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Controlling an Invasive Tree with a Native Fungus: Inoculating *Ailanthus altissima* (Tree-of-Heaven) with *Verticillium nonalfalfae* in Highly Disturbed Appalachian Forests of Ohio

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Abstract

Highly disturbed forests are commonplace throughout the eastern United States and their residing composition and structure is reflective of their past land use. Management and restoration efforts are complicated by diverse and abundant nonnative invasive plants, including *Ailanthus altissima*. *Verticillium nonalfalfae* has been identified as a potential native mycoherbicide option for *Ailanthus*. To test the efficacy of *Verticillium* on *Ailanthus* we designed a study in highly disturbed forests of southern Ohio. At each of five sites, we monitored symptomology, mortality, and rate of spread of stem-inoculated *Verticillium* on *Ailanthus* in four inoculated plots and compared it to a control plot. We also monitored native plants for *Verticillium* symptomology and community responses to *Ailanthus* control. Our results suggest that *Verticillium* is an effective tool for controlling *Ailanthus* with no observed effect on native flora. Further, *Verticillium* naturally spreads through stands and mortality is slow enough that other resident nonnative invasive plants do not rapidly increase.

Study Implications: Managing problematic invasive plants is a costly and time-consuming endeavor that quickly overwhelms resources. The identification and development of native biocontrols will help to suppress invasive plants, especially when considered in conjunction with other control options. Native biocontrols are pests or diseases that are typically nonlethal residents of the local environment but have significant and detrimental impact on nonnative plants. The native fungus *Verticillium nonalfalfae* along with several other *Verticillium* species has been identified to kill the invasive *Ailanthus altissima*. *Verticillium* can be applied to a subset of *Ailanthus* stems, and through time, will spread naturally with minimal impact to native species.

Keywords: Mycoherbicide, biological control, tree reproduction, restoration, invasive species management

Ailanthus altissima (Mill.) Swingle (commonly referred to as tree-of-heaven but *Ailanthus* hereafter) has been present in North American landscapes for over two hundred years (Hu, 1979). Its current invasive range extends from Connecticut south to Georgia, west to Oklahoma and north to Iowa and Wisconsin (Figure

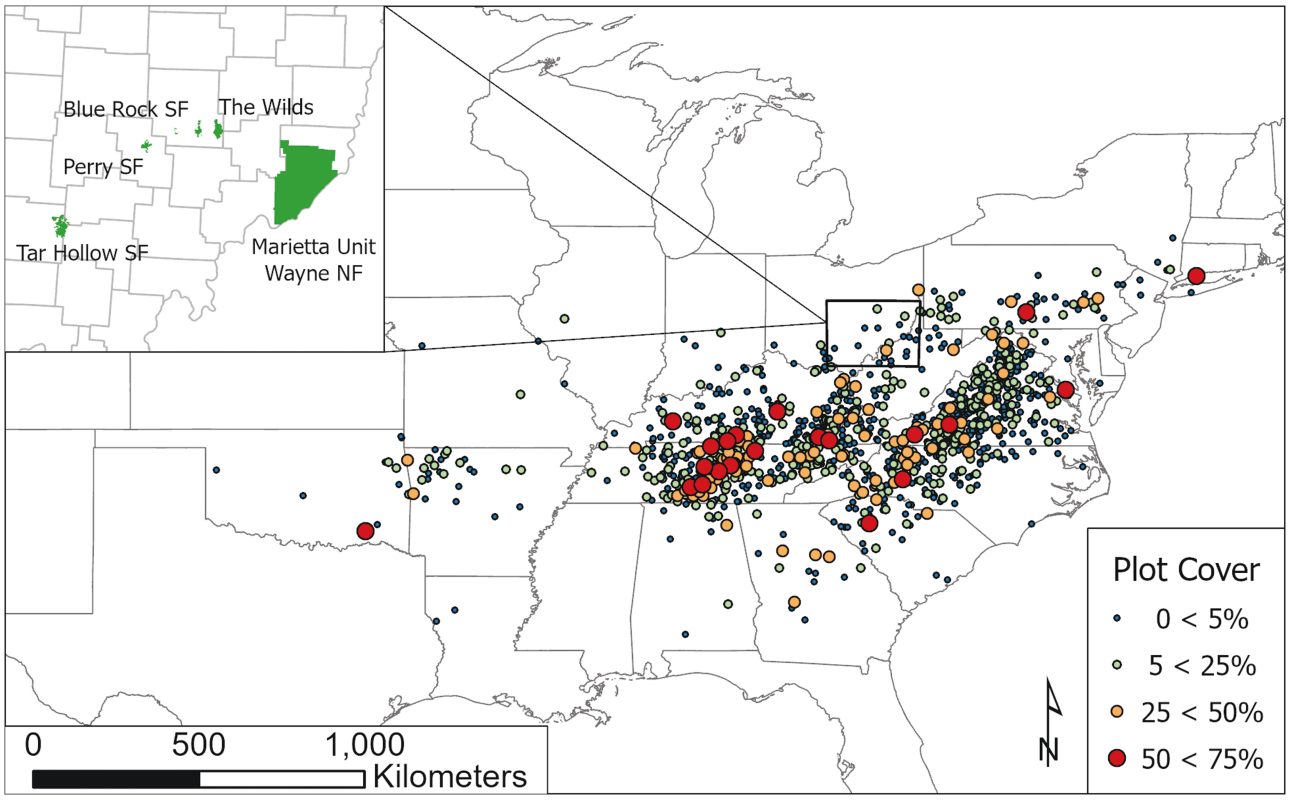

Figure 1. Distribution and abundance of *Ailanthus altissima* in the eastern United States based on Forest Inventory and Analysis (FIA) data. Map of sites for *Verticillium*-inoculation trials of *Ailanthus* in Ohio (inset), including Tar Hallow State Forest, Perry State Forest, Blue Rock State Forest, The Wilds, and the Marietta Unit of the Wayne National Forest. Four stands within each site were inoculated with *Verticillium* in May 2015 and monitored through 2020. An additional site at each location was used as a water-inoculated control.

1). *Ailanthus* subsequently ranked as one of the worst invasive plants (USDA Forest Service 2018, Ridley and Oswalt 2021) due to its wide environmental tolerance (Miller 1990), allelopathic properties that limit native flora (Mergen 1959, Lawrence et al. 1991), and high reproductive capacity resulting in rapid colonization following disturbance. Because of its widespread distribution and high abundance at local scales, eradication is no longer possible. However, limiting its abundance and impact through effective management is important and requires a diversity of control options, especially those that could be implemented at broad spatial scales. Currently, chemical herbicide is the primary method used to control *Ailanthus*; however, its use is often both expensive and labor intensive. Biological control agents that are minimally impactful to native communities but harmful to target nonnative invasive plants such as *Ailanthus* may provide an additional management approach.

Ailanthus is most often abundant in open sites such as roadsides, but its presence is increasing within disturbed forested sites, especially within mixed hardwood forests of the eastern United States. *Ailanthus* possesses numerous characteristics associated with highly invasive and transformative plants. It is extremely fast growing, with sprouts achieving 3 to 4 m of height growth within the first year and heights reaching 25–30 m (Swingle 1916). It is dioecious and a prolific seeder, producing up to 350,000 seeds/tree/year (Pannell 2002) that can remain viable in the soil beyond 5 years (Rebbeck and Jolliff 2018, Redwood et al. 2019). In addition, *Ailanthus* is capable of aggressive clonal spread, often creating dense thickets that can outcompete native trees. Although considered shade-intolerant, clonal sprouts can develop up to 15–30 m from a parent tree (Illick and Brouse 1926) and can persist in a shaded forest understory for 20 years or more (Kowarik 1995). These characteristics allow *Ailanthus* to spread and persist in forested landscapes, displacing native species. Although the long-term direct effects of *Ailanthus* on native tree regeneration are not known, its highly competitive traits and production of the allelopathic compound ailanthone (Heisey 1996) likely perpetuate barriers to natural regeneration in

eastern United States forests (Dey et al. 2019, Miller and McGill 2019, Vickers et al. 2019).

The vigorous sprouting capacity of *Ailanthus* is also challenging to management, whereby mechanical treatments alone usually lead to higher stem densities. To increase efficacy, mechanical methods are often combined with chemical herbicides. However, chemical control is often costly and requires multiple applications (Smith and Smith 2009). Approaches that combine individual stem control with other site-level treatments such as mastication or prescribed fire may also be effective for reducing fire-sensitive invasive trees (Pile et al. 2017b). For example, combining stem-injection treatments and prescribed burning was > 99% effective in killing *Ailanthus* trees and saplings (Rebbeck et al. 2019). Unfortunately, implementation of these control measures has limitations given the wide-spread distribution of *Ailanthus* within many forested landscapes in the eastern United States and limitations on the use of prescribed fire. Given these management obstacles, the use of the highly specific biological control agent, *Verticillium* wilt, a native soilborne fungus, is highly desirable.

