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Field-inoculated *Ailanthus altissima* stands reveal the biological control potential of *Verticillium nonalfalfae* in the mid-Atlantic region of the United States

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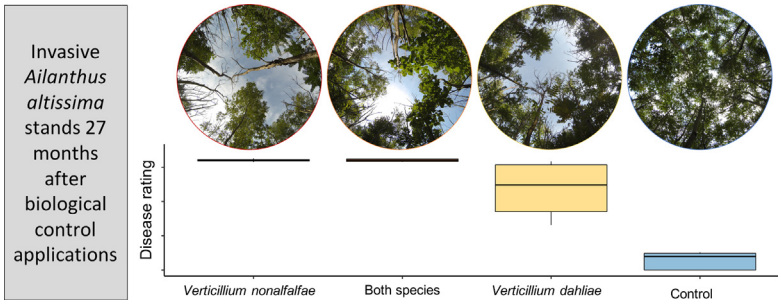
GRAPHICAL ABSTRACT



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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*.

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1. 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*.

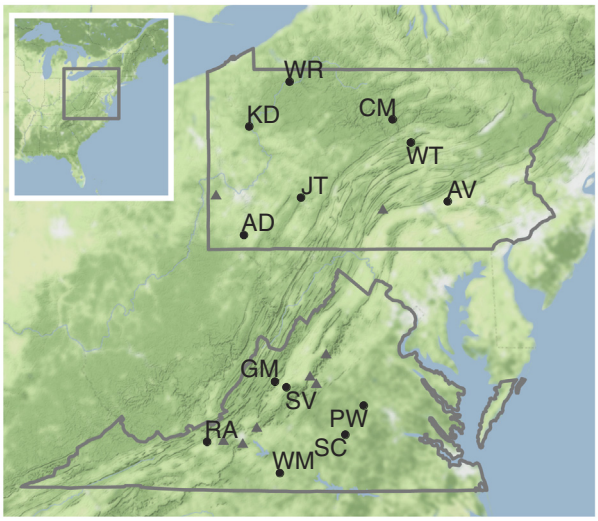

Fig. 1. Location and site code of all sites in Pennsylvania (top) and Virginia (bottom) included in the regional field analysis marked by circular points. Previously located areas with natural *V. nonalfalfae* impacting *A. altissima* within these two states are indicated by gray triangles (Kahle and 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.

2. Materials and methods

2.1. Site selection and 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) ≥ 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 FTA™ 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 MyTaq™ 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 (UltraPure™ Agarose, Invitrogen, Carlsbad, CA), and 5 μL/100 μL of SmartGlow™ Pre Stain (Accuris, Edison, NJ). Successful amplification was confirmed by electrophoresis at 90 V for 70 min and DNA bands were visualized on a SmartDoc™ 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 ($\Delta AICc < 2$) 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. 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. 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.

