. altissima* (Gioria and Osborne, 2010; Hejda et al., 2009). Accordingly, paired plots were chosen based on the presence of a discrete *A. altissima*-dominated area within a stand that contained no other *A. altissima* and whether they appeared homogeneous with respect to disturbance history. To achieve the same disturbance history, sites were only selected that were adjacent to a public roadway with similar aspects and slopes that were highly likely disturbed at the same time during past construction. To minimize any direct influences of *A. altissima* to uninvaded plots, paired plots were separated by a distance of at least 2x the height of the tallest *A. altissima* tree present but by no more than 100 m apart. Sites were accessed following all requirements of the VA Department of Transportation Land Use Permit 018-6614.

All plot centers were identified, mapped (Fig. 1), and marked with metal tree tags. At invaded plots, the plot center was placed at the estimated invasion origin (the area containing the densest and largest *A. altissima* stems), while at uninvaded plots, the plot center was chosen in a location an appropriate distance away with similar terrain. To estimate the age of the invasion, the *A. altissima* with the largest diameter at breast height (DBH) was cored and the growth rings were counted (Kasson et al., 2013).

2.2 Established vegetation measurements

To quantify the stand composition, a 10-degree (basal area factor) prism (Cruise-Master Prisms Inc., Sublimity, OR) was used to estimate the basal area (BA, m^2) of the dominant tree species while standing at all plot centers. In total, 24 different species had measurable basal area at all of these sites (Fig. 2). Of these, 13 species were measured at invaded sites and 21 species at uninvaded sites. The only nonnative species present were *A. altissima* and *Lonicera maackii*. *Ailanthus altissima* was only identified at invaded plots, making up 80% of their measurable basal area, while, *L. maackii* represented less than 2% of all measurable basal area at of both invaded and uninvaded plots.

The established understory species composition of all plots was measured on 23 – 25 July 2018 during peak growing season. Site 3 was excluded due to destruction by roadside construction. To quantify the understory plants present, three locations in each plot were selected by running a meter tape from the plot center to its edge at 0, 120, and 240 degrees away from the roadway. Then, at the location one-third of the way from the plot center to the stand edge a 1 x 1 m quadrat was placed. If the stand edge was not obvious in uninvaded plots, the samples were taken at the distance comparable to that of the paired invaded plot samples. A visual estimation of percent cover of all herbaceous plants and a count of any woody plants (excluding canopy trees) was recorded in each quadrant. All understory species present in this area were either identified in the field or sampled and taken back to the lab for later identification. All plant identifications, scientific and common names, and nativity (native, nonnative, unknown) were confirmed using the Flora of Virginia App (Weakley et al., 2017). All taxa were identified to the highest possible taxonomic resolution. If this identification was not to species, the plant's nativity was determined based on all possible species it might be. Individuals with unclear or unknown nativity were designated as such.

2.3 Seedbank collection, germination, and identification

To determine what germinable propagules were in the soils, a seedbank emergence method was used (Brown, 1992). This method focuses on what seedlings germinate and ignores any propagules that are not viable or remain dormant. To sample the seedbank, three soil samples at each plot were collected between 19 February and 6 March 2018 and placed into separate Ziplock bags. Each soil sample, 2 L in volume, was excavated just inside of where the understory measurements were taken. These samples were excavated from a 20 cm diam and 5 cm deep area using a 70% EtOH cleaned spade. All organic materials besides large sticks (> 20 cm) were included in the samples. This collection time allowed seeds from the previous growing season to go through a natural cold dormancy while limiting any germination caused by the next growing season. Samples were stored at 4°C until planted.

On 18 May 2018, each soil sample was moved to a greenhouse and mixed with both 4 L of potting soil (Miracle-Gro® Potting Mix 0.21-0.11-0.16) to ensure drainage and nutrient needs of all seeds were met, and with 120 ml of powdered activated charcoal (Biogize-SD™ Soil Detox, Dovecreek, CO) to limit the influence of any allelochemicals present (Callaway, 2003). Activated charcoal was added to allow a comparison of invaded and uninvaded seedbanks regardless of allelochemical influences and because *A. altissima* allelochemicals disappear quickly in the soil once *A. altissima* has been removed (Heisey, 1990). Each soil-seedbank mixture was spread in a tray (47 x 33 cm) and watered as needed. Corresponding invaded-uninvaded germination trays were placed adjacent to each other and switched twice a month to limit the uneven influence of any environmental gradients within the greenhouse.

Seedling germination was monitored weekly. Once seedlings were identified to species, they were removed from the tray to minimize competition. If numerous identical seedlings germinated at the same time, they were counted and thinned to three representative seedlings until identification could be confirmed. If seedling identification did not occur within a reasonable amount of time, the plant was repotted to minimize its influence on the rest of the seedbank until identification could occur. Seedling identification and related information was based on the information provided in the Flora of Virginia App (Weakley et al., 2017). Seedling germination was allowed to occur for 34 weeks (until November 1, 2018).

2.4 Analysis

2.4.1 Invasion's influence on nativity

To determine the influence of *A. altissima* invasion on the herbaceous understory, the woody understory, and the seedbank, all taxa (excluding *A. altissima*) in each category were analyzed. The understory vegetation was analyzed in two data subsets due to the differences in field quantification (individual counts vs. percent cover) and *A. altissima* was excluded to ensure the response variables were independent of *A. altissima* presence (Vilà et al., 2006; Wearne and Morgan, 2006). These three vegetation categories were quantified using four different response variables: i) proportion native plants, ii) proportion native species, iii) native diversity, and iv) non-native diversity.

The "proportion native plants" measured the proportion of native individuals out of the total individuals counted (or in the case of the herbaceous understory, native percent cover out of the total percent cover). Similarly, the "proportion native species" calculated the number of native taxa present out of the total number of taxa identified.

The "native diversity" and "nonnative diversity" was measured by calculating a diversity index for either the native or the nonnative subset of the data. The chosen diversity index, the probability of an interspecific encounter (PIE), measures the probability that two individuals randomly drawn from a population represent different taxa (Hurlbert, 1971). This diversity index ranges from zero to one, with larger numbers indicating a higher diversity. PIE is calculated with the following equation,

$$PIE = \left(\frac{N}{N-1}\right)\left[1 - \sum_{i-1}^{S}(p_i)^2\right]$$

where p_i is the fraction of the total number of individuals that consists of taxon *i, N* is the total number of individuals, and *S* is the total number of taxa. This diversity index was chosen because it can be meaningfully interpreted, it is insensitive to small sample sizes, and it can be modified to include non-count data (Gotelli, 2008). For example, when used for percent cover, PIE calculates the probability that two randomly chosen points in an area land on different taxa.

Within each of these vegetation categories, these four response variables were fit to linear mixed models containing two additive effects. The first being invasion status (invaded or uninvaded) as a fixed effect and the second being site as a random effect. These were fit using the lmer function from the "lme4" package in R (Bates et al., 2015; R Core Team, 2018). Chi^2 and p-values were then calculated using the likelihood ratio test comparing each model to a null model containing only the random effect and considered significant if $p < 0.05$ (Bates et al., 2015). Data were transformed as needed to meet parametric assumptions. Values that could not be calculated due to the lack of any vegetation present in a plot were excluded. To minimize excluded data, this analysis was completed on response variables pooled to the plot level for all calculations.

2.4.2 Age of the invasion's influence on nativity

The proportion of native individuals and proportion of native species were compared over time to determine if stand age helps account for the variation observed in any of the above analyses. These response variables for each of the three vegetation categories were analyzed using a linear mixed model. This model contained two interactive fixed effects (invasion status and age of the invasion) and an additive random site variable. These were again fit using the lmer function from the lme4 package in R (Bates et al., 2015; R Core Team, 2018). Chi^2 and p-values were then calculated using the likelihood ratio test comparing each model to a null additive model containing invasion status as a fixed effect and site as a random effect, with $p < 0.05$ indicating significance. If the model was determined to be significant, it was also compared to a second null model which contained three additive effects: age of the invasion and invasion status as fixed effects and site as a random effect. This additional comparison was completed to determine which effect or interaction was driving the significance observed.

2.4.3 Seedbank and resident vegetation similarity

The taxa found in the seedbank and the resident vegetation (including the herbaceous understory, woody understory, and canopy taxa) were compared at each plot using the Sørensen–Dice similarity index (Sørensen, 1948). This index calculates the similarity between the compared communities using the following equation:

$$\text{Sørensen–Dice similarity index} = \frac{2(c)}{a+b}$$

where *a* represents the total number of taxa found in one group, *b* represents the total number of taxa found in the other group, and *c* represents the number of taxa that are shared between the two groups. *Ailanthus altissima* was excluded from this calculation.

To determine if the age of the invasion or the invasion status influenced this similarity index, their relationship was analyzed using a linear regression containing two interactive predictor variables: invasion status and age of the invasion. The significance ($p < 0.05$) of the model, the two predictor variables, and their interaction was used to reach conclusions about this relationship.

2.4.4 Ailanthus altissima seedbank presence

The prevalence of *A. altissima* seeds in the seedbank were analyzed separately to help predict long-term species-specific management needs. The proportion of *A. altissima* in the seedbank at invaded plots was analyzed using a linear regression which contained only invasion age as a predictor effect (R Core Team, 2018). Uninvaded plots were excluded from this analysis since their *A. altissima* seedbank numbers were minimal. The significance of the regression and its adjusted R^2 value was used to validate this relationship.

3.0 Results

3.1 Invasion's influence on nativity

All herbaceous understory plants were identified to species excluding those in the *Carex*, *Hackelia*, *Solidago*, and *Triosteum* genera. Of these, the nativity of the genus could be confirmed for everything but *Carex* spp. In total, 62 taxa were identified, of which 38.7% were nonnative (Table 1, Supplemental Table A.1).

The likelihood ratio tests used to determine if the invasion of *A. altissima* influenced the herbaceous understory's proportion native plants and the proportion native species were both significant. In both cases, these proportions were negatively influenced by *A. altissima* invasion, with the proportion of native individuals or species ranging from 0 - 1.0 at invaded sites compared to always being over 0.5 at uninvaded sites. The overall mean of the proportion of native plants was reduced from 0.82 to 0.56 while the proportion native species was similarly reduced from 0.72 to 0.52. In contrast, there was no indication that the invasion of *A. altissima* influenced the native diversity nor the nonnative diversity of these plots (Fig. 3).

In total 1,437 woody understory plants were counted, comprised of 35 species. Of these 35 species, only five were nonnative (*A. altissima*, *Celastrus orbiculatus*, *Ligustrum obtusifolium*, *Lonicera japonica*, and *Lonicera maackii*) (Table 1, Supplemental Table A.1).

The likelihood ratio test that analyzed how the woody understory (excluding *A. altissima*) was influenced by *A. altissima* invasion was significant for three of the four response variables. This output indicated that the proportion native individuals, the proportion native species, and the nonnative diversity are all influenced by the invasion of *A. altissima*. Both proportions decreased with invasion while nonnative diversity increased. The proportion of native individuals was very variable, ranging from almost 0 to 1, while the proportion of native species was always at or above .5. The overall mean of the proportion of native plants was reduced from 0.56 to 0.33, with the mean proportion of native species reduced similarly from 0.84 to 0.74. The woody understory's nonnative diversity index hovered roughly around 60% at both sites (increasing just slightly with *A. altissima* invasion), compared to the native diversity index which tended to be closer to 90%. The overall mean of the nonnative diversity index was increased from 0.54 to 0.65 (Fig. 3).