Because of restrictions on the movement of biological materials across state lines, the identification of naturally occurring disease of invasive plants is important for developing and testing its efficacy as a biocontrol agent and for identifying potential nontarget impacts. Subsequently, these studies are often restricted to the state where the pathogen is identified until they are approved as a mycoherbicide. *Verticillium nonalfalfae,* a fungus native to the eastern United States, was first observed in the early 2000s by managers in Pennsylvania forests who noticed wilting *Ailanthus* and in 2002, it was isolated from the dead and dying trees (Schall and Davis 2009a). Symptoms of *Ailanthus* infected with *Verticillium* include wilting leaves, premature defoliation, terminal dieback, yellow vascular discoloration, and subsequent mortality. In 2009, *V. nonalfalfae* was isolated from wilting *Ailanthus* in Virginia (Snyder et al. 2013). From these findings, observational and experimental studies on *Verticillium* as a biocontrol were initiated in both Pennsylvania and Virginia. Following rigorous testing and numerous trials, *V. nonalfalfae* has been found to be very specific and deadly to *Ailanthus* and more effective than a similar *Verticillium* (*V. dahlia*) species across varying environmental conditions in Pennsylvania and Virginia (Brooks et al. 2020d). Several years later, in 2012, wilting *Ailanthus* trees were discovered in Ohio forests. Samples from Ohio were collected from stems of three symptomatic *Ailanthus* and all isolates were putatively identified as *V. nonalfalfae* based on the presence of verticillate conidiophores and formation of melanized hyphae. DNA was extracted and molecular analyses confirmed taxonomic placement of the Ohio *Ailanthus* isolates (VnAaOH1-VnAaOH3) among those recovered from *Ailanthus* in Pennsylvania and Virginia (Rebbeck et al. 2013). *Verticillium* wilt on *Ailanthus* has also been reported in Italy, Austria, Spain, and Hungary (Maschek and Halmschlager 2017, Izsépi et al. 2018, Pisuttu et al. 2020, Moragrega et al. 2021).

In addition to testing the efficacy of *Verticillium* on *Ailanthus* as a biological control agent, understanding its potential negative effects on native and agricultural plants is critical before widespread management applications. Field and greenhouse tests of over seventy-one species have resulted in only poison ivy (*Toxicodendron radicans* [L.] Kuntze), staghorn sumac (*Rhus typhina* L.), red elderberry (*Sambucus racemosa* L.), and striped maple (*Acer pensylvanicum* L.) displaying mortality following *Verticillium* inoculation (Kasson 2012, Kasson et al. 2014, Kasson et al. 2015). Additionally, only three species appeared to acquire the fungus from natural spread (*Ailanthus*, staghorn sumac, and striped maple); and the actual incidence was very low (3% for striped maple and 16% for staghorn sumac, but 100% for *Ailanthus*). These results suggest low risk to native tree and shrubs in forest communities and the promising potential of *Verticillium* as a mycoherbicide, although further nontarget testing is ongoing (Brooks et al. 2020b).

Ailanthus is widespread in Ohio forests and is a significant concern for forest management and restoration in the state. As *Verticillium* was found to be naturally occurring in populations of *Ailanthus* in Ohio forests, developing and testing the efficacy of *Verticillium* as a potential native mycoherbicide was undertaken using one of the Ohio fungal isolates (VnAaOH1). In addition to this fungus being native to North America, once introduced into a stand, it can spread to noninoculated *Ailanthus* trees through root grafts and clonal connections (O'Neal and Davis 2015b). This may result in the building up of inoculum to potentially spread throughout a forested landscape without further human intervention. In five Ohio forests with *Ailanthus* infestations, our study objectives were (1) to quantify the effectiveness of *Verticillium* inoculation to kill *Ailanthus* trees and saplings and prevent root and basal sprouting, (2) quantify the rate of disease spread to noninoculated *Ailanthus*, and (3) determine the impact of fungal induced *Ailanthus* mortality on forest

structure, tree regeneration, and native and nonnative understory vegetation. We also monitored the incidence of any potential nontarget effects of *Verticillium* on coexisting vegetation.

Materials and Methods

Study Sites

This study was conducted on forested sites where *Ailanthus* was relatively abundant, comprising on average 33.6% of the total basal area. All sites were located within the Southern Unglaciated Allegheny Plateau in southeastern Ohio: (1) Blue Rock State Forest (B; 39.8412° N, 81.8585° W); (2) Perry State Forest (P; 39.7695° N, 82.2082° W); (3) Tar Hollow State Forest (T; 39.3549° N, 82.7671° W); (4) Wayne National Forest, Athens District, Marietta Unit (M; 39.527° N, 81.1809° W); and (5) The Wilds, (W; 39.8295° N, 81.7330° W), a private conservation center and nonprofit safari park (Figure 1). The topography of these sites is characterized by high hills, sharp ridges, and narrow valleys (Beatley and Bartley 1959, Iverson et al. 2019). After European settlement, level lands were cleared for agriculture, especially on ridgetops and creek bottoms (Cleland et al. 2007). Historically, oaks and hickories dominated the landscape (Dyer and Hutchinson 2019). Sites with either or both poor soils and severe erosion were typically abandoned and left to natural succession. Most slopes have been repeatedly logged. Strip mining for coal as well as oil and gas exploration and production were common in some areas. As past land use can influence invasive plants and successional trajectories (Brudvig et al. 2013, Pile et al. 2017a, Holmes et al. 2021), it is important to highlight the disturbance history of our sites and plots. Even within study sites (see below), plots differed in disturbance histories, especially relating to mining and reclamation. Laws passed in 1977 required site reclamation following strip mining, but typically reclamation leads to highly compacted soils that severely limit tree establishment and growth. At Blue Rock State Forest, two plots had a history of mining underneath the soil surface in the 1940s. At Perry State Forest, all plots were strip mined around the late 1950s through the early 1960s, which was prior to requirements for reclamation. Tar Hollow State Forest and the Marietta Unit on the Wayne National Forest had no history of strip mining. The Wilds is a highly disturbed landscape with extensive strip mining prior to reclamation and thereafter. Most of the landscape is now reclaimed grassland, although the forested study plots appear to be on sites strip mined prior to reclamation (Michael Bowden, Ohio Department of Natural Resources, Division of Mineral Resources Management, personal communication via email).

Inoculation Preparation

Protocols for *Verticillium* culture maintenance and inoculum preparation followed those reported by O'Neal and Davis (2015a) and Kasson et al. (2014). *Verticillium nonalfalfae* was grown in petri dishes containing plum extract agar (PEA) amended with streptomycin sulfate and neomycin sulfate (PEA + SN) (Kasson et al. 2014). To produce inoculum, 6-week-old *Verticillium* isolate VnAaOH1 cultures were flooded with 10 ml sterile distilled water and scraped with a sterile spatula to loosen mycelia and conidia. The resultant suspension was collected, vortexed, and passed through a milk filter (KenAg, Ashland, OH) to remove mycelial fragments. Conidial concentration was determined using a hemocytometer and adjusted to 10^7 conidia ml^{-1} by adding sterile distilled water to the suspension. Viability of resulting inoculum was determined by counting colony forming units from ten-fold serial dilutions on PEA + SN dishes. Inoculum exhibiting > 80% conidial viability was stored at 4°C until used in the field.

Data Collection and Treatments

One control and four *Verticillium* treatment plots were established as 20 × 50 m sampling units at each of the five sites, resulting in a total of twenty-five plots. As *Ailanthus* is a gap colonizing species, areas of high abundance are often localized to pockets of varying densities. Because our study objectives were to determine the effect of *Verticillium* on naturally occurring populations of *Ailanthus*, locations within stands that had large, reproductively mature *Ailanthus* and high densities were prioritized for *Verticillium* treatment plots. This resulted in control plots having lower *Ailanthus* stem densities with the objective of control plots to ascertain whether mechanical stem wounding alone ("water-inoculated", see description below) had an impact on the health of individual *Ailanthus* stems. Plots were spaced at a minimum distance of 2 to 3 km apart to ensure no plot-to-plot fungal contamination occurred.

We collected preinoculation data in the summer of 2014 with postinoculation surveys repeated in the summers of 2016, 2018, and 2020 across all plots. Within each 20 × 50 m plot, overstory data collection included tallies and diameter at breast

height (DBH) measurements of all trees ≥ 6 cm DBH; *Ailanthus* trees were tracked to follow individuals through time. Across the 20 × 50 m plots, we also tallied all *Ailanthus* saplings > 139 cm tall and < 6 cm DBH. Non-*Ailanthus* saplings were tallied by species in a 2 × 50 m belt transect. Within each of five 2 × 5 m subplots along the belt transect, *Ailanthus* seedling (50–139 cm tall) density and that of other species were recorded, as was the percent coverage of grasses, herbs and forbs, vines, shrubs, and tree seedlings for native and nonnative species (Figure S1).

In late May through early June 2015, ten randomly selected *Ailanthus* trees (≥ 6 cm DBH) within each *Verticillium* plot were inoculated with three evenly spaced injection sites at the base of the trunk using a hatchet. Each cut was injected with 1 ml of 1×10^7 conidia ml^{-1} using a sterilized 3 ml syringe (Kasson et al. 2014). To serve as a reference condition, at each control plot, five randomly selected *Ailanthus* trees were wounded with a sterile hatchet at three points at the stem base and treated with 1 ml of sterile distilled water per injection site ("water-inoculated").