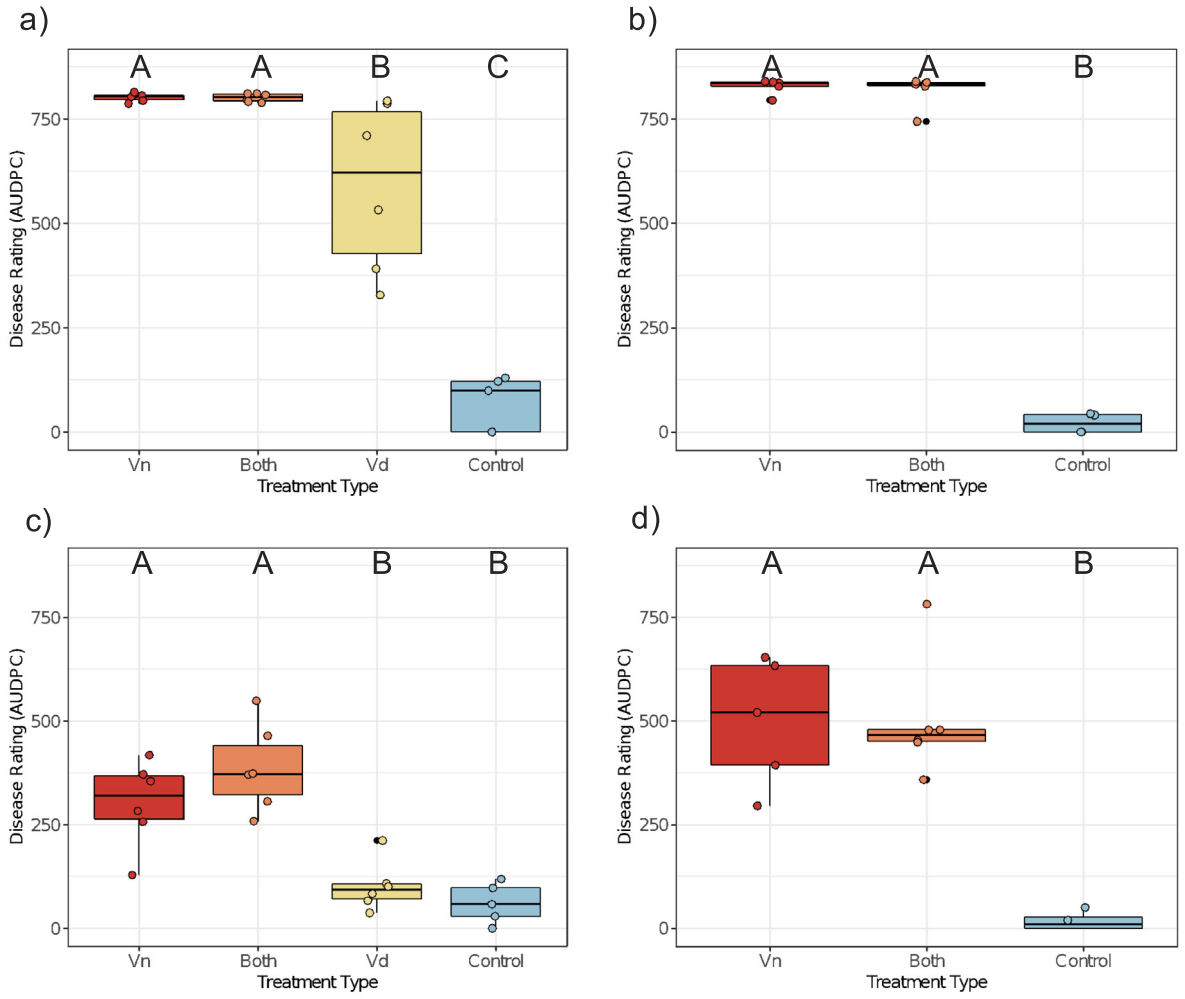

Fig. 2. Boxplot of the area under the disease progress curve (AUDPC) values for each treatment (Vn = *Verticillium nonalfalfae*, Vd = *V. dahliae*) split by state and by inoculation status. Model and predictor significance for the additive linear model (Treatment + Site) is reported for each. Significant 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).

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

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

(*continued on next page*)

Table 1 (*continued*)

State	Model information					
Type	***i***	**Model**	***k***	**AICc**	**ΔAICc**	**Weight**
	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), AvgDBH (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), AvgDBH (p = 0.221).
[4] Model: F(3,11) = 977, p = 1.26e-13, Treatment (p = 4.52e-14), AvgTemp (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), AvgTemp (p = 0.0232).
[7] Model: F(3,11) = 20.75, p = 7.80e-5, Treatment (p = 4.64e-05), Rainfall (p = 0.0354).