In total, 1,771 seedlings germinated and were all identified to species, excluding those in the *Carex* and *Solidago* genera. Of the two genera, the nativity could only be determined for species in the *Solidago* genus. These seedlings included 77 different taxa, of which almost half (48%) were nonnative (Table 1, Supplemental Table A.1).

The likelihood ratio test, which analyzed how the seedbank (excluding *A. altissima*) was influenced by the presence of *A. altissima*, showed that none of the four response variables were significantly changed (Fig. C). In all cases, seedbank samples from plots were never exclusively native nor nonnative, with the diversity indexes always higher than 50%.

3.3 Age of the invasion's influence on nativity

When considering the age of the invasion, the linear regressions were significant for both the woody understory's proportion native plants and its proportion native species (Fig. 4). Comparing both of these models to the second null model, the inclusion of the age variable ($p=0.01$ and 0.0001, respectively) appeared to be driving the significance over the interaction ($p=0.051$ and $p=0.21$, respectively). No other models were significant.

3.4 Seedbank and resident vegetation similarity

In the seedbank and vegetation (including canopy) combined, 144 different taxa were identified. Of these, 67 taxa were found only in the resident vegetation, 47 taxa were found only in the seedbank, and 30 taxa were found in both. The Sørensen–Dice similarity index was calculated for all plots, excluding those at site 3 which were destroyed before established vegetation could be quantified. The overall similarity index was 0.30 in invaded plots and 0.29 in uninvaded plots, with individual plots ranging in value between 0.00 and 0.30 (Fig. 5).

The interactive linear model relating the similarity index to both the invasion age and invasion status was not significant ($F(3, 14) = 2.173$, $p = 0.1368$) (Fig. 5), nor were either predictor variables or their interaction.

4.5 *Ailanthus altissima* seedbank presence

In total, 179 *A. altissima* seedlings germinated from the collected soils. Not surprisingly, of these, 85.5% germinated from soil collected at invaded sites. *Ailanthus altissima* represented 10.1% of the total seedbank, or 14.1% of the invaded seedbank and 3.8% of the uninvaded seedbank.

Regarding the relationship between the proportion of *A. altissima* in seedbank samples at invaded plots and the age of the invasion, the selected linear model was significant ($F(1, 28)$ 16.26, $p = 0.00039$), and showed a positive relationship between the two variables (Fig. 6). The model's adjusted R^2 indicated that the age of the invasion explained over 34% of the variations seen. To meet parametric assumptions, the percentage of *A. altissima* in seedbanks was log transformed prior to this analysis.

4.0 Discussion

4.1 *Ailanthus altissima* impacts understory nativity, not seedbank nativity

Our results found that *A. altissima* has a negative impact on the proportion of native plants (individuals and species) in the understory, but not the seedbank (Fig. 3). This impact on the understory was expected, as it had been previously shown that invasive trees cause high levels of decline in resident native plant richness (Gaertner et al., 2009). It is therefore possible that the previous *A. altissima* impact studies that did not consider nativity of the resident species may have found similar impacts if nativity was included in their analysis (Fotiadis et al., 2011; Motard et al., 2011; Traveset et al., 2008). For example, when Fotiadis et al. (2011) found an increase in plant diversity in areas invaded by *A. altissima*, they suggested that this increase was caused by the presence of synanthropic species, but did not specifically look at which species were nonnative. It is also possible that their results differ not just due to the difference in their methods regarding plant nativity, but due to the difference in location or habitat assessed.

The nonnative diversity of the woody understory significantly increased with *A. altissima* invasion (Fig. 3). This may indicate that the decreases observed in the proportion of native woody plants in the understory was driven by a change in nonnative plants, not native plants. This potential facilitation between an invasive species and other nonnative species, referred to as an invasion meltdown, has been found in other systems as well (Simberloff, 2006; Simberloff and Von Holle, 1999). *Ailanthus altissima's* impact on the understory diversity was also documented by Vilà et al. (2006), who recorded the corresponding decrease in native plant diversity with *A. altissima* invasion, which we did not find. Unlike the woody understory, we saw no change in diversity indices in the herbaceous understory. This might indicate that invasive species' impacts can be best observed when the nativity categories are compared as a whole, not considered independently.

We found that the invasion of *A. altissima* had no impact on the seedbank's nativity or diversity. Since no previous studies have looked at this relationship, it remains to be seen if this lack of impact is consistently found. Particularly because in other systems invasive plants have been associated with decreased native seedbank species richness and diversity, while increasing nonnative seedbank richness and abundance (Gioria et al., 2014). However, we are not the first to find that invasive plants impact the resident vegetation and not the seedbank. For example, Wearne and Morgan (2006) who looked at an invasive shrub, *C. scoparius,* found few changes in seedbank composition or richness, while the above ground species richness was reduced in older stands. Similarly, Vilà and Gimeno (2007) noted that the similarity between areas uninvaded and invaded by the invasive *Oxalis pes-caprae* (the Bermuda buttercup) was higher in the seedbank samples than the vegetation samples, indicating seedbanks were not as influenced by invasion. Therefore, our results support the theory that seedbanks change more slowly than above ground vegetation in response to an invasive plant (Wearne and Morgan, 2006).

Though we did not design this study to determine the mechanisms causing *A. altissima* influence, previous studies indicate that invasive species' impacts to co-occurring vegetation are usually caused by competitive effects (Levine et al., 2003). This might be especially true for *A.*

altissima, as its competitive ability includes outgrowing native species (Martin et al., 2010), producing allelopathic chemicals that influence the growth of other species (Heisey, 1996; Mergen, 1959; Nilsen et al., 2018), and directly influencing soil properties (Gaertner et al., 2009; Vilà et al., 2006). All of these competitive mechanisms were excluded from our seedbank emergence sampling by removing seedlings after germination, adding activated charcoal, and including nutrient-rich potting soil. This was done in order to quantify the entire germinable seedbank. However, these mechanisms would have all influenced the understory vegetation, and therefore competitive effects could account for the difference in *A. altissima* impact between the understory and the seedbank observed in this study. If this is the case, seedbank composition may actually be irrelevant in regard to future stand composition, unless *A. altissima* with its competitive ability is eradicated from the site.

4.2 As *A. altissima* invasions age, their influence on the woody understory increases

We found that as the age of *A. altissima* invasions increases, the proportion of native plants in woody understory decreases (Fig. 4). This change was observed over an invasion age range of 2 - 53 years. This positive relationship of impact and invasion age matches the now popularized "invasion curve," which shows that the area infested, impacts, and management costs all theoretically increase with time (Blackwood et al., 2010; Pyšek and Prach, 1993; Strayer et al., 2006; Yokomizo et al., 2009). Though not tested in this experimental design, invasion age may not be the only variable driving this change, as invasion age is also likely correlated with other variables such as invasion size and abundance of the invasive species.

This finding demonstrates how important invasion age can be when considering impacts, though few other studies have focused on age. Again, matching our results, the Australian study comparing the time since invasion (ranging between 8-25 years) of the woody shrub *C. scoparius*, showed that native species richness and cover declined over the length of the invasion (Wearne and Morgan, 2004). Similarly, Ortega et al. (2019) found that as the time since invasion increased for the woody exotic *Rhamnus cathartica*, overstory cover of native woody species decreased. However, invasion age can often be hard to determine. The meta-analysis by Vilà et al. (2011) was unable to determine how impacts might change in regard to plant abundance, let alone invasion age, due to lack of data. And even previous studies looking at *A. altissima* impacts, which have the potential to characterize invasions with respect to age, have not accounted for it in their analyses (Fotiadis et al., 2011; Motard et al., 2011; Traveset et al., 2008; Vilà et al., 2006).

The fact that this relationship is seen in the woody understory and not the herbaceous understory or the seedbank, may just be a matter of temporal scale, and given enough time, other categories may also be impacted (Gaertner et al., 2009). For example, it has been suggested that there is some sort of temporal impact tipping point that must be crossed for impact to increase (Wearne and Morgan, 2004). If this is the case, it is possible that our study did not encompass the time frame needed to see increasing impacts to the herbaceous understory or the seedbank. Alternatively, *A. altissima*'s impact on the herbaceous understory might occur almost immediately after establishment, similar to what has been found with *Microstegium vimineum* (Japanese stilt grass) (Tekiela and Barney, 2017). Either way, we found that at least part of *A. altissima*'s impact increased with the age of the invasion.

4.3 Site restoration recommendations

Land managers should be prepared to manage more nonnative plants in *A. altissima* invaded areas than in uninvaded areas. This is because with *A. altissima* invasion, the proportion of native understory plants decreased, both in terms of total plants and individual species (Fig. 3). Since small-scale eradication of *A. altissima* is now possible using either chemical methods (Asaro et al., 2009; Gover et al., 2013) or a proposed biopesticide (Schall and Davis, 2009), restoration plans post-management are becoming a reality. This potential for a secondary invasion after management of the targeted invasive species has been shown to be a global problem (Pearson et al., 2016), and we therefore suggest monitoring all plants for other nonnative species both prior to and after management.

Given that *A. altissima*'s impact on the woody understory increased with invasion age (Fig. 4), management of *A. altissima* infestations should be completed early to help limit impacts to the woody understory. This is especially true since *A. altissima* appears to impact not just the woody understory's proportion of native plants and species, but also increases the nonnative woody diversity (Fig. 3). Therefore, any restoration plans focused on restoring the area's native woody canopy may face increasing difficulties as these invasions age. It is possible that in certain places *A. altissima* management can result in the reestablishment of native woody species, as seen by O'Neal and Davis (2015). However, in other areas it appears that *A. altissima* removal does not change the mean number of native woody plants (Kasson et al., 2014). It is possible that the difference in post-management woody composition observed may be due to the *A. altissima* invasion age at both of these sites. A study including pre-treatment data would better determine the outcome of the nonnative and native species post *A. altissima* restoration.

Though we found that *A. altissima* invasions did not impact the seedbank nativity or diversity (Fig. 3, Fig. 4), it is likely that additional nonnative species are present within the seedbank. This is because in all the seedbank soils collected, nonnative plants other than *A. altissima* were present. Additionally, like most forests, we found that these seedbanks and the resident vegetation had very low levels of similarity (Fig. 5) (Hopfensperger, 2007). Though we found that *A. altissima* invasions did not impact the seedbank, land managers should still be prepared for additional nonnative species not currently established to germinate during management activities.

Since we found the seedbanks uninfluenced by *A. altissima* invasion, we do not know if supplemental seeding post-management would be helpful. Seeding has been previously considered (Burch and Zedaker, 2003; Harris et al., 2013); however, to date, no experiments looking at supplemental seeding in this system have been reported. In fact, Burch and Zedaker (2003) found that when *A. altissima* was removed, reseeding with native herbaceous species was not necessary and therefore they did not include it in their study. Similar accounts of seedbanks being sufficient to recolonize with native vegetation after clearing have also been found in other systems, including with the invasive tree *Acacia longifolia* in South Africa (Fourie, 2008).