To evaluate disease progression and mortality, *Ailanthus* trees were observed during the active growing season (June through October) each year from 2015 through 2020 using a modification of the *Ailanthus* wilt disease severity scale developed by O'Neal and Davis (2015a). The disease severity rating relates to symptom progression: 0 = healthy foliage; 1 = chlorotic and/or necrotic margins on leaves; 2 = slight wilt (<15% wilting foliage with no or slight defoliation); 3 = moderate wilt (15 to < 50% wilting foliage with no or slight defoliation); 4 = severe wilt [50% to 100% wilting foliage with no or slight defoliation [<15%]); 5 = moderate defoliation (15% to < 50%); 6 = severe defoliation (50% to 90%); 7 = very severe defoliation (90% to 100%) with epicormic sprouting; and 8 = dead. Other non-*Ailanthus* tree species, woody shrubs, and vines were also monitored for symptom development within inoculation areas.

To estimate light transmission to the forest floor, a hemispheric canopy photograph was taken at the center of each plot in summer 2015, 2017, and 2020. We used a Nikon Coolpix 8700 digital camera with a hemispherical lens levelled on a tripod at 1.5 m height. Gap Light Analyzer software (GLA, version 2.0, Simon Fraser University) was used to calculate the percentage of open sky.

Data Analyses

To address objective 1, quantifying the effectiveness of *Verticillium* to kill *Ailanthus,* we used generalized linear repeated measures models to quantify changes in overstory *Ailanthus* density (trees per hectare, TPH) and basal area (m^2/ha, BA), as well as the density of *Ailanthus* saplings, and seedlings/seedling sprouts over time (2014–2020). Values were compared using plot level means with model factors including treatment, site, and the interaction of treatment by site. Treatment and site were considered fixed factors within the model PROC GLM statement and each dependent variable was assessed for normality prior to analysis using PROC UNIVARIATE. To assess the relationship of tree size (DBH) to the probability it would survive, we compared *Ailanthus* status (dead or alive) in 2020 to its DBH in 2014 for stems that were not inoculated with *Verticillium* in treatment plots (n = 767 stems) using a binary logistic regression (PROC LOGISTIC). We did the same analysis to assess survival as a function of *Ailanthus* density (TPH) and BA. To assess disease progression in *Ailanthus* in treatment plots, we compared *Ailanthus* status (dead or alive) to the prior year's disease rating. We grouped disease scores into three general categories for analysis: none (no symptoms observed, severity scale 0), moderate (severity scale 1–3), and severe (severity scale 4–7). We constructed a logistic model assessing the binary response of dead (0) or living (1) at time 2 to the disease category at time 1 for all the time periods from 2015 to 2020. Analyses were conducted in SAS Institute version 9.4 software (Cary, NC) with significance determined at an alpha of 0.05.

To address objective 2, quantifying the rate of *Verticillium* spread, we mapped each *Ailanthus* overstory stem in 2019 using methods and the INTERPNT© program developed by Boose (1997) and Boose et al. (1998). Plot corners served as benchmarks, with additional benchmarks located at 5 and 10 m along the transverse sides of the plot to create a 3-4-5 right angle. Each *Ailanthus* tree was mapped based on the distance from DBH to the nearest neighbor or benchmark and DBH recorded. Distances for each plot were entered into the INTERPNT program and assessed for possible field errors. The final XY coordinates and annual status (alive or dead) was used to track the rate of spread within *Verticillium* plots. We used the package "spatstat" (Baddeley and Turner 2005) within the R programming environment version 4.1.1 (R Core Team 2021) to conduct spatial analyses on the coordinate data. The spatial distribution of *Ailanthus*

stems in 2014 was analyzed using Ripley's *K(d)* function on measured stem locations. The *K(d)* function counts the number of other observed points within a radius of *d* from each point. The observed measure of *K(d)* is compared against an envelope representing a null model of complete spatial randomness generated by ninety-nine simulations of a point pattern with an equal number of points to test for spatial clumping or uniform distribution across the range of *d* (Ripley 1998). For each inventory year, we calculated the mean distance between newly symptomatic stems and the two nearest previously symptomatic stems.

To address objective 3, determining the impact of fungal induced *Ailanthus* mortality on forest structure, tree regeneration, and native and nonnative understory vegetation, we used the same model approach that was employed for *Ailanthus* abundance. We used generalized linear repeated measures models to quantify changes in overstory non-*Ailanthus* TPH and BA, as well as the density of non-*Ailanthus* saplings, and seedlings/seedling sprouts over time (2014, 2016, 2018, and 2020). We also used the same approach to assess changes in the percentage of open sky and functional group coverage (woody species, herbaceous species, and nonnative invasive species) of the understory plant community. Values were compared using plot level means with model factors including treatment, site, and the interaction of treatment by site. Treatment and site were considered fixed factors within the model PROC GLM statement and each dependent variable was assessed for normality prior to analysis using PROC UNIVARIATE. Analyses were conducted in SAS Institute version 9.4 software (Cary, NC) with significance determined at an alpha of 0.05.

Results

Common overstory species at the sites reflected prior intensive land use with ash, cherry, elm, maple, yellow-poplar, and other non-oak-hickory species being abundant (Table 1). Although overstory *Ailanthus* contributed a large proportion of the basal area and stem abundance across the study sites, both its contribution and that of other species were highly variable. Prior to treatment, average overstory density of *Ailanthus* was 500 ± 306 TPH with a range from 140 to 1,510 TPH, and basal area per hectare averaged 8.9 ± 6.4 m^2/ha and ranged from 0.6 to 25.7 m^2/ha. *Ailanthus* mean TPH and BA were greater in the *Verticillium* wilt plots than in the control plots except for plots at the Wilds (Table 1). Most *Ailanthus* trees occupied the 10 to 25 cm diameter classes, with DBHs up to 45 cm and averaging 14.4 cm (Figure 2).

Table 1. Pretreatment 2014 density (trees/ha) and basal area (m^2/ha) (in parentheses) of trees ≥ 6.0 cm DBH, among the five study sites and the assigned treatment.

Site	Treatment	*Ailanthus*	Maple[1]	Elm-ash-cherry[2]	Yellow-poplar	Oak-hickory[3]	Other[4]	Total
Blue Rock	Control	150 (1.7)	140 (1.0)	190 (4.2)	0 (0.0)	20 (0.1)	400 (24.7)	900 (31.7)
	Wilt	440 (8.3)	238 (4.8)	95 (2.2)	40 (0.4)	18 (0.4)	230 (6.7)	1060 (22.7)
Marietta	Control	300 (13.7)	820 (9.7)	30 (1.8)	10 (1.0)	20 (0.3)	20 (1.4)	1200 (27.8)
	Wilt	413 (8.7)	295 (3.9)	218 (6.0)	23 (1.1)	3 (0.02)	115 (3.4)	1065 (23.4)
Perry	Control	180 (2.0)	200 (19.5)	160 (5.6)	40 (0.6)	210 (15.8)	110 (2.1)	900 (45.6)
	Wilt	658 (12.5)	73 (1.7)	158 (3.9)	13 (0.9)	13 (0.5)	295 (10.7)	1208 (30.1)
Tar Hollow	Control	140 (0.6)	20 (0.7)	70 (5.1)	140 (7.8)	0 (0.0)	120 (3.9)	490 (18.1)
	Wilt	550 (10.9)	88 (4.2)	98 (1.6)	123 (5.6)	15 (2.7)	50 (1.1)	923 (26.1)
The Wilds	Control	540 (9.5)	30 (0.1)	160 (5.7)	0 (0.0)	0 (0.0)	110 (2.3)	840 (17.6)
	Wilt	440 (8.2)	137 (1.7)	170 (3.2)	40 (4.2)	13 (0.6)	127 (3.2)	927 (21.1)
Total	Control	262 (5.5)	242 (6.2)	122 (4.5)	38 (1.9)	50 (3.2)	152 (6.9)	866 (28.2)
	Wilt	500 (9.7)	166 (3.2)	148 (3.4)	48 (2.4)	12 (0.9)	163 (5.0)	1036 (24.7)

Within the following groups, species are listed in decreasing order of abundance (trees/ha).

[1]sugar maple, red maple, box elder, silver maple.

[2]American elm, black cherry slippery elm, ash (*Fraxinus* spp.).

[3]northern red oak, white oak, hickory (*Carya* spp.), black oak, chestnut oak, scarlet oak, pignut hickory, shagbark hickory, mockernut hickory.

[4]black locust, shortleaf pine, sassafras, eastern white pine, yellow birch, black walnut, American beech, eastern cottonwood, flowering dogwood, blackgum, eastern redbud, sourwood, crabapple (*Malus* spp.), Osage-orange, American basswood, red pine, pawpaw, yellow buckeye, pitch pine, butternut.