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-an-percip/), 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

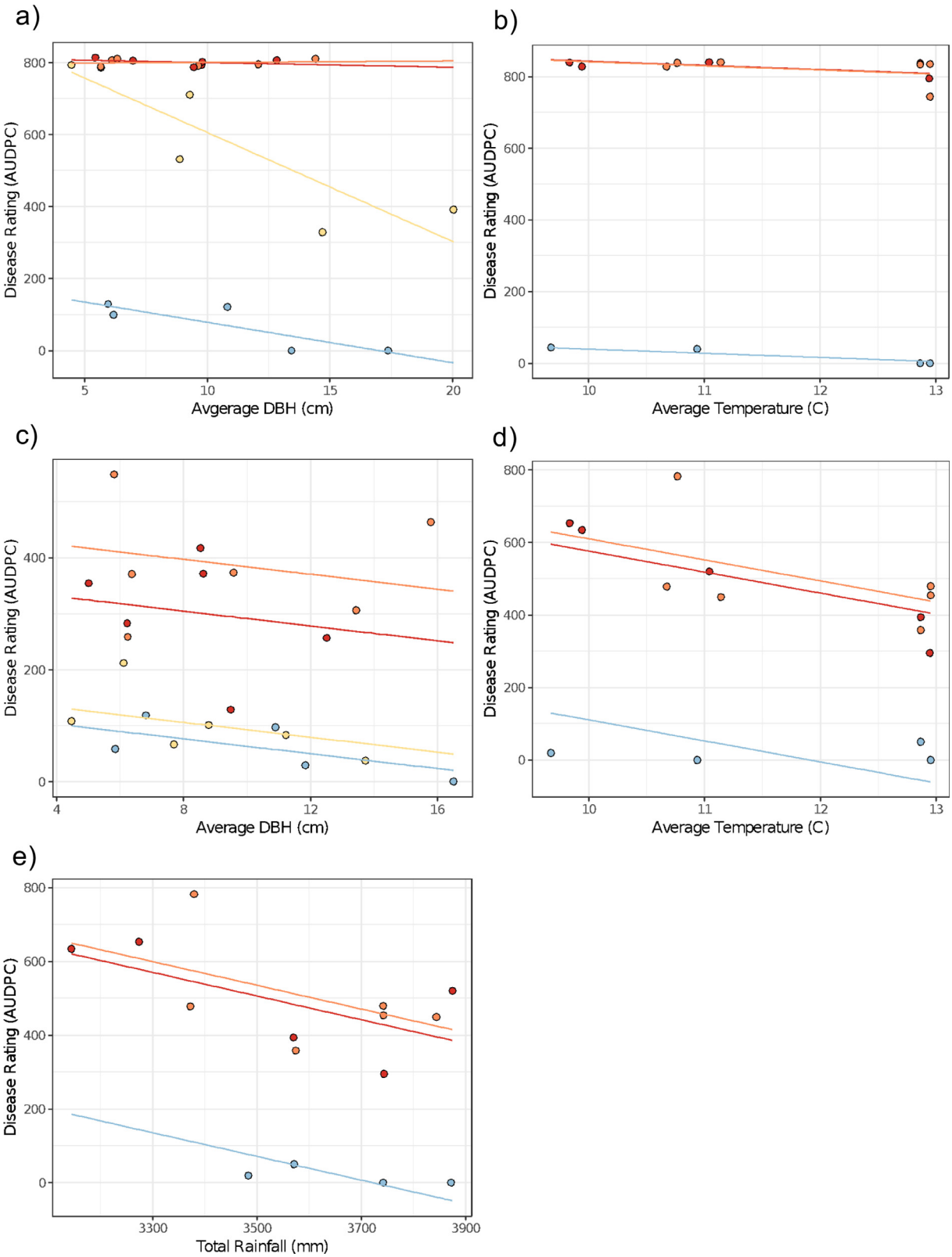

Fig. 3. The best fitting selected linear regression models determined by AICc shown as lines and actual data displayed as points for Pennsylvania or Virginia data split by inoculation status. Treatment type indicated by color: *V. nonalfalfae* (red), combination (orange), *V. dahliae* (yellow), or the 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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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 potentially 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.

CRediT authorship contribution statement

Rachel K. Brooks: Conceptualization, Data curation, Methodology, Formal analysis, Visualization, Writing - original draft. **Kristen L. Wickert:** Conceptualization, Data curation, Methodology, Writing - original draft. **Anton Baudoin:** Methodology, Supervision, Writing - review & editing. **Matt T. Kasson:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing. **Scott Salom:** Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2020.104298.

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