These management suggestions should be relevant to anyone wishing to control *A. altissima* along corridors in Virginia, one of the most highly infested areas in the country (EDDmapS, 2019; McAvoy et al., 2012). However, since *A. altissima* can invade many different habitat types (Traveset et al., 2008), and the impacts of invasive plants tend to vary by habitat (Burch and Zedaker, 2003), these findings might not be applicable everywhere. Nevertheless, with this knowledge of both the established vegetation and the seedbank, land managers should be able to better support the timely management of *A. altissima* and predict post-removal restoration needs.

4.4 The added risk of other invasive species

Since our work did not distinguish high-risk invasive species from naturalized nonnative species, we cannot be certain if *A. altissima*'s impact differs between the two. We did however find that many other significant invasive species were both present and abundant within the understory and the seedbank (Table 1 & Supplemental Table A.1). Depending on management goals, these species likely deserve their own attention.

For example, the highly impactful invasive Japanese stilt grass, *Microstegium vimineum* (Adams and Engelhardt, 2009), was found in considerable quantities in both the understory and the seedbank samples, with highest numbers at *A. altissima*-invaded plots (with average understory cover increasing three-fold and average seedbank emergence counts increasing almost ten-fold in invaded plots compared to uninvaded plots) (Table 1 & Supplemental Table A.1). Additionally, *Pueraria montana var. lobata*, the highly impactful invasive kudzu vine that can outcompete trees (Forseth and Innis, 2004), was found only in the seedbank, with highest numbers also in the invaded sites (with average emergence numbers increasing from 0.1 per uninvaded plot to 1.1 per invaded plot) (Table 1 & Supplemental Table A.1). Other highly impactful invasive species were also prevalent in the understory including *Alliaria petiolata* (garlic mustard)*, Lonicera maackii* (amur honeysuckle), and *Lonicera japonica* (Japanese honeysuckle; Table 1). The presence of these particular invasive species stresses the need for land managers to be able to identify and include numerous invasive species in the same management plan.

4.5 Manage *A. altissima* early to limit its seedbank presence

Early intervention in *A. altissima* control is highly suggested to reduce the need for future *A. altissima* seedbank management. This is because *A. altissima*'s seedbank presence increased with *A. altissima* stand age (Fig. 6). With this increase in propagule pressure, the species' ability to reinvade an area after management activities will likely increase over time (Lockwood et al., 2005). Since *A. altissima* is an early-successional, gap-obligate tree, even if the established *A. altissima* were successfully removed, the seedbank could take advantage of any disturbance caused by the initial control (Carter and Fredericksen, 2007; Kasson et al., 2013; Knapp and Canham, 2000). With female *A. altissima* able to produce 10 million seeds over 40 years, it is not surprising that this increase of seed prevalence over time was found (Wickert et al., 2017).

We also found *A. altissima* seeds at low levels in the seedbanks of uninvaded plots. This finding was not unexpected, since paired sites were located no more than 100 m from each other to minimize differences, and *A. altissima* seeds are dispersed by wind (Kowarik and Von der Lippe, 2011). It is therefore important to remember that if *A. altissima* is present in your region, any disturbance may result in a release of *A. altissima* seedbank seeds. This concern is amplified by the recent work that found *A. altissima* seeds able to survive in the soil for much longer than initially expected (over five years) (Rebbeck and Jolliff, 2018).

4.6 Conclusions

We found that *A. altissima* invasion reduces the proportion of native plants and species in the understory, but not the seedbank. And this impact on the woody understory becomes more severe as the invasion ages. These findings can help direct *A. altissima* management programs and post-removal restoration needs. This is the first study to determine if *A. altissima* impacts the seedbank and to look at how its impact on established vegetation and the seedbank changes over time.

Acknowledgements

We thank Tom Wieboldt, Jordan Metzgar, Tom McAvoy, Vasiliy Lakoba, Gourav Sharma, and Becky Fletcher for help identifying plants. Additional thanks to Matt Kasson, Ashley Toland, Holly Wantuch, Caleb Gore, and Ariel Heminger for their support. This work was supported by USDA Forest Service Grant # 15-CA-11420004-161 to SMS and ABB.

References

Adams, S.N., Engelhardt, K.A., 2009. Diversity declines in *Microstegium vimineum* (Japanese stiltgrass) patches. Biol. Conserv. 142**,** 1003-1010. https://doi.org/10.1016/j.biocon.2009.01.009.

Asaro, C., Becker, C., Creighton, J., 2009. Control and utilization of tree-of-heaven: A guide for Virginia landowners. Virginia Department of Forestry Publication P00144, Charlottesville, VA.

Barney, J.N., Tekiela, D.R., Barrios-Garcia, M.N., Dimarco, R.D., Hufbauer, R.A., Leipzig-Scott, P., Nunez, M.A., Pauchard, A., Pyšek, P., Vítková, M., 2015. Global Invader Impact Network (GIIN): toward standardized evaluation of the ecological impacts of invasive plants. Ecol. Evol. 5**,** 2878-2889. https://doi.org/10.1002/ece3.1551.

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67**,** 1-48. https://doi.org/10.18637/jss.v067.i01.

Blackwood, J., Hastings, A., Costello, C., 2010. Cost-effective management of invasive species using linear-quadratic control. Ecol. Econ. 69, 519-527. https://doi.org/10.1016/j.ecolecon.2009.08.029.

Brown, D., 1992. Estimating the composition of a forest seed bank: a comparison of the seed extraction and seedling emergence methods. Can. J. Bot. 70, 1603-1612. https://doi.org/10.1139/b92-202.

Buckley, Y.M., Bolker, B.M., Rees, M., 2007. Disturbance, invasion and re-invasion: Managing the weed-shaped hole in disturbed ecosystems. Ecol. Lett. 10, 809-817. https://doi.org/10.1111/j.1461-0248.2007.01067.x.

Burch, P.L., Zedaker, S.M., 2003. Removing the invasive tree *Ailanthus altissima* and restoring natural cover. J. Arboric. 29, 18-24.

Callaway, R.M., 2003. Experimental designs for the study of allelopathy. Plant Soil. 256, 1-11. https://doi.org/10.1023/A:1026242418333.

Carter, W.K., Fredericksen, T.S., 2007. Tree seedling and sapling density and deer browsing incidence on recently logged and mature non-industrial private forestlands in Virginia, USA. For. Ecol. Manage. 242, 671-677. https://doi.org/10.1016/j.foreco.2007.01.086.

EDDmapS. 2019. Tree-of-heaven *Ailanthus altissima* (P. Mill.) Swingle. Early Detection & Distribution Mapping System. The University of Georgia, Center for Invasive Species and Ecosystem Health. https://www.eddmaps.org/distribution/uscounty.cfm?sub=3003 (accessed Nov 2019).

Forseth, I.N., Innis, A.F., 2004. Kudzu (*Pueraria montana*): history, physiology, and ecology combine to make a major ecosystem threat. Crit. Rev. Plant Sci. 23, 401-413. https://doi.org/10.1080/07352680490505150.

Fotiadis, G., Kyriazopoulos, A., Fraggakis, I., 2011. The behaviour of *Ailanthus altissima* weed and its effects on natural ecosystems. J. Environ. Ecol. 32, 801.

Fourie, S., 2008. Composition of the soil seed bank in alien-invaded grassy fynbos: potential for recovery after clearing. S. Afr. J. Bot. 74, 445-453. https://doi.org/10.1016/j.sajb.2008.01.172.

Gaertner, M., Den Breeyen, A., Hui, C., Richardson, D.M., 2009. Impacts of alien plant invasions on species richness in Mediterranean-type ecosystems: a meta-analysis. Prog. Phys. Geog. 33, 319-338. https://doi.org/10.1177/0309133309341607.

Gioria, M., Jarošík, V., Pyšek, P., 2014. Impact of invasions by alien plants on soil seed bank communities: emerging patterns. Perspect. Plant Ecol. Evol. Syst. 16, 132-142. https://doi.org/10.1016/j.ppees.2014.03.003.

Gioria, M., Osborne, B., 2010. Similarities in the impact of three large invasive plant species on soil seed bank communities. Biol. Invasions. 12, 1671-1683. https://doi.org/10.1007/s10530-009-9580-7.

Gotelli, N.J., 2008. A Primer of Ecology. 26-40. ISSN 978-0-87893-318-1.

Gover, A., Johnson, J., Lloyd, K., Sellmer, J., 2013. Tree-of-heaven (*Ailanthus altissima*), Quicksheet 5. Wildland Weed Management, Penn State, College of Agricultural Sciences.

Harris, P.T., Cannon, G.H., Smith, N.E., Muth, N.Z., 2013. Assessment of plant community restoration following tree-of-heaven (*Ailanthus altissima*) control by *Verticillium albo-atrum*. Biol. Invasions. 15, 1887-1893. https://doi.org/10.1007/s10530-013-0430-2.

Heisey, R.M., 1990. Evidence for allelopathy by tree-of-heaven (*Ailanthus altissima*). J. Chem. Ecol. 16, 2039-2055. https://doi.org/10.1007/BF01020515.

Heisey, R.M., 1996. Identification of an allelopathic compound from *Ailanthus altissima* (Simaroubaceae) and characterization of its herbicidal activity. Am. J. Bot. 83**,** 192-200. https://doi.org/10.1002/j.537-2197.1996.tb12697.x.

Hejda, M., Pyšek, P., Jarošík, V., 2009. Impact of invasive plants on the species richness, diversity and composition of invaded communities. J. Ecol. 97**,** 393-403. https://doi.org/10.1111/j.1365-2745.2009.01480.x.

Hepting, G., 1971. *Ailanthus altissima*. In: Diseases of forest and shade trees in the United States, Handbook 386. USDA Forest Service, Washington, D.C. pp. 63-64.

Hoebeke, E.R., Jendek, E., Zablotny, J.E., Rieder, R., Yoo, R., Grebennikov, V.V., Ren, L., 2017. First North American records of the Eastasian metallic wood-boring beetle *Agrilus smaragdifrons* Ganglbauer (Coleoptera: Buprestidae: Agrilinae), a specialist on tree of heaven (*Ailanthus altissima*, Simaroubaceae). Proc. Entomol. Soc. Wash. 119**,** 408-422. https://doi.org/10.4289/0013-8797.119.3.408.

Hopfensperger, K.N., 2007. A review of similarity between seed bank and standing vegetation across ecosystems. Oikos. 116**,** 1438-1448. https://doi.org/10.1111/j.0030-1299.2007.15818.x.

Hu, S.Y., 1979. *Ailanthus*. Arnoldia. 39**,** 29-50.

Hurlbert, S.H., 1971. The nonconcept of species diversity: a critique and alternative parameters. Ecology. 52**,** 577-586. https://doi.org/10.2307/1934145.

Kahle, D., Wickham, H., 2013. ggmap: Spatial visualization with ggplot2. R J. 5**,** 144-161. https://doi.org/10.32614/RJ-2013-014.