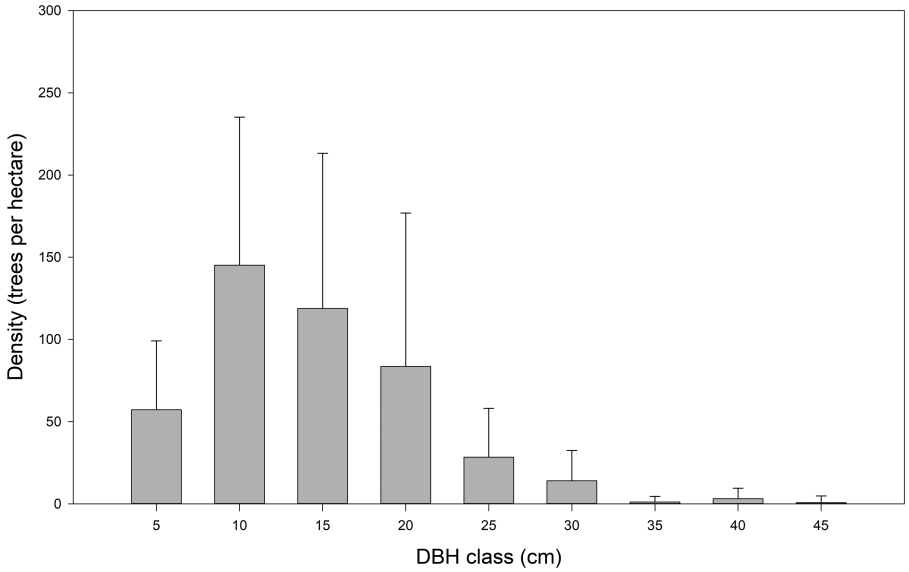

Figure 2. Pretreatment (2014) diameter distribution of overstory (>6 cm diameter at breast height [DBH]) *Ailanthus* trees across experimental sites in Ohio.

Verticillium Effectiveness on *Ailanthus*

For overstory *Ailanthus*, TPH and BA in *Verticillium* plots decreased sharply through time, although the overall effect of treatment was nonsignificant (Table 2; Figure 3). However, 3 to 4 years (2018–2019) after *Verticillium* inoculation, both overstory and midstory *Ailanthus* densities approximated that of the controls, which had been much lower pretreatment, with continued reductions in 2020 (Figures 3 and 4). For overstory *Ailanthus*, the control plots averaged 308 ± 158 TPH and 5.5 ± 5.8 m^2/ha in 2014 with relatively little change to 274 ± 110 TPH and 6.1 ± 5.8 m^2/ha by 2020. In contrast, on the *Verticillium* plots, *Ailanthus* averaged 548 ± 317 TPH and 9.8 ± 6.4 m^2/ha in 2014, decreasing sharply to 176 ± 135 TPH and 4.7 ± 3.8 m^2/ha by 2020. The findings are similar for the density of *Ailanthus* saplings (Table 2; Figure 4). *Ailanthus* saplings in the control plots averaged 354 ± 311 saplings/ha in 2014 and 242 ± 376 saplings/ha in 2020. On the *Verticillium* plots, *Ailanthus* saplings averaged 730 ± 657 saplings/ha in 2014 decreasing to 200 ± 270 saplings/ha in 2020. Although we observed reductions of *Ailanthus* in *Verticillium* wilt plots, this lack of treatment significance for *Ailanthus* trees and saplings is likely due to our unbalanced experimental design, with five control plots that are highly variable between sites with fewer *Ailanthus* in the control plots compared to the twenty *Verticillium* plots. Seedling-sized *Ailanthus* were also highly variable, with site being an important factor (Table 2; Figure 4). There was no significant change over time in the number of seedling-sized *Ailanthus* in either control or *Verticillium* plots (Table 2; Figure 4). *Ailanthus* seedlings averaged 720 ± 1301 and 421 ± 596 seedlings/ha in 2014 to 400 ± 616 and 411 ± 469 seedlings/ha in 2020 in control and *Verticillium* plots, respectively.

Mortality was greatest for inoculated *Ailanthus* stems, with over 60% of inoculated trees dead after 2016 and 86% dead by 2020 (Figure 5). By 2020, more than 60% of overstory noninoculated *Ailanthus* in the *Verticillium* plots had also died. However, sites were highly variable. Blue Rock (76%) and Marietta (78%) had the highest mortality of noninoculated stems in *Verticillium* plots by 2020, with the lowest mortality observed at The Wilds (35%; Figure 5). The Wilds also had the lowest (64%) mortality of *Verticillium*-inoculated stems, with the other sites ranging from 84% to 98% mortality. The Wilds was also the only site with water-inoculation mortality in a control plot.

Tree size (DBH) and *Ailanthus* density were significant predictors for survival of noninoculated *Ailanthus* in *Verticillium* plots. As DBH increased, the probability of survival also increased ($\chi^2 = 19.8$; $P < 0.01$; intercept = –1.3758, DBH = 0.0508; Figure 6). Fifty percent survival was estimated when noninoculated *Ailanthus* were over 27 cm DBH. The probability of survival for an individual stem of *Ailanthus* decreased as *Ailanthus* density increased ($\chi^2 = 4.6$; $P = 0.03$; intercept = -0.3165, TPH = –0.00041; Figure 6). There was no relationship detected for plot-level *Ailanthus* BA and survival. Visual symptoms of *Verticillium* were significant for predicting mortality the following

Table 2. Model effects for *Ailanthus* and non-*Ailanthus* species, including overstory, midstory, and understory density metrics and the percentage of open sky, comparing treatment (control versus *Verticillium*-inoculated), site, time, and their interactions using generalized linear repeated measures analysis. Model results are from between-subject effects for treatment, site, and their interaction and within-subject effects for time, treatment, site, and their interactions. Significance determined at an alpha of 0.05 is indicated in bold text.

Dependent Variable	Treatment	Site	Treatment × Site	Time	Time × Treatment	Time × Site	Time × Site × Treatment
Ailanthus altissima							
Overstory density	F = 0.5; *P* = 0.47	F = 0.4; *P* = 0.82	F = 0.3; *P* = 0.85	**F = 7.4; *P* < 0.01**	**F = 5.9; *P* < 0.01**	F = 0.1; *P* = 1.00	F = 0.4; *P* = 0.99
Overstory basal area	F = 0.5; *P* = 0.50	F = 0.6; *P* = 0.66	F = 1.0; *P* = 0.45	**F = 3.6; *P* < 0.01**	**F = 5.5; *P* < 0.01**	F = 0.2; *P* = 1.00	F = 0.4; *P* = 1.00
Midstory sapling density	F = 0.6; *P* = 0.44	F = 1.9; *P* = 0.18	F = 0.1; *P* = 0.97	**F = 5.6; *P* < 0.01**	F = 2.4; *P* = 0.08	F = 0.4; *P* = 0.97	F = 0.4; *P* = 0.85
Understory reproduction density	F = 0.2; *P* = 0.66	**F = 4.4; *P* = 0.02**	F = 2.0; *P* = 0.15	F = 2.0; *P* = 0.13	F = 1.5; *P* = 0.23	F = 1.7; *P* = 0.11	F = 1.3; *P* = 0.24
non-*Ailanthus* species							
Overstory density	F = 0.1; *P* = 0.70	F = 1.3; *P* = 0.34	F = 0.3; *P* = 0.90	F = 1.4; *P* = 0.26	F = 0.1; *P* = 0.94	F = 1.0; *P* = 0.50	F = 0.3; *P* = 0.99
Overstory basal area	F = 3.9; *P* = 0.07	F = 3.1; *P* = 0.06	F = 2.0; *P* = 0.16	F = 0.8; *P* = 0.51	F = 0.1; *P* = 0.96	F = 0.4; *P* = 0.95	F = 0.2; *P* = 0.99
Midstory sapling density	F = 1.1; *P* = 0.31	F = 3.0; *P* = 0.06	F = 2.8; *P* = 0.07	F = 1.2; *P* = 0.31	F = 1.4; *P* = 0.26	F = 1.4; *P* = 0.22	F = 0.8; *P* = 0.67
Understory reproduction density	F = 0.8; *P* = 0.40	F = 2.6; *P* = 0.09	F = 0.5; *P* = 0.73	F = 1.0; *P* = 0.40	F = 0.2; *P* = 0.89	F = 0.6; *P* = 0.81	F = 0.5; *P* = 0.90
Percent open sky	F = 4.5; *P* = 0.05	F = 0.5; *P* = 0.74	F = 0.1; *P* = 0.99	F = 1.8; *P* = 0.17	F = 1.4; *P* = 0.26	F = 0.4; *P* = 0.90	F = 0.4; *P* = 0.92
Woody understory coverage	F = 1.4; *P* = 0.27	F = 1.0; *P* = 0.43	F = 0.7; *P* = 0.62	**F = 4.6; *P* < 0.01**	F = 2.0; *P* = 0.13	F = 1.1; *P* = 0.38	F = 1.5; *P* = 0.18
Herbaceous understory coverage	F = 4.1; *P* = 0.06	**F = 5.5; *P* < 0.01**	**F = 3.1; *P* = 0.05**	**F = 11.4; *P* < 0.01**	F = 1.1; *P* = 0.37	**F = 2.7; *P* = 0.01**	F = 1.1; *P* = 0.43
Nonnative invasive understory coverage	F = 2.5; *P* = 0.14	F = 3.0; *P* = 0.06	F = 0.2; *P* = 0.93	F = 2.2; *P* = 0.11	F = 0.6; *P* = 0.59	F = 0.7; *P* = 0.74	F = 1.2; *P* = 0.35

year (χ^2 = 490.2; P < 0.001), particularly if the symptoms were severe (Figure 6). Trees with both no and moderate symptoms had over 90% survival the following year, but trees with severe wilt predicted a 42% chance of mortality. Spatial analyses of stem locations indicated that most plots had *Ailanthus* stems with a clumped distribution with a search radius of 2 m or greater, apart from two plots at Tar Hollow State Forest that were randomly distributed at all distances (Table S1). However, using stem mapped data, we were not able to determine rate of spread patterns within plots, likely due to small plot size and the high degree of heterogeneity of our plots and sites (Figure 7).

Community Response to *Verticillium* Wilt

Throughout our study we observed no wilting of non-*Ailanthus* species on *Verticillium* plots. Further, there were no significant treatment effects on the density (TPH) or BA of overstory non-*Ailanthus* (Figure 3; Table 2). Although there was a reduction in overstory *Ailanthus* in the *Verticillium* plots, there was no significant change in the percentage of open sky, which averaged < 8% each sample year after treatment (Figure 3; Table 2). There were also no significant changes to the density of saplings or seedling-sized reproduction of non-*Ailanthus* species either by treatment, site, time, or their interactions (Figure 4). The understory coverage of woody species, herbaceous species, and nonnative invasive plants was not affected by *Verticillium* inoculation and *Ailanthus* mortality (Table 2; Figure 8). The coverage of herbaceous plants was different between sites, with sites responding differently through time. The Wilds, Blue Rock, and Tar Hollow generally had higher coverage of herbaceous plants than the other study sites. Trends in herbaceous coverage were not generalizable through time nor by treatment (Figure S2). There were also no discernable changes in nonnative

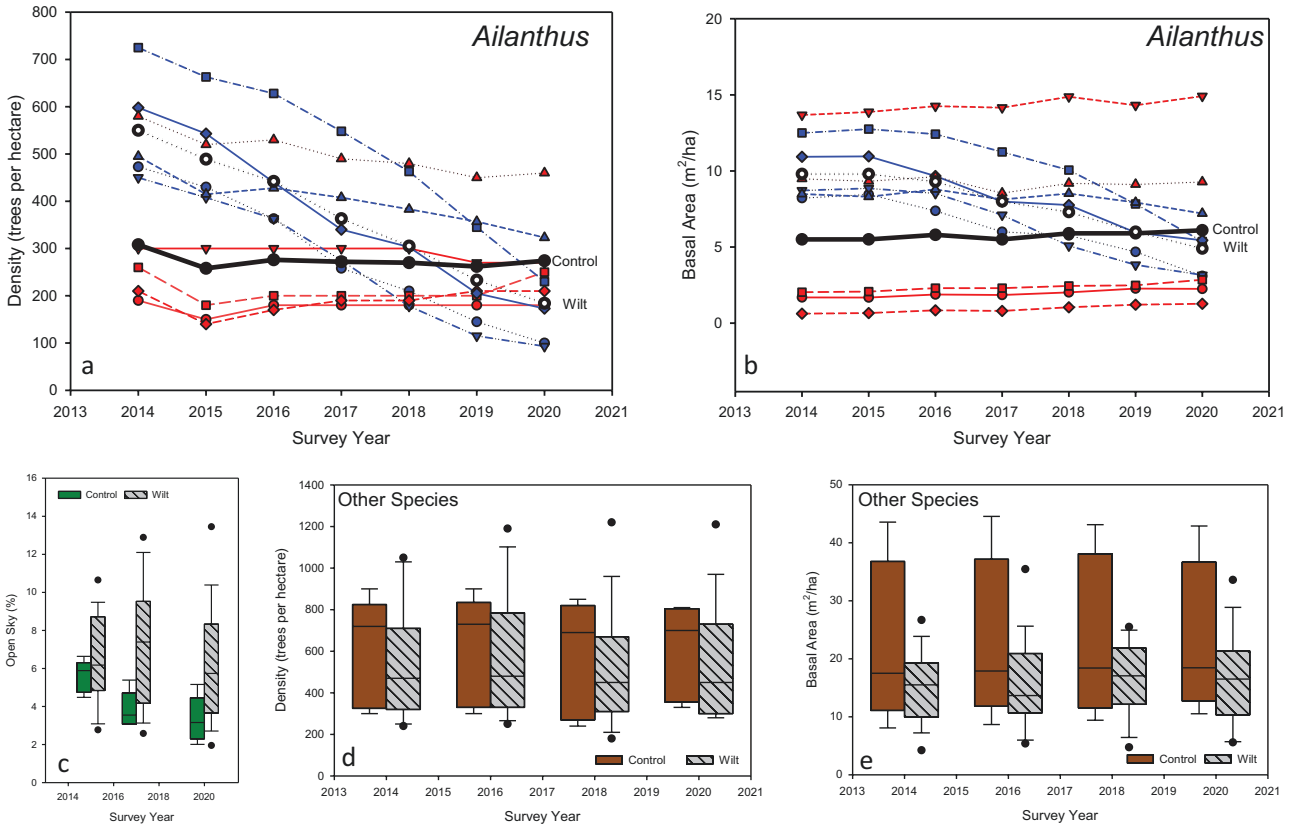

Figure 3. Change in the density (trees per hectare) and basal area (m²/ha) of overstory (> 6 cm DBH) *Ailanthus* (panels a and b) and non-*Ailanthus* species (panels d and e) and the percentage of open sky (panel c) by treatment. Blue symbols in *Ailanthus* panels represent *Verticillium* inoculated ("wilt") plots and red symbols represent water inoculated control plots. Treatment means for *Ailanthus* panels are represented by black symbols and lines (wilt plots = open circle, dashed line; control plots = closed circle, solid line). Box plots represent the interquartile range with solid line within the box indicating the median value. Whiskers represent 10th and 90th percentiles with all outliers indicated as points outside of the whiskers.

invasive species abundance. However, it is worth noting that similar to the differences seen between our control and treatment plots for *Ailanthus* abundance, treatment plots generally had greater nonnative invasive plant coverage than our control plots (32 ± 34% compared to 19 ± 21%, respectively).

Discussion

Managers need a variety of tools to manage widespread and locally abundant invasive plants. Based on a growing number of studies assessing the efficacy of *Verticillium* for controlling *Ailanthus*, this native fungus holds great promise for reducing *Ailanthus* abundance and impact through purposeful inoculation and natural spread. Understanding its use and applicability for forest management is an important next step, and this is the first study to show that the Ohio strain of *Verticillium* is effective for managing *Ailanthus* infestations. Our findings suggest (1) *Verticillium* greatly reduces overstory *Ailanthus* without increases in the abundance of *Ailanthus* saplings or reproduction; (2) with the inoculation of a few individuals, *Verticillium* is able to spread and kill over 50% of noninoculated *Ailanthus* trees within 4 years, with greater effectiveness in patches with high densities and smaller individuals; (3) there is no observed effect of the *Verticillium* treatment on non-*Ailanthus* species in a "typical" highly disturbed, *Ailanthus*-invaded forest; and (4) because *Ailanthus* mortality is gradual, there were few changes in understory conditions, including in canopy openness or response from other species, especially nonnative invasive plants.