Kasson, M.T., O'Neal E.S., Davis, D.D., 2015. Expanded host range testing for *Verticillium nonalfalfae*: potential biocontrol agent against the invasive *Ailanthus altissima*. Plant Dis. 99, 823-835. https://doi.org/10.1094/PDIS-04-14-0391-RE.

Kasson, M.T., Davis, M.D., Davis, D.D., 2013. The invasive *Ailanthus altissima* in Pennsylvania: A case study elucidating species introduction, migration, invasion, and growth patterns in the Northeastern US. Northeast Nat. 20**,** 1-60. https://doi.org/10.1656/045.020.m101.

Kasson, M.T., Short, D.P., O'Neal, E.S., Subbarao, K.V., Davis, D.D., 2014. Comparative pathogenicity, biocontrol efficacy, and multilocus sequence typing of *Verticillium nonalfalfae* from the invasive *Ailanthus altissima* and other hosts. Phytopathology. 104**,** 282-292. https://doi.org/10.1094/PHYTO-06-13-0148-R.

Kinkel, L.L., Bakker, M.G., Schlatter, D.C., 2011. A coevolutionary framework for managing disease-suppressive soils. Annu. Rev. Phytopathol. 49, 47-67. https://doi.org/10.1146/annurev-phyto-072910-095232.

Knapp, L.B., Canham, C.D., 2000. Invasion of an old-growth forest in New York by *Ailanthus altissima*: sapling growth and recruitment in canopy gaps. J. Torrey Bot. Soc.**,** 307-315. https://doi.org/10.2307/3088649.

Kowarik, I., Säumel, I., 2007. Biological flora of Central Europe: *Ailanthus altissima* (Mill.) Swingle. Perspect. Plant Ecol. Evol. Syst. 8**,** 207-237. https://doi.org/10.1016/j.ppees.2007.03.002.

Kowarik, I., Von der Lippe, M., 2011. Secondary wind dispersal enhances long-distance dispersal of an invasive species in urban road corridors. NeoBiota. 9**,** 49. https://doi.org/10.3897/neobiota.9.1469.

Kuebbing, S.E., Nuñez, M.A., Simberloff, D., 2013. Current mismatch between research and conservation efforts: the need to study co-occurring invasive plant species. Biol. Conserv. 160**,** 121-129. https://doi.org/10.1016/j.biocon.2013.01.009.

Levine, J.M., Vila, M., Antonio, C.M., Dukes, J.S., Grigulis, K., Lavorel, S., 2003. Mechanisms underlying the impacts of exotic plant invasions. P. Roy. Soc. Lond. B Biol. Sci. 270**,** 775-781. https://doi.org/10.1098/rspb.2003.2327.

Lockwood, J.L., Cassey, P., Blackburn, T., 2005. The role of propagule pressure in explaining species invasions. Trends Ecol. Evol. 20**,** 223-228. https://doi.org/10.1016/j.tree.2005.02.004.

Martin, P.H., Canham, C.D., Kobe, R.K., 2010. Divergence from the growth-survival trade-off and extreme high growth rates drive patterns of exotic tree invasions in closed-canopy forests. J. Ecol. 98**,** 778-789. https://doi.org/10.1111/j.1365-2745.2010.01666.x.

McAvoy, T.J., Snyder, A.L., Johnson, N., Salom, S.M., Kok, L.T., 2012. Road survey of the invasive tree-of-heaven (*Ailanthus altissima*) in Virginia. Invas. Plant. Sci. Manage. 5**,** 506-512. https://doi.org/10.1614/Ipsm-D-12-00039.1.

Mergen, F., 1959. A toxic principle in the leaves of Ailanthus. Botanical Gaz. 121**,** 32-36. https://doi.org/10.1086/336038.

Motard, E., Muratet, A., Clair-Maczulajtys, D., MacHon, N., 2011. Does the invasive species *Ailanthus altissima* threaten floristic diversity of temperate peri-urban forests? Cr. Biol. 334**,** 872-879. https://doi.org/10.1016/j.crvi.2011.06.003.

Nilsen, E.T., Huebner, C.D., Carr, D.E., Bao, Z., 2018. Interaction between *Ailanthus altissima* and native *Robinia pseudoacacia* in early succession: Implications for forest management. Forests. 9**,** 221. https://doi.org/10.3390/f9040221.

O'Neal, E.S., Davis, D.D., 2015. Biocontrol of *Ailanthus altissima*: Inoculation protocol and risk assessment for *Verticillium nonalfalfae* (Plectosphaerellaceae: Phyllachorales). Biocontrol Sci. Techn. 25**,** 950-969. https://doi.org/10.1080/09583157.2015.1023258.

Ortega, Y.K., Valliant, M.T., Pearson, D.E., 2019. To list or not to list: using time since invasion to refine impact assessment for an exotic plant proposed as noxious. Ecosphere. 10, e02961. https://doi.org/10(11):e02961. 10.1002/ecs2.2961.

Pearson, D.E., Ortega, Y.K., Runyon, J.B., Butler, J.L., 2016. Secondary invasion: the bane of weed management. Biological Conservation. 197**,** 8-17.

Pimentel, D., Zuniga, R., Morrison, D., 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol. Econ.

Pyšek, P., Prach, K., 1993. Plant invasions and the role of riparian habitats: a comparison of four species alien to central Europe. In: Ecosystem Management. Springer. pp. 254-263. https://doi.org/10.1007/978-1-4612-4018-1_23.

Pyšek, P., Richardson, D.M., 2010. Invasive species, environmental change and management, and health. Annu. Rev. Environ. Resour. 35**,** 25-55. https://doi.org/10.1146/annurev-environ-033009-095548.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Rebbeck, J., Jolliff, J., 2018. How long do seeds of the invasive tree, *Ailanthus altissima* remain viable? For. Ecol. Manage. 429**,** 175-179. https://doi.org/10.1016/j.foreco.2018.07.001.

Schall, M.J., Davis, D.D., 2009. *Ailanthus altissima w*ilt and mortality: etiology. Plant Dis. 93**,** 747-751. https://doi.org/10.1094/Pdis-93-7-0747.

Simberloff, D., 2006. Invasional meltdown 6 years later: important phenomenon, unfortunate metaphor, or both? Ecol. Lett. 9, 912-919. https://doi.org/10.1111/j.1461-0248.2006.00939.x.
Simberloff, D., Von Holle, B., 1999. Positive interactions of nonindigenous species: invasional meltdown? Biol. Invasions. 1, 21-23. https://doi.org/10.1023/A:1010086329619.
Song, S., Kim, S., Kwon, S.W., Lee, S.-I., Jablonski, P.G., 2018. Defense sequestration associated with narrowing of diet and ontogenetic change to aposematic colours in the spotted lanternfly. Sci. Rep. 8, 16831. https://doi.org/10.1038/s41598-018-34946-y.
Sørensen, T., 1948. A method of establishing groups of equal amplitude in plant sociology based on similarity of species content and its application to analyses of the vegetation on Danish commons. Kongelige Danske Videnskabernes Selskab, Biologiske Skrifter. 5, 1-34.
Strayer, D.L., Eviner, V.T., Jeschke, J.M., Pace, M.L., 2006. Understanding the long-term effects of species invasions. Trends Ecol. Evol. 21, 645-651. https://doi.org/10.1016/j.tree.2006.07.007.
Tekiela, D.R., Barney, J.N., 2017. Invasion Shadows: The Accumulation and Loss of Ecological Impacts from an Invasive Plant. Invas. Plant. Sci. Manage. 10, 1-8.
Traveset, A., Brundu, G., Carta, L., Mprezetou, I., Lambdon, P., Manca, M., Médail, F., Moragues, E., Rodríguez-Pérez, J., Siamantziouras, A.-S.D., 2008. Consistent performance of invasive plant species within and among islands of the Mediterranean basin. Biol. Invasions. 10, 847-858. https://doi.org/10.1007/s10530-008-9245-y.
Vilà, M., Espinar, J.L., Hejda, M., Hulme, P.E., Jarošík, V., Maron, J.L., Pergl, J., Schaffner, U., Sun, Y., Pyšek, P., 2011. Ecological impacts of invasive alien plants: a meta-analysis of their effects on species, communities and ecosystems. Ecol. Lett. 14, 702-708. https://doi.org/10.1111/j.1461-0248.2011.01628.x.
Vilà, M., Gimeno, I., 2007. Does invasion by an alien plant species affect the soil seed bank? J. Veg. Sci. 18, 423-430. https://doi.org/10.1111/j.1654-1103.2007.tb02554.x.
Vilà, M., Tessier, M., Suehs, C.M., Brundu, G., Carta, L., Galanidis, A., Lambdon, P., Manca, M., Médail, F., Moragues, E., 2006. Local and regional assessments of the impacts of plant invaders on vegetation structure and soil properties of Mediterranean islands. J. Biogeogr. 33, 853-861. https://doi.org/10.1111/j.1365-2699.2005.01430.x.
Walker, L.R., Smith, S.D., 1997. Impacts of invasive plants on community and ecosystem properties. In: Assessment and Management of Plant Invasions. Luken, J.O., Thieret, J.W., Eds.), Springer, New York, NY. pp. 69-86. https://doi.org/10.1007/978-1-4612-1926-2_7.
Wallner, A.M., Hamilton, G.C., Nielsen, A.L., Hahn, N., Green, E.J., Rodriguez-Saona, C.R., 2014. Landscape factors facilitating the invasive dynamics and distribution of the brown marmorated stink bug, *Halyomorpha halys* (Hemiptera: Pentatomidae), after arrival in the United States. PLoS One. 9, e95691. https://doi.org/10.1371/journal.pone.0095691.
Weakley, A.S., Ludwig, J.C., Townsend, J.F., 2017. Flora of Virginia. Flora of Virginia Project and High Country Apps LLC, Richmond, VA.
Wearne, L.J., Morgan, J., 2006. Shrub invasion into subalpine vegetation: implications for restoration of the native ecosystem. Plant Ecol. 183, 361-376. https://doi.org/10.1007/s11258-005-9046-7.

Wearne, L.J., Morgan, J.W., 2004. Community-level changes in Australian subalpine vegetation following invasion by the non-native shrub *Cytisus scoparius*. J. Veg. Sci. 15**,** 595-604. https://doi.org/10.1658/1100-9233(2004)015[0595:CCIASV]2.0.CO;2.
Wickert, K.L., O'Neal, E.S., Davis, D.D., Kasson, M.T., 2017. Seed production, viability, and reproductive limits of the invasive *Ailanthus altissima* (tree-of-heaven) within invaded environments. Forests. 8. https://doi.org/10.3390/f8070226.
Yokomizo, H., Possingham, H.P., Thomas, M.B., Buckley, Y.M., 2009. Managing the impact of invasive species: the value of knowing the density–impact curve. Ecol. Appl. 19**,** 376-386. https://doi.org/10.1890/08-0442.1.