The use of native biological control agents may cause undesired effects; however, their risk is often transient when compared to the potential risks associated with nonnative control agents (De Clercq et al. 2011). Recent evidence is mounting that long established and widely distributed invasive plants are experiencing reductions in growth and performance as a result of native resident pathogen accumulation (Flory et al. 2018). This phenomenon may be attributed to

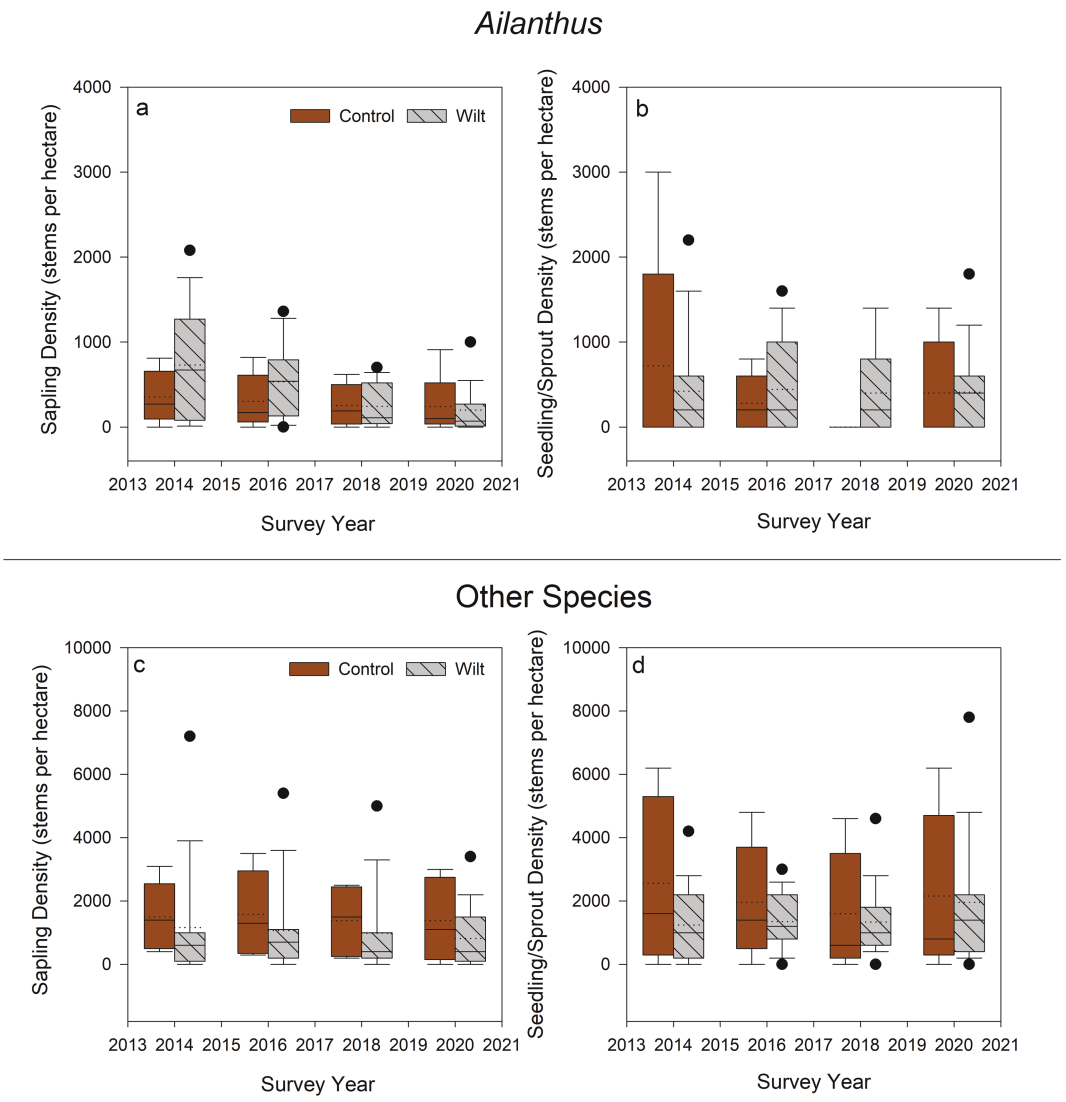

Figure 4. Density of saplings (<6 cm DBH and > 1.4 m tall) and seedlings (<1.4 m tall) for *Ailanthus* (upper panels) and non-*Ailanthus* species (lower panels) by survey year in control and *Verticillium* wilt plots. Box plots represent the interquartile range with the solid line within the box marking the median and the dotted line representing the mean. Whiskers represent 10th and 90th percentiles with all outliers indicated as points outside of the whiskers.

the recently observed impact of native *Verticillium* on *Ailanthus*. *Verticillium* is not alone in its proposed use as a native fungal biocontrol (Kasson et al. 2014). In North America, native fungal species have been used to control unwanted persimmons (*Diospyros virginiana* L.) (Wilson 1965), red alder (*Alnus rubra* Bong.) and aspens (*Populus* spp.) (Harper et al. 1999), and the nonnative, invasive Brazilian peppertree (*Schinus terebinthifolius* Raddii) (Shetty et al. 2011). With increasing evidence on the efficacy of *Verticillium* to control *Ailanthus* with minimal nontarget impacts to native or agricultural species (Schall and Davis 2009b), scientists are compiling and submitting evidence to register *Verticillium* as a biocontrol agent to the US Environmental Protection Agency (Brooks et al. 2020d). With adequate funding to complete the EPA registration process, the product should be commercially available in the United States within 3 to 4 years (Brooks et al. 2020b). In Austria, an approved herbicide developed using a *Verticillium nonalfalfae* isolate is commercially available as *Ailantex*®, (Dubach et al. 2021). It is important to note that other biological control agents are being tested for the control of *Ailanthus*, with over sixty natural enemies identified. An eriophyid mite, *Aculus mosoniensis*, has been shown to have high specificity for *Ailanthus* (Marini et al. 2021).

Native biological herbicides offer an option to control nonnative invasive plants, but their use will

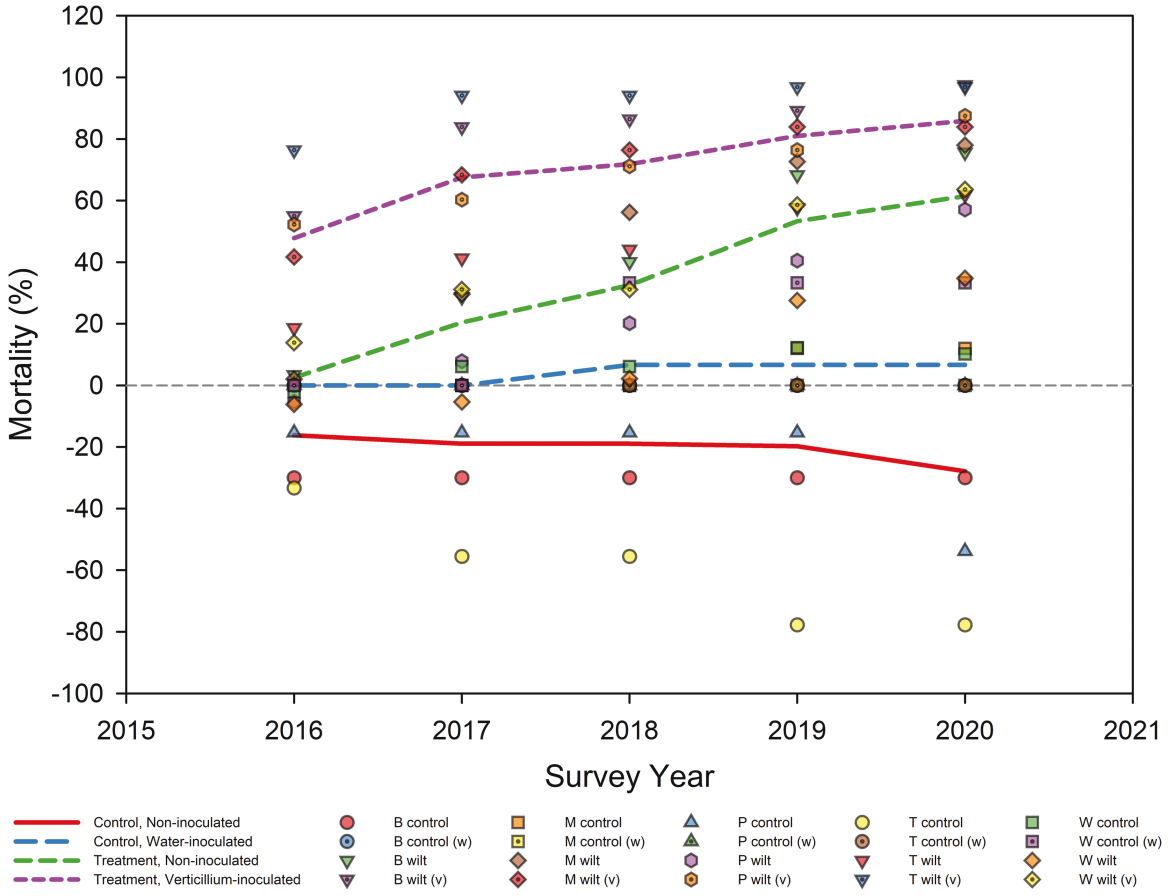

Figure 5. Cumulative percentage of *Ailanthus* overstory (>6 cm DBH) mortality by year compared to pretreatment data collected in 2014 by plot treatment type (control and *Verticillium* wilt plots) and by stem inoculation status (noninoculated, water- inoculated, or *Verticillium*-inoculated) through the study period. Lines represent overall means by treatment and inoculation status and symbols represent site-level means (w = water-inoculated; v = *Verticillium*-inoculated).

require framing their efficacy separately from that of chemical herbicides. Chemical herbicides are evaluated for their ability to kill individual stems and reduce re-invasion. For example, chemical herbicides are highly effective at killing *Ailanthus* (DiTomaso and Kyser 2007), but depending on the chemical formulation and application method used, can also result in nontarget impacts to neighboring non-*Ailanthus* trees (Lewis and McCarthy 2008). However, the intended outcome of biological herbicides is to reduce the impact of the invasive species by allowing for other species to have a competitive advantage (Ghosheh 2005). Results from our study show *Verticillium*-induced mortality from natural spread is not a rapid process and that stem size and infestation density are important. However, for stems that were directly inoculated with *Verticillium*, mortality exceeds 65% within a year of inoculation and nears 90% within 6 years. Comparably, in other studies assessing *Verticillium* control of *Ailanthus*, density appears to be greater and stem size smaller than the *Ailanthus* populations at our study sites (Maschek and Halmschlager 2017, Brooks et al. 2020d). These demographic differences may contribute to the reduced mortality and slower disease progression found in our study. However, disease spread through the soil might be more limited than spread by root contact (Brooks et al. 2020a, Dubach et al. 2021), leading to denser stands of *Ailanthus* having greater transmission between stems. Similar to our results, a recent field evaluation of naturally infected *Ailanthus* in Virginia indicates that even with *Verticillium* presence and *Ailanthus* declines for over 5 years, complete eradication of localized populations did not occur (Brooks et al. 2020a). Land use history may play a role in the efficacy of *Verticillium* to control *Ailanthus* and may have contributed to some of the differences in mortality observed. In particular, iron hyperaccumulation from strip mining may increase the tolerance of *Ailanthus* to *Verticillium* (Wickert 2019) and may be attributed to the reduced efficacy of *Verticillium* at The Wilds.