Figures

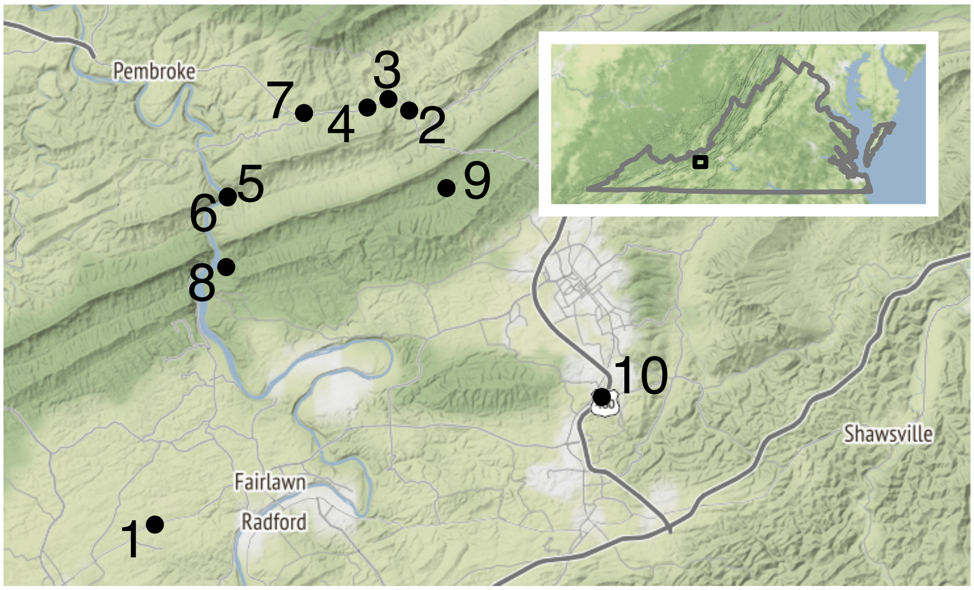


Figure 1: Site location of all invaded-uninvaded paired plots used in this study located in southwestern Virginia (map produced using ggmap and ggplot2, Kahle and Wickham, 2013; R Core Team, 2018).

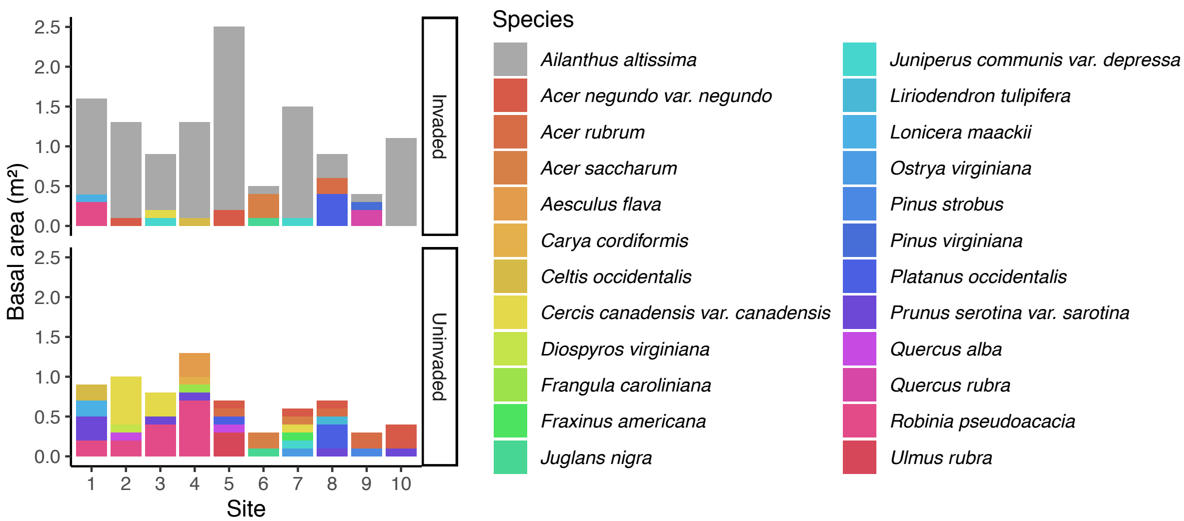


Figure 2: Basal area (m^2) of all canopy level plants estimated using a wedge 10-degree prism. Data are separated by plot and invasion status (invaded or uninvaded) and species are distinguished by color.

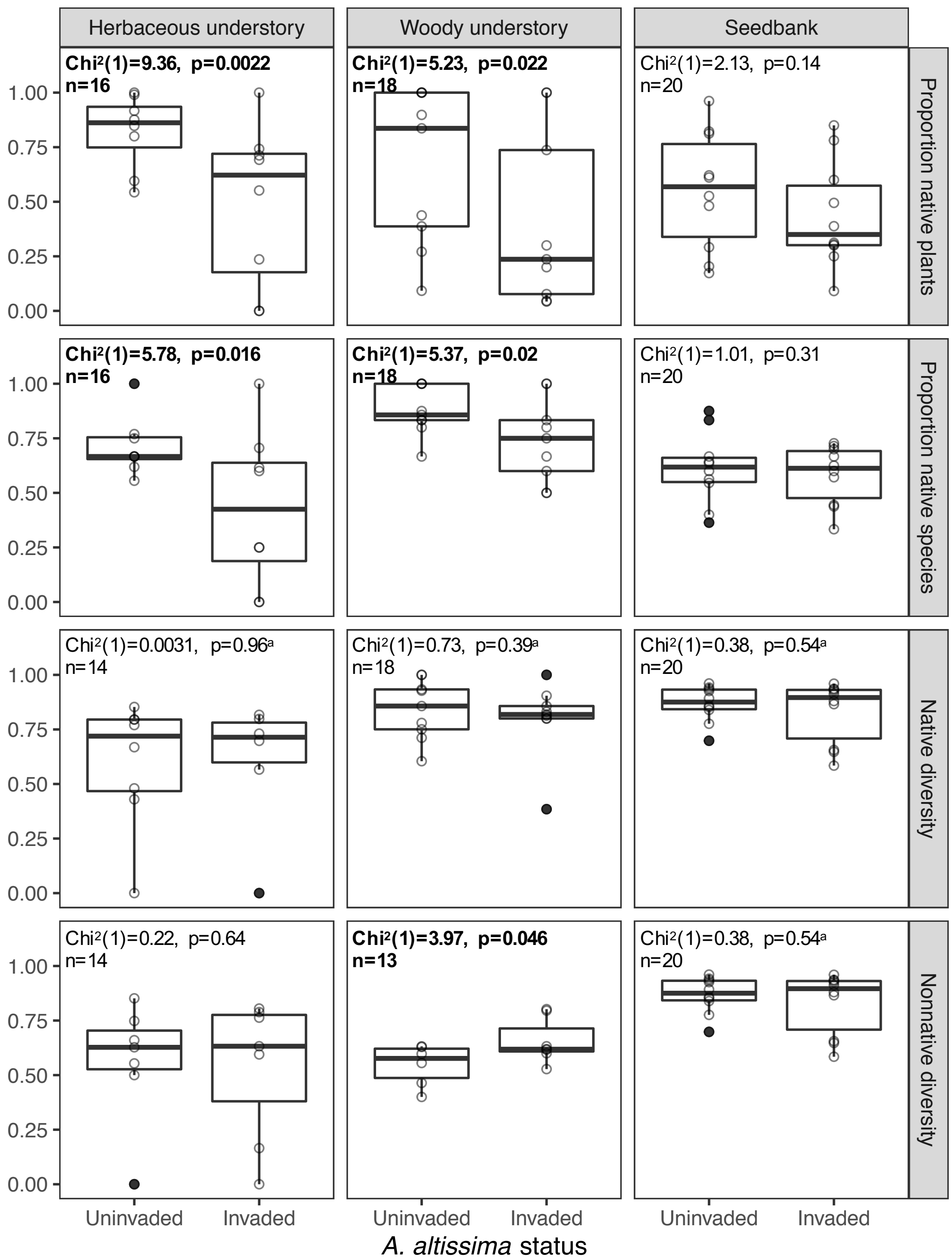


Figure 3: For the herbaceous understory, the woody understory, and the seedbank, the proportion of total individuals that are native ("proportion native plants"), the proportion of species that are native ("proportion native species"), the diversity index for native plants ("native diversity"), and the diversity index for nonnative plants ("nonnative diversity") are shown split by invasion status (uninvaded or invaded). All calculations are made on data pooled to the plot level with *A. altissima* excluded. The proportion native individuals and diversity indices for the herbaceous understory were calculated using percent cover, not counts. The diversity index chosen for this calculation is the "probability of interspecific encounters" or PIE, which measures the

probability that two individuals randomly drawn from a population represent different taxa or, in the case of the herbaceous data, the probability that two randomly selected points land on two different taxa (Gotelli, 2008; Hurlbert, 1971). Center line on the boxplot represents the median of the data. Each relationship was analyzed using a linear mixed model with invasion status (invaded or uninvaded) as a fixed effect and site as a random effect. P-values reported are from a likelihood ratio test comparing each model to a null model containing only the random effect, with bolded values indicating $p < 0.05$. Sample size (n) for each analysis is shown. Plots containing no data for a specific nonnative or native vegetation category have been excluded.

[a] response variable arcsine square root transformed prior to analysis

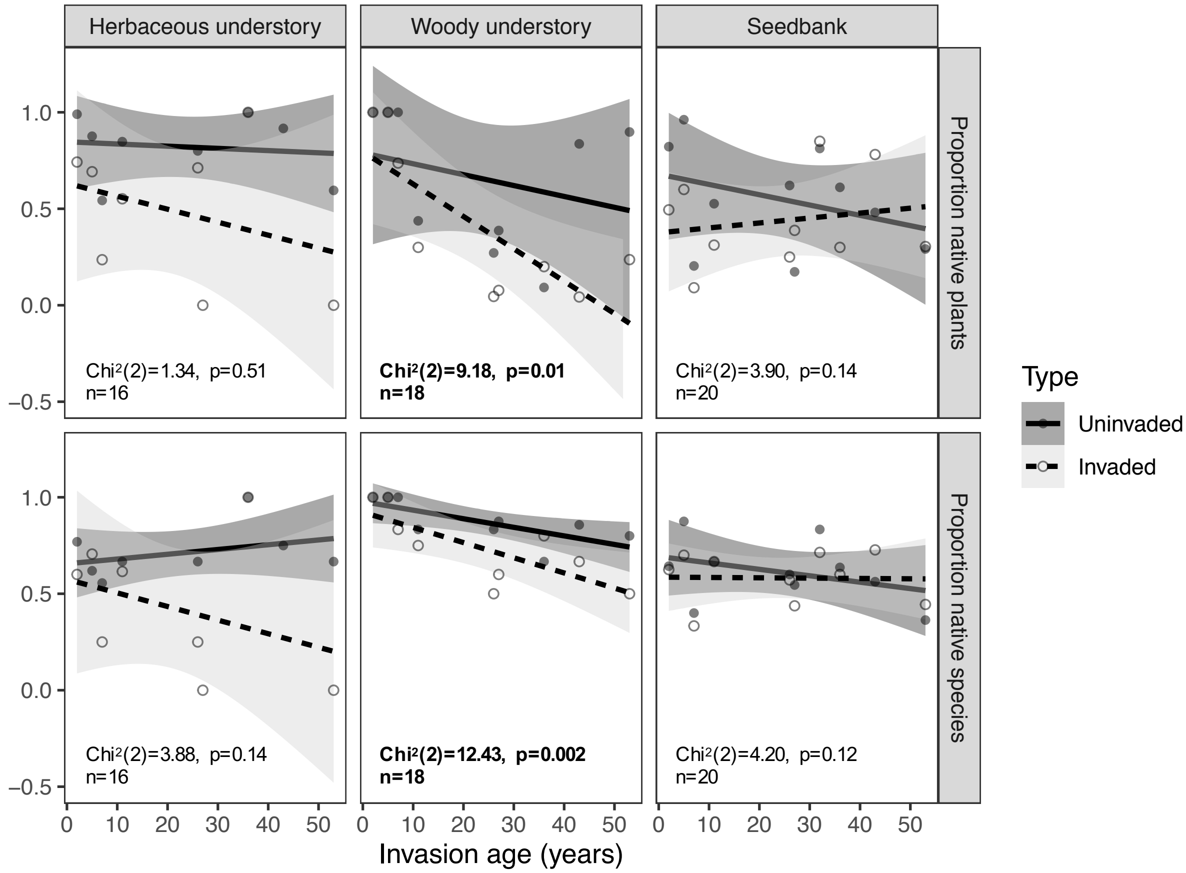


Figure 4: The relationships between the invasion age, invasion status (uninvaded or invaded), and the proportion of total counts or species that are native ("proportion native plants" and "proportion native species") for the three vegetation categories ("herbaceous understory", "woody understory", and "seedbank"). All calculations are made on data pooled to the plot level with *A. altissima* excluded. Proportion native individuals for the herbaceous understory were calculated using percent cover, not counts. Significance was determined using a likelihood ratio test, in which the interactive model (age of invasion * invasion status + (1|site)) was compared to the null model (invasion status + (1|Site)) and shown bolded if significant ($p < 0.05$). Sample size (n) for each analysis is also displayed. Plots containing no data for a specific nonnative or native vegetation category have been excluded.

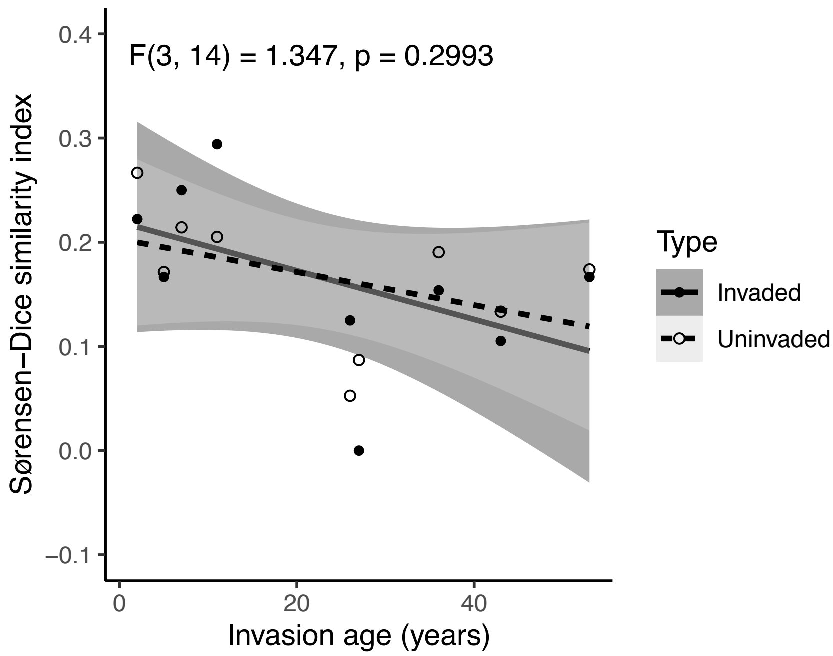


Figure 5: The Sørensen–Dice similarity index comparing the below-ground (seedbank) and above-ground (understory and canopy) species for each plot compared to the age of the *A. altissima* invasion (n=18). *Ailanthus altissima* was excluded from this analysis. The linear mixed models (lines) analyzing the interaction between the invasion status (invaded or uninvaded) and the age of the invasion with site as a random factor and their 95% confidence intervals (shaded areas) are shown. The regression, its predictors, and their interactions are all non-significant ($p > 0.05$). The regression details are displayed (R Core Team, 2018).

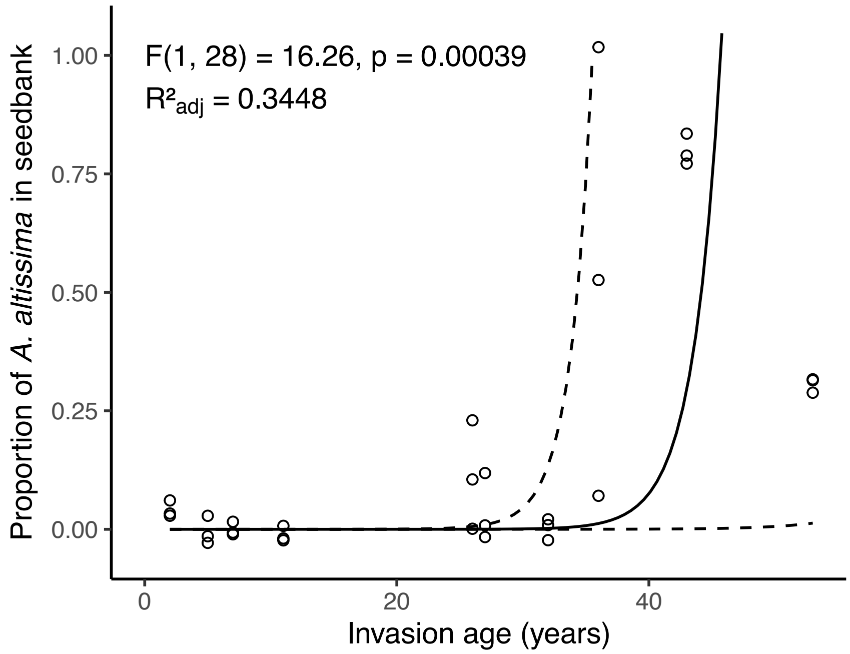


Figure 6: Proportion of *A. altissima* in the seedbank sample as related to the age in years of the *A. altissima* invasion (estimated by determining the age of the oldest *A. altissima*) is shown (n = 27). The linear regression (solid line) analyzing the log of the *A. altissima* proportion given invasion age and its 95% confidence interval (dashed line) is shown back transformed to the response space. Points have been jittered vertically by 0.03 so overlapping points can be observed. The regression significance and adjusted R^2 values are displayed (R Core Team, 2018).

Tables

Table 1: The 20 most abundant taxa as determined by the total percent cover (PC) or total count (count) of the herbaceous understory, woody understory, and the seedbank split by invasion status (invaded or uninvaded) is shown. The nativity (nonnative or native) of each taxa is also recorded. Identification and nativity was determined using the Flora of Virginia App (Weakley et al., 2017).

	Herbaceous understory			Woody understory			Seedbank		
	Taxa	**PC**	**Nativity**	**Taxa**	**Count**	**Nativity**	**Taxa**	**Count**	**Nativity**
Invaded	*Eupatorium godfreyanum*	29.0	native	*Lonicera japonica*	341	nonnative	*Microstegium vimineum*	225	nonnative
	Microstegium vimineum	23.5	nonnative	*Ailanthus altissima*	53	nonnative	*Muhlenbergia frondosa*	178	native
	Rubus phoenicolasius	23.0	nonnative	*Acer negundo var. negundo*	37	native	*Ailanthus altissima*	153	nonnative
	Polystichum acrostichoides	16.5	native	*Lonicera maackii*	30	nonnative	*Oxalis stricta*	97	native
	Amphicarpaea bracteata	16.0	native	*Toxicodendron radicans*	21	native	*Glechoma hederacea*	64	nonnative
	Geum canadense	15.0	native	*Parthenocissus quinquefolia*	16	native	*Veronica hederifolia*	33	nonnative
	Rubus allegheniensis	10.5	native	*Celastrus orbiculatus*	15	nonnative	*Galinsoga quadriradiata*	25	nonnative
	Alliaria petiolata	7.8	nonnative	*Prunus serotina var. serotina*	8	native	*Ranunculus abortivus*	25	native
	Leersia virginica	7.5	native	*Liriodendron tulipifera*	7	native	*Cardamine flexuosa*	24	nonnative
	Hylodesmum nudiflorum	7.0	native	*Hypericum prolificum*	3	native	*Erechtites hieraciifolius*	15	native
	Centaurea stoebe ssp. micranthos	6.1	nonnative	*Smilax rotundifolia*	3	native	*Securigera varia*	15	nonnative
	Ipomoea pandurata	6.0	native	*Betula alleghaniensis*	2	native	*Galium tinctorium*	14	native
	Persicaria maculosa	6.0	nonnative	*Ligustrum obtusifolium*	2	nonnative	*Verbascum thapsus*	14	nonnative
	Galium mollugo	5.0	nonnative	*Lindera benzoin*	2	native	*Bidens bipinnata*	13	native
	Solidago spp.	5.0	native	*Acer rubrum*	1	native	*Acalypha virginica*	12	native
	Verbesina occidentalis	5.0	native	*Acer saccharum*	1	native	*Acer negundo var. negundo*	12	native
	Persicaria virginiana	4.0	native	*Carya ovata*	1	native	*Geum virginianum*	11	native
	Dactylis glomerata	3.0	nonnative	*Cornus florida*	1	native	*Pueraria montana var. lobata*	11	nonnative
	Carex spp.	3.0	unknown	*Fraxinus nigra*	1	native	*Solanum ptychanthum*	11	native
	Cryptotaenia canadensis	3.0	native	*Ulmus rubra*	1	native	*Ageratina altissima var. altissima*	9	native
Uninvaded	*Amphicarpaea bracteata*	92.1	native	*Lonicera japonica*	120	nonnative	*Ranunculus abortivus*	99	native
	Phryma leptostachya	32.0	native	*Acer negundo*	49	native	*Muhlenbergia frondosa*	77	native

var. leptostachya			*var. negundo*					
Sporobolus compositus var. compositus	31.5	native	*Toxicodendron radicans*	45	native	*Oxalis stricta*	72	native
Leersia virginica	26.0	native	*Lonicera maackii*	44	nonnative	*Verbascum thapsus*	35	nonnative
Ipomoea pandurata	16.0	native	*Staphylea trifolia*	21	native	*Cardamine hirsuta*	31	nonnative
Rubus allegheniensis	15.5	native	*Parthenocissus quinquefolia*	20	native	*Ailanthus altissima*	26	nonnative
Alliaria petiolata	14.9	nonnative	*Celastrus orbiculatus*	18	nonnative	*Acalypha virginica*	25	native
Solidago spp.	14.2	native	*Ulmus rubra*	8	native	*Microstegium vimineum*	23	nonnative
Polystichum acrostichoides	13.0	native	*Viburnum prunifolium*	7	native	*Veronica hederifolia*	22	nonnative
Hylodesmum nudiflorum	12.0	native	*Celtis occidentalis*	6	native	*Glechoma hederacea*	21	nonnative
Collinsonia canadensis	10.5	native	*Acer saccharum*	4	native	*Solanum ptychanthum*	19	native
Microstegium vimineum	10.3	nonnative	*Celastrus scandens*	4	native	*Barbarea vulgaris*	15	nonnative
Geum canadense	10.0	native	*Juglans cinerea*	4	native	*Erechtites hieraciifolius*	15	native
Symphyotrichum cordifolium	9.0	native	*Prunus serotina var. serotina*	4	native	*Rubus phoenicolasius*	14	nonnative
Galium mollugo	6.0	nonnative	*Diospyros virginiana*	3	native	*Carex spp.*	11	unknown
Securigera varia	5.4	nonnative	*Cercis canadensis var. canadensis*	2	native	*Geum virginianum*	10	native
Verbesina occidentalis	5.0	native	*Fraxinus americana*	2	native	*Nepeta cataria*	10	nonnative
Tussilago farfara	4.5	nonnative	*Liriodendron tulipifera*	2	native	*Acer negundo var. negundo*	9	native
Cirsium arvense	3.5	nonnative	*Pinus strobus*	2	native	*Ageratina altissima var. altissima*	9	native