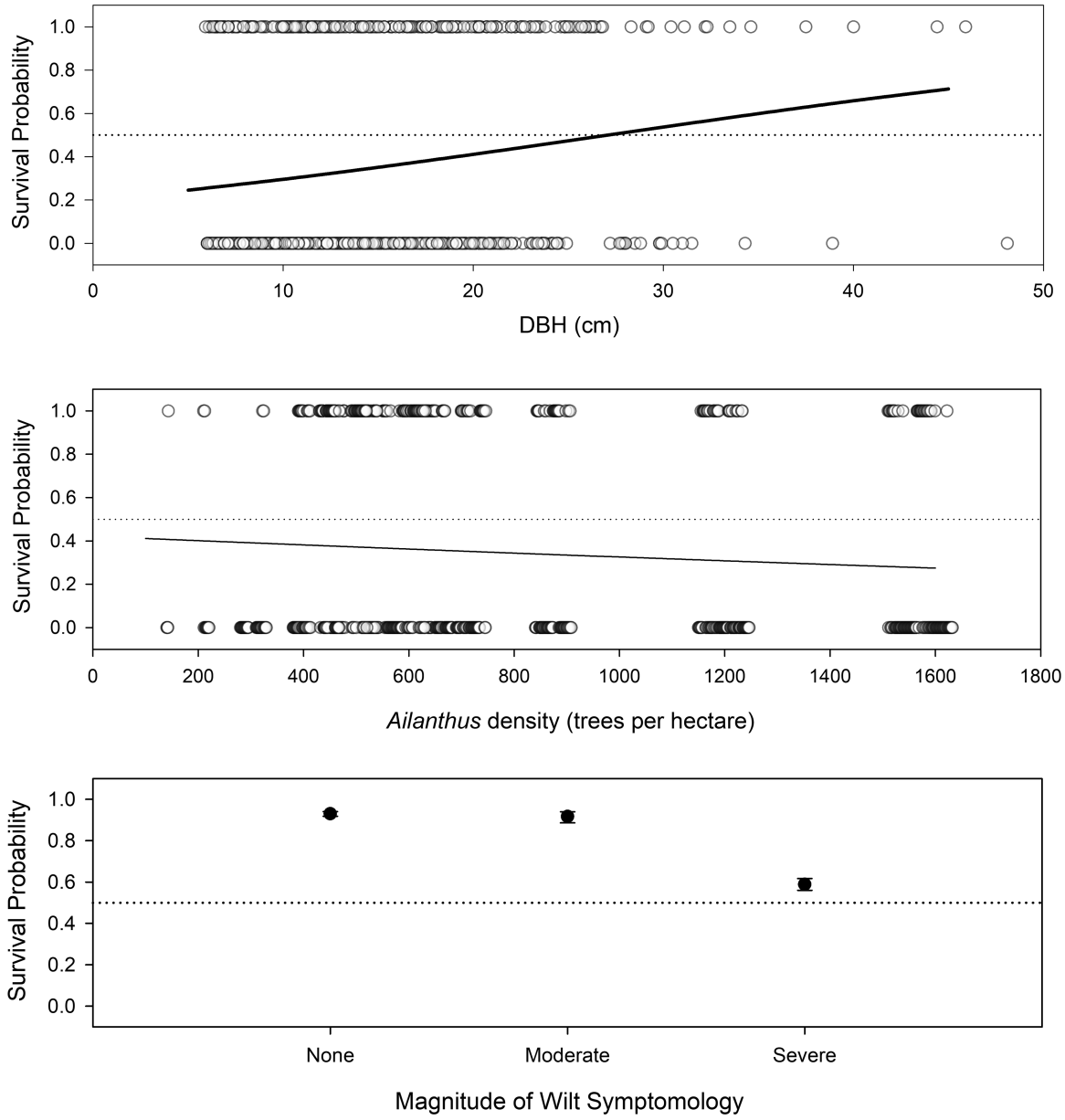

Figure 6. Probability of survival for *Ailanthus* trees exposed to *Verticillium* through natural spread in treatment plots based (1) DBH (upper panel), (2) plot density of *Ailanthus* on a per hectare basis (TPH; middle panel), and (3) on prior year observed symptoms (lower panel). Open circles represent raw data points, with plot-level TPH jittered to reduce overlap. Reference lines are plotted at 50% survival.

Following invasive plant management, the response of the residing plant community can range from positive to negative and can depend on the control tactic, timing, and duration along with effects of site, species, and environmental attributes. Our findings are similar to that of Harris et al. (2013) in central Pennsylvania, where reductions in overstory *Ailanthus* due to *Verticillium* did not result in significant changes to the understory community. However, the slow rate of spread of *Verticillium* with minor changes in light transmittance, likely due to *Ailanthus*' narrow and sparse crown shape and gap-infilling by adjacent trees, reduces the response of cooccurring invasive plants or the secondary invasion by other invasive plants. Our sites varied in land-use and disturbance histories and plant communities, especially in the degree of invasion by other nonnative invasive species. On sites where native forests are desired but understories lack the necessary advanced reproduction and have minimal competition from other invasive plants, treatment of

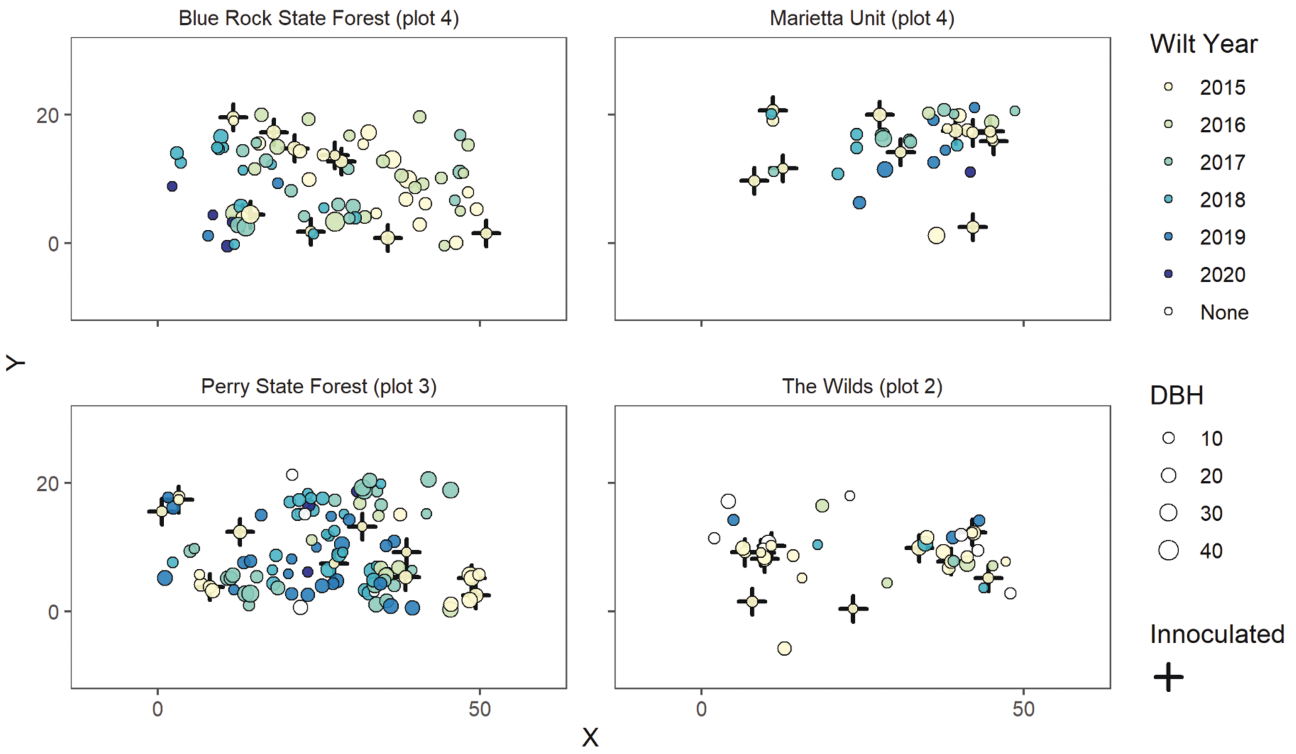

Figure 7. Example stem plots documenting *Verticillium* induced symptoms on *Ailanthus*. A circle represents an individual overstory (>6 cm diameter at breast height [DBH]) *Ailanthus*, with circle size corresponding to DBH (larger the circle the greater the DBH). Circle color indicates year that a disease severity rating of two or higher was recorded. Unfilled circles were recorded as unaffected in all survey years. Circles with a plus inside (+) were among the ten randomly selected, *Verticillium*-inoculated stems in 2014; presence of symptoms in other *Ailanthus* stems is assumed to be through natural spread.

overstory *Ailanthus* with *Verticillium* may provide opportunities for artificial regeneration. However, many of the sites in our study, like many disturbed forests in the eastern United States, will require managing not only *Ailanthus* but also the other invasive shrubs, forbs, and grasses that further impede restoration efforts.