Supplementary material

Supplemental Table A.1: The complete list of all identified taxa. Taxa are listed by family, then to species (or genus if species could not be determined). Nativity to the region is also displayed. The mean counts (or mean percent cover for the herbaceous understory data), standard error (SE), and number of plots that taxa was present in (n) is shown for the herbaceous understory, woody understory, and seedbank split by invasion status (invaded or uninvaded). Nativity and identification are based on the Flora of Virginia App (Weakley et al., 2017).

			Herbaceous understory						Woody understory						Seedbank					
			Uninvaded			Invaded			Uninvaded			Invaded			Uninvaded			Invaded		
Family	**Species**	**Nativity**	**Mean[1]**	**SE[1]**	**n**	**Mean[1]**	**SE[1]**	**n**	**Mean[1]**	**SE[1]**	**n**	**Mean[1]**	**SE[1]**	**n**	**Mean[2]**	**SE[2]**	**n**	**Mean[2]**	**SE[2]**	**n**
Adoxaceae	*Viburnum prunifolium*	native	-	-	-	-	-	-	0.778	0.572	2	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0
Amaryllidaceae	*Allium vineale*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	0	-	-	0	0.400	0.400	1	0.000	0.000	0
Anacardiaceae	*Toxicodendron radicans*	native	-	-	-	-	-	-	5.000	3.283	4	2.333	0.726	7	0.000	0.000	0	0.000	0.000	0
Apiaceae	*Cryptotaenia canadensis*	native	0.111	0.111	1	0.333	0.333	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Apiaceae	*Daucus carota*	nonnative	0.033	0.033	1	0.044	0.034	2	-	-	-	-	-	-	0.600	0.427	2	0.200	0.133	2
Apiaceae	*Osmorhiza claytonii*	native	0.000	0.000	0	0.222	0.222	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Asteraceae	*Ageratina altissima var. altissima*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.900	0.547	3	0.900	0.482	3
Asteraceae	*Ambrosia artemisiifolia*	native	0.033	0.033	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Asteraceae	*Bidens bipinnata*	native	0.056	0.056	1	0.000	0.000	0	-	-	-	-	-	-	0.600	0.499	2	1.300	1.193	2
Asteraceae	*Centaurea stoebe ssp. micranthos*	nonnative	0.178	0.178	1	0.678	0.678	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Asteraceae	*Cirsium arvense*	nonnative	0.000	0.000	0	0.478	0.388	2	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Asteraceae	*Cirsium vulgare*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.100	0.100	1	0.000	0.000	0
Asteraceae	*Conyza canadensis var. canadensis*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.700	0.335	4	0.800	0.359	4
Asteraceae	*Erechtites hieraciifolius*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	1.500	0.401	8	1.091	0.222	10
Asteraceae	*Erigeron annuus*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.500	0.307	3	0.300	0.213	2
Asteraceae	*Erigeron philadelphicus var. philadelphicus*	native	0.000	0.000	0	0.056	0.056	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Asteraceae	*Eupatorium godfreyanum*	native	0.000	0.000	0	3.444	2.734	3	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Asteraceae	*Galinsoga quadriradiata*	nonnative	0.011	0.011	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	2.500	1.408	3
Asteraceae	*Hieracium pilosella*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.100	0.100	1	0.000	0.000	0
Asteraceae	*Leucanthemum vulgare*	nonnative	0.000	0.000	0	0.222	0.222	1	-	-	-	-	-	-	0.300	0.300	1	0.000	0.000	0
Asteraceae	*Polymnia canadensis*	native	0.000	0.000	0	0.222	0.222	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Asteraceae	*Smallanthus uvedalia*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.800	0.533	2
Asteraceae	*Solidago spp.*	native	0.022	0.022	1	2.111	1.359	3	-	-	-	-	-	-	0.900	0.407	5	0.400	0.306	2
Asteraceae	*Sonchus asper*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.200	0.133	2	0.100	0.100	1
Asteraceae	*Symphyotrichum cordifolium*	native	0.111	0.111	1	1.111	0.889	2	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Asteraceae	*Taraxacum officinale*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.400	0.221	3	0.100	0.100	1
Asteraceae	*Tussilago farfara*	nonnative	0.500	0.500	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Asteraceae	*Verbesina occidentalis*	native	1.000	0.667	2	0.111	0.111	1	-	-	-	-	-	-	0.100	0.100	1	0.000	0.000	0
Asteraceae	*Youngia japonica*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.100	0.100	1	0.000	0.000	0
Balsaminaceae	*Impatiens capensis*	native	0.122	0.110	2	0.278	0.222	2	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Betulaceae	*Betula alleghaniensis*	native	-	-	-	-	-	-	0.111	0.111	1	0.222	0.222	1	0.300	0.213	2	0.200	0.200	1
Boraginaceae	*Hackelia spp.*	native	0.000	0.000	0	0.056	0.056	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Brassicaceae	*Alliaria petiolata*	nonnative	1.389	0.446	8	1.133	1.109	2	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Brassicaceae	*Barbarea vulgaris*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	1.500	1.500	1	0.000	0.000	0
Brassicaceae	*Cardamine flexuosa*	nonnative	0.033	0.033	1	0.000	0.000	0	-	-	-	-	-	-	0.400	0.306	2	2.400	1.384	5
Brassicaceae	*Cardamine hirsuta*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	3.100	2.775	3	0.500	0.500	1
Brassicaceae	*Cardamine impatiens*	nonnative	0.001	0.001	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Campanulaceae	*Lobelia inflata*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.100	0.100	1
Campanulaceae	*Lobelia spicata*	native	0.000	0.000	0	0.089	0.061	2	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Cannabaceae	*Celtis occidentalis*	native	-	-	-	-	-	-	0.444	0.444	1	0.000	0.000	0	0.600	0.267	4	0.100	0.100	1
Caprifoliaceae	*Lonicera japonica*	nonnative	-	-	-	-	-	-	13.333	6.521	5	37.889	17.215	6	0.200	0.200	1	0.400	0.400	1
Caprifoliaceae	*Lonicera maackii*	nonnative	-	-	-	-	-	-	4.889	3.438	2	3.333	3.333	1	0.000	0.000	0	0.000	0.000	0
Caprifoliaceae	*Triosteum spp.*	native	0.000	0.000	0	0.056	0.056	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Caryophyllaceae	*Silene dichotoma ssp. dichotoma*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.700	0.597	2
Caryophyllaceae	*Stellaria media*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.200	0.133	2	0.500	0.500	1
Celastraceae	*Celastrus orbiculatus*	nonnative	-	-	-	-	-	-	2.000	2.000	1	1.667	1.546	2	0.000	0.000	0	0.000	0.000	0
Celastraceae	*Celastrus scandens*	native	-	-	-	-	-	-	0.444	0.444	1	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0
Commelinaceae	*Commelina communis*	nonnative	0.000	0.000	0	0.044	0.044	1	-	-	-	-	-	-	0.400	0.306	2	0.000	0.000	0
Convolvulaceae	*Ipomoea pandurata*	native	1.778	1.778	1	0.667	0.667	1	-	-	-	-	-	-	0.000	0.000	0	0.400	0.306	2
Cornaceae	*Cornus alternifolia*	native	-	-	-	-	-	-	0.111	0.111	1	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0
Cornaceae	*Cornus florida*	native	-	-	-	-	-	-	0.000	0.000	0	0.111	0.111	1	0.000	0.000	0	0.000	0.000	0
Crassulaceae	*Sedum ternatum*	native	0.000	0.000	0	0.333	0.333	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Cupressaceae	*Juniperus virginiana var. virginiana*	native	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0	0.100	0.100	1
Cyperaceae	*Carex spp.*	unknown	0.333	0.333	1	0.344	0.344	1	-	-	-	-	-	-	1.100	0.795	3	0.000	0.000	0
Dryopteridaceae	*Polystichum acrostichoides*	native	1.444	1.444	1	1.833	1.833	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0