Management Considerations

Verticillium may offer the opportunity to develop control strategies and prioritization zones across a landscape for *Ailanthus* in combination with other treatments. Strategies that distribute efforts over time and space by integrating patch size, demographic information (e.g., age structure, number of fruiting individuals, reproduction strategy) and landscape position may increase effectiveness and reduce cost (Eppinga et al. 2021). *Verticillium* would have the potential for long-term reductions in *Ailanthus* abundance where patch sizes are large and the age structure is relatively young. Further, a significant degree of spatial clumping of *Ailanthus* stems occurred at our study sites and has been reported elsewhere (Brooks et al. 2020a). Because *Verticillium* appears to spread more effectively through root-grafting than by soil, this spatial clumping may limit spread to disconnected *Ailanthus* clumps. Therefore, the purposeful inoculation of *Verticillium* stems should be well distributed across a population to ensure movement of the fungus to uninfected individuals in distinct clumps (Brooks et al. 2020a). Subsequently, remote or aerial detection of satellite patches or large, fruiting females could be prioritized for chemical treatment (Rebbeck et al. 2015). It is recommended that *Verticillium* inoculation occur in the spring, as high temperatures may impede fungal colonization or even kill *Verticillium*, and late season applications could be affected by early frost events that may reduce hyphal growth, conidial viability, and ultimately limiting fungal spread (Isaac 1949, Galanopoulos and Tribe 1974, Maschek and Halmschlager 2017). Unfortunately, the spotted lanternfly (*Lycorma delicatula*), a phloem-feeding species with a high preference for *Ailanthus* that threatens both natural and agricultural systems, was not found to spread *Verticillium* in *Ailanthus* populations, at least in a laboratory setting (Brooks et al. 2020c).

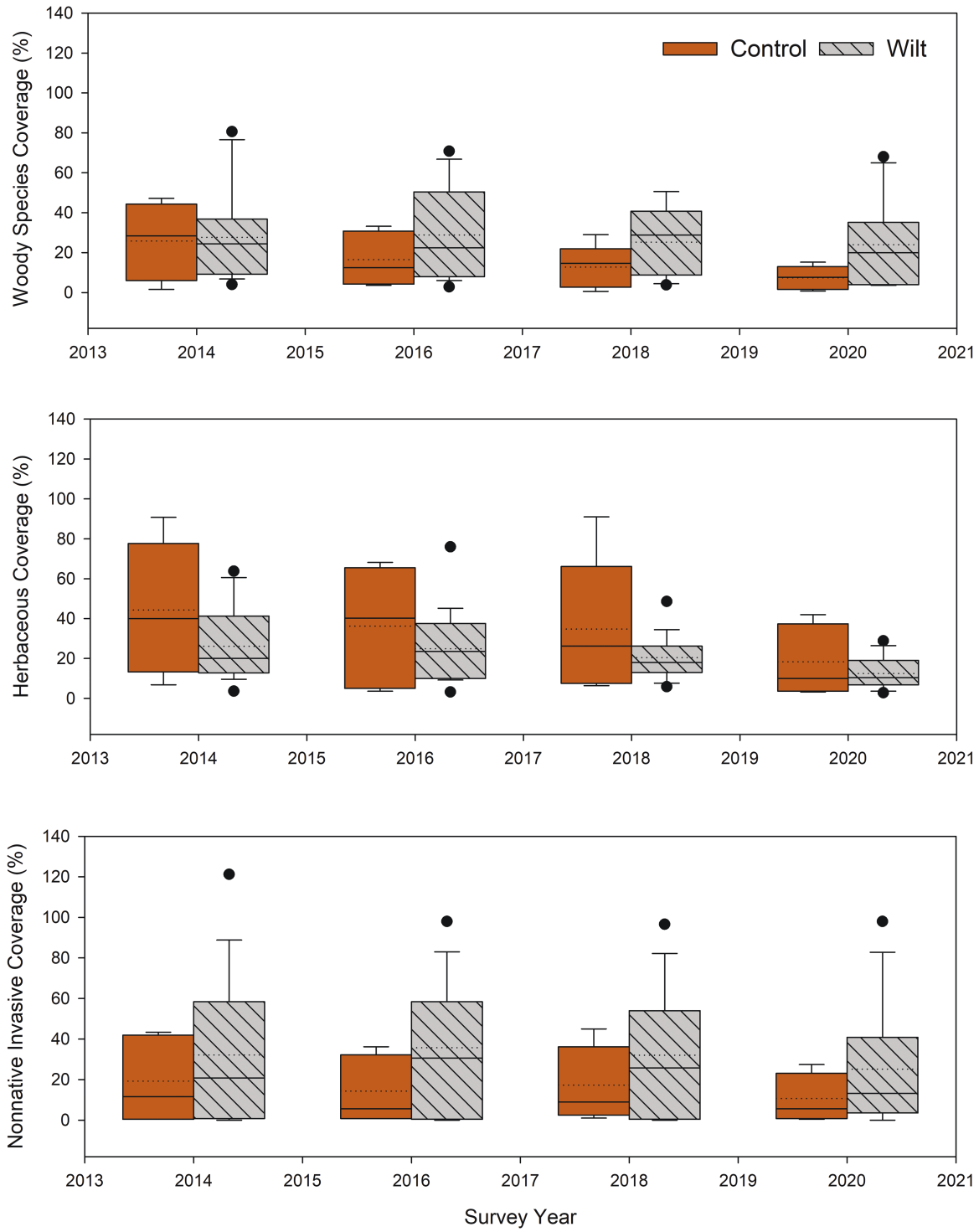

Figure 8. Coverage by functional group for control and *Verticillium* wilt plots recorded in 2014, 2016, 2018, and 2020. The 2014 survey year represents preinoculation treatment coverages. Box plots represent the interquartile range with the solid line within the box marking the median and the dotted line representing the mean. Whiskers represent 10th and 90th percentiles with all outliers indicated as points outside of the whiskers.

Minimizing the impact of *Ailanthus* from a forest stand is often only the first phase of restoration on highly disturbed forests sites. Restoration requires both the eradication of invasive nonnative plants *and* restoring a fully functioning and diverse native plant community (D'Antonio and Meyerson 2002). However, planting desirable native species is often necessary when invasive plants have long occupied a site and land use history has depleted the seed bank (Collier et al. 2002). Reintroduced propagules of native species

may reduce reinvasion or secondary invasions by other nonnative plants (Shea and Chesson 2002, Kettenring and Adams 2011). Further, the integration of biological controls and native seeding may have an additive effect on reducing invader abundance (Cutting and Hough-Goldstein 2013). *Ailanthus* is predominately found on relatively mesic disturbed forest sites (Rebbeck et al. 2017). Enhancing the competitiveness of native associates that grow well on these sites through management (Murphy 2005, Pile et al. 2019), including maples, yellow-poplar, or shrubs such as spicebush (Lindera benzoin [L.] Blume), may reduce growing space for further plant invasions. Also, the reintroduction of species such as disease-resistant American elm may serve to enhance forest restoration projects by helping to exclude the invasion of nonnative plants by rapidly capturing available resources (Lake et al., 2014).

The long-term fate of *Verticillium* for controlling *Ailanthus* is unknown. However, the persistence of *Verticillium* in the soil appears to be limited without a viable population of *Ailanthus* and may result in reinvasion (Brooks et al. 2020a). We followed *Ailanthus* response for 6 years after treatment and did not see increases in the density of *Ailanthus* reproduction, which is often a concern with this species' aggressive capacity to root-sucker following management and to establish from seed after canopy disturbance. Many of the *Ailanthus* populations in our study sites are in actively managed timberlands. The effect of any operation (thinning or regeneration harvests) or other disturbance agents and the efficacy of *Verticillium* to control subsequent local increases in density or spread of *Ailanthus* will require further study.

Supplementary Materials

Supplementary data are available at *Journal of Forestry* online.

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