Ebenaceae	*Diospyros virginiana*	native	-	-	-	-	-	-	0.333	0.333	1	0.000	0.000	0	0.300	0.300	1	0.000	0.000	0
Ericaceae	*Pyrola americana*	native	0.056	0.056	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Ericaceae	*Vaccinium pallidum*	native	-	-	-	-	-	-	0.000	0.000	0	0.111	0.111	1	0.000	0.000	0	0.000	0.000	0
Euphorbiaceae	*Acalypha viginica*	native	0.311	0.146	4	0.200	0.166	2	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Euphorbiaceae	*Acalypha virginica*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	2.500	0.719	8	1.200	0.629	4
Fabaceae	*Amphicarpaea bracteata*	native	6.789	5.062	3	5.222	5.222	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Fabaceae	*Cercis canadensis var. canadensis*	native	-	-	-	-	-	-	0.222	0.222	1	0.000	0.000	0	0.200	0.133	2	0.000	0.000	0
Fabaceae	*Hylodesmum nudiflorum*	native	1.333	1.333	1	0.778	0.778	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Fabaceae	*Pueraria montana var. lobata*	nonnative	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0	0.100	0.100	1	1.100	0.526	4
Fabaceae	*Securigera varia*	nonnative	0.000	0.000	0	0.711	0.548	3	-	-	-	-	-	-	0.300	0.153	3	1.500	0.654	5
Fabaceae	*Trifolium repens*	nonnative	0.000	0.000	0	0.022	0.022	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Fagaceae	*Quercus alba*	native	-	-	-	-	-	-	0.111	0.111	1	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0
Geraniaceae	*Geranium maculatum*	native	0.411	0.278	2	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Hypericaceae	*Hypericum prolificum*	native	-	-	-	-	-	-	0.000	0.000	0	0.333	0.333	1	0.000	0.000	0	0.000	0.000	0
Juglandaceae	*Carya ovata*	native	-	-	-	-	-	-	0.000	0.000	0	0.111	0.111	1	0.000	0.000	0	0.000	0.000	0
Juglandaceae	*Juglans cinerea*	native	-	-	-	-	-	-	0.444	0.294	2	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0
Juglandaceae	*Juglans nigra*	native	-	-	-	-	-	-	0.111	0.111	1	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0
Lamiaceae	*Collinsonia canadensis*	native	1.167	1.167	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Lamiaceae	*Glechoma hederacea*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	2.100	0.657	7	5.636	2.001	10
Lamiaceae	*Nepeta cataria*	nonnative	0.056	0.056	1	0.000	0.000	0	-	-	-	-	-	-	1.000	0.683	4	0.100	0.100	1
Lauraceae	*Lindera benzoin*	native	-	-	-	-	-	-	0.111	0.111	1	0.222	0.222	1	0.000	0.000	0	0.000	0.000	0
Magnoliaceae	*Liriodendron tulipifera*	native	-	-	-	-	-	-	0.222	0.147	2	0.778	0.778	1	0.500	0.167	5	0.500	0.269	3
Menispermaceae	*Menispermum canadense*	native	-	-	-	-	-	-	0.111	0.111	1	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0
Oleaceae	*Fraxinus americana*	native	-	-	-	-	-	-	0.222	0.147	2	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0
Oleaceae	*Fraxinus nigra*	native	-	-	-	-	-	-	0.000	0.000	0	0.111	0.111	1	0.000	0.000	0	0.000	0.000	0
Oleaceae	*Ligustrum obtusifolium*	nonnative	-	-	-	-	-	-	0.000	0.000	0	0.222	0.222	1	0.000	0.000	0	0.000	0.000	0
Oxalidaceae	*Oxalis florida*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.700	0.260	5	0.900	0.504	4
Oxalidaceae	*Oxalis stricta*	native	0.422	0.205	4	0.122	0.110	2	-	-	-	-	-	-	6.455	3.263	10	6.182	3.352	10
Paulowniaceae	*Paulownia tomentosa*	nonnative	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0	0.500	0.269	3	0.200	0.133	2
Phrymaceae	*Phryma leptostachya var. leptostachya*	native	0.000	0.000	0	3.556	3.556	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Phytolaccaceae	*Phytolacca americana var. americana*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.500	0.224	4	0.800	0.291	5
Pinaceae	*Pinus strobus*	native	-	-	-	-	-	-	0.222	0.222	1	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0
Plantaginaceae	*Chaenorrhinum minus*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.100	0.100	1
Plantaginaceae	*Plantago rugelii*	native	0.000	0.000	0	0.111	0.111	1	-	-	-	-	-	-	0.500	0.401	2	0.300	0.213	2
Plantaginaceae	*Veronica hederifolia*	nonnative	0.078	0.036	4	0.056	0.056	1	-	-	-	-	-	-	2.200	1.467	4	3.300	1.783	5
Plantaginaceae	*Veronica officinalis*	unknown	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.400	0.306	2
Platanaceae	*Platanus occidentalis*	native	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0	0.100	0.100	1	0.000	0.000	0
Poaceae	*Andropogon virginicus var. virginicus*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.500	0.224	4	0.000	0.000	0
Poaceae	*Dactylis glomerata*	nonnative	0.001	0.001	1	0.333	0.333	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Poaceae	*Digitaria ischaemum*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.100	0.100	1	0.000	0.000	0
Poaceae	*Echinochloa crusgalli var. crusgalli*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.200	0.200	1
Poaceae	*Elymus hystrix*	native	0.111	0.111	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Poaceae	*Leersia virginica*	native	0.000	0.000	0	3.722	2.905	2	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Poaceae	*Microstegium vimineum*	nonnative	0.956	0.776	2	2.800	1.519	4	-	-	-	-	-	-	2.300	1.446	3	22.500	8.814	8
Poaceae	*Muhlenbergia frondosa*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	7.700	4.709	3	17.800	10.459	3
Poaceae	*Panicum miliaceum*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.400	0.221	3	0.900	0.706	2
Poaceae	*Poa compressa*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.100	0.100	1
Poaceae	*Schedonorus arundinaceus*	nonnative	0.333	0.333	1	0.333	0.333	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Poaceae	*Setaria viridis var. viridis*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.100	0.100	1
Poaceae	*Sporobolus compositus var. compositus*	native	3.500	3.500	1	0.333	0.333	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Poaceae	*Tridens flavus*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.200	0.133	2	0.000	0.000	0
Polygonaceae	*Persicaria maculosa*	nonnative	0.667	0.667	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Polygonaceae	*Persicaria virginiana*	native	0.000	0.000	0	0.444	0.444	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Polygonaceae	*Rumex crispus ssp. crispus*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.500	0.500	1	0.000	0.000	0
Portulacaceae	*Portulaca oleracea*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.300	0.300	1	0.000	0.000	0

Ranunculaceae	*Anemone americana*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.100	0.100	1	0.000	0.000	0
Ranunculaceae	*Clematis viorna*	native	0.167	0.167	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Ranunculaceae	*Ranunculus abortivus*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	9.900	9.247	3	2.500	2.391	2
Rosaceae	*Geum canadense*	native	1.944	1.655	2	0.833	0.471	3	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Rosaceae	*Geum virginianum*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	1.000	0.394	6	1.100	0.781	4
Rosaceae	*Potentilla indica*	nonnative	0.000	0.000	0	0.056	0.056	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Rosaceae	*Potentilla simplex*	native	0.011	0.011	1	0.067	0.067	1	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Rosaceae	*Prunus serotina var. serotine*	native	-	-	-	-	-	-	0.333	0.333	1	0.889	0.455	4	0.000	0.000	0	0.000	0.000	0
Rosaceae	*Rosa multiflora*	nonnative	0.222	0.222	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Rosaceae	*Rubus allegheniensis*	native	1.167	1.167	1	1.722	1.096	3	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Rosaceae	*Rubus phoenicolasius*	nonnative	1.889	1.889	1	0.667	0.667	1	-	-	-	-	-	-	1.400	0.427	7	0.400	0.221	3
Rubiaceae	*Galium mollugo*	nonnative	1.011	0.673	2	0.211	0.140	2	-	-	-	-	-	-	0.100	0.100	1	0.000	0.000	0
Rubiaceae	*Galium tinctorium*	native	0.000	0.000	0	0.044	0.044	1	-	-	-	-	-	-	0.200	0.200	1	1.400	0.653	5
Ruscaceae	*Maianthemum racemosum ssp. racemosum*	native	0.011	0.011	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0
Sapindaceae	*Acer negundo var. negundo*	native	-	-	-	-	-	-	5.444	3.473	4	4.111	2.366	3	0.900	0.277	6	1.200	0.512	5
Sapindaceae	*Acer rubrum*	native	-	-	-	-	-	-	0.000	0.000	0	0.111	0.111	1	0.000	0.000	0	0.000	0.000	0
Sapindaceae	*Acer saccharum*	native	-	-	-	-	-	-	0.444	0.338	2	0.111	0.111	1	0.000	0.000	0	0.000	0.000	0
Saxifragaceae	*Hydatica petiolaris*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.500	0.401	2
Saxifragaceae	*Micranthes pensylvanica*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.100	0.100	1	0.100	0.100	1
Scrophulariaceae	*Verbascum blattaria*	nonnative	0.022	0.022	1	0.000	0.000	0	-	-	-	-	-	-	0.000	0.000	0	0.300	0.153	3
Scrophulariaceae	*Verbascum thapsus*	nonnative	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	3.500	2.296	8	1.400	0.872	5
Simaroubaceae	*Ailanthus altissima*	nonnative	-	-	-	-	-	-	0.000	0.000	0	3.778	2.565	5	1.833	0.711	11	9.267	5.561	14
Smilacaceae	*Smilax hispida*	native	-	-	-	-	-	-	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0	0.100	0.100	1
Smilacaceae	*Smilax rotundifolia*	native	-	-	-	-	-	-	0.222	0.147	2	0.333	0.236	2	0.000	0.000	0	0.000	0.000	0
Solanaceae	*Physalis longifolia var. subglabrata*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.200	0.200	1	0.000	0.000	0
Solanaceae	*Solanum dulcamara*	nonnative	0.000	0.000	0	0.056	0.056	1	-	-	-	-	-	-	0.100	0.100	1	0.000	0.000	0
Solanaceae	*Solanum ptychanthum*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	1.900	1.090	5	1.100	0.605	4
Staphyleaceae	*Staphylea trifolia*	native	-	-	-	-	-	-	2.333	2.333	1	0.000	0.000	0	0.000	0.000	0	0.000	0.000	0
Ulmaceae	*Ulmus rubra*	native	-	-	-	-	-	-	0.889	0.455	4	0.111	0.111	1	0.000	0.000	0	0.000	0.000	0
Urticaceae	*Pilea pumila*	native	0.000	0.000	0	0.000	0.000	0	-	-	-	-	-	-	0.100	0.100	1	0.100	0.100	1
Violaceae	*Viola sororia*	native	0.000	0.000	0	0.067	0.055	2	-	-	-	-	-	-	0.200	0.200	1	0.200	0.200	1
Vitaceae	*Parthenocissus quinquefolia*	native	-	-	-	-	-	-	2.222	1.115	6	1.778	0.760	5	0.000	0.000	0	0.000	0.000	0
Vitaceae	*Vitis vulpina*	native	-	-	-	-	-	-	0.000	0.000	0	0.111	0.111	1	0.300	0.213	2	0.500	0.342	2

[1]Sample sized used in calculations was n=9
[2]Sample sized used in calculations was n=10

Chapter 6: Final conclusions

With the identification of the naturally occurring *Verticillium nonalfalfae*, the future success of *Ailanthus altissima* invasions over large scales is no longer certain. Indicative of a successful biological control agent, we found that in naturally infested *A. altissima* stands, *V. nonalfalfae* persisted over long periods of time consistently reducing *A. altissima* numbers and volumes. Additionally, when artificially inoculated into healthy stands, it rapidly killed *A. altissima* and spread to surrounding *A. altissima* regardless of climate variables, tree size, or *V. dahliae* presence. Because of this, we continue to support its biopesticide registration with the US Environmental Protection Agency and foresee that a limited number of applications to certain trees within *A. altissima* stands could effectively remove *A. altissima* from large areas.

In comparison to *V. nonalfalfae*, it does not appear that *V. dahliae* is a very promising biological control agent for *A. altissima*. Though we confirmed its presence and pathogenicity to *A. altissima* in Virginia, it performed poorly in artificially inoculated stands, where it only caused disease on smaller stems and did not spread efficiently. We therefore do not believe continued research focusing on *V. dahliae* as a biological control agent of *A. altissima* is necessary at this time.

With the promise of *V. nonalfalfae* controlling *A. altissima* over larger areas, we found that early *A. altissima* management should be encouraged to minimize the tree's impact on the woody understory nativity and a buildup of *A. altissima* seeds in the seedbank over time. Once removed, we found that additional site restoration may need to be considered, since *A. altissima* stands appear to have lower proportions of native understory species established.