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The bioherbicide *Verticillium nonalfalfae* effectively removes tree-of-heaven (*Ailanthus altissima*) but leaves many other non-native plants

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Abstract

Tree-of-heaven [*Ailanthus altissima* (Mill.) Swingle] readily exploits disturbances, grows quickly into dense monocultures, and suppresses native plant species. The vascular wilt pathogen, *Verticillium nonalfalfae*, native to the eastern United States, has been proposed as a biocontrol agent for the invasive *A. altissima*. Studies consistently demonstrate the safety and efficacy of the bioherbicide, but they also note that the selective nature of the fungus does not preclude other invasive plants that commonly co-occur with *A. altissima* from occupying the site. We quantified the standing plant community and seedbank at several sites across Virginia 5 yr after inoculation with *V. nonalfalfae* to understand which species are present or being naturally recruited. *Ailanthus altissima* remained dominant in untreated areas but was nearly eradicated from the treatment plots. Other non-native species made up a large portion of the plant community and seedbank across all study areas, with no differences in their respective cover and count between treatments. While variability in plant community composition is high and site-specific context is important for establishing effective management strategies, planting native species and mitigating other invasives will be crucial to ensuring native species successfully establish in bioherbicide-treated areas.

Introduction

Several recent studies have demonstrated the safety and efficacy of using *Verticillium nonalfalfae* (*Plectosphaerellaceae*), as a bioherbicide to treat and kill tree-of-heaven [*Ailanthus altissima* (Mill.) Swingle, Simaroubaceae] (Brooks et al. 2020b; Pile Knapp et al. 2022; Schall and Davis 2009a). The native, soil-borne fungal pathogen induces a vascular wilt disease, often killing its host within 2 to 4 mo, spreading to neighboring stems through clonal and grafted roots (O'Neal and Davis 2015), and preventing regeneration for several years following inoculation (Brooks et al. 2020a; Kasson et al. 2014). Natural infections of *A. altissima* by *V. nonalfalfae* have been reported in three U.S. states (Rebeck et al. 2013; Schall and Davis 2009a; Snyder et al. 2013) and two European nations (Maschek and Halmshlager 2017; Moragrega et al. 2021), so augmentation biocontrol is a promising management strategy across much of *A. altissima*'s invaded range, particularly because it poses very little or no risk to other native woody species (Kasson et al. 2015; Schall and Davis 2009b).

Ailanthus altissima is typically only one of several non-native plants in heavily invaded sites characterized by fewer plant species (Constán-Nava et al. 2015; Motard et al. 2011) and lower native plant diversity (Vilá et al. 2006). Invasion by *A. altissima* is also associated with increased non-native plant diversity and decreased ratios of native to non-native plant cover and species richness, effects that worsen over time (Brooks et al. 2021). Interestingly, selective removal of *A. altissima* has shown some variability in its effects on the local plant community. Harris et al. (2013) and Pile Knapp et al. (2022) both found that while *A. altissima* was effectively eradicated from sites treated with *V. nonalfalfae*, understory vegetative communities did not change; percent cover by life-form and origin in inoculated areas did not differ from the controls several years postinoculation. In a shorter-term study, Burch and Zedaker (2003) treated *A. altissima* with a chemical herbicide and observed an understory shift toward native species dominance in both established plots and the surrounding area. Meanwhile, native woody species appear able to regenerate in the understories of both *A. altissima*-dominated and *V. nonalfalfae*-treated areas (Kasson et al. 2014; Kowarik 1995) despite an herbaceous component typically rife with invasive species (O'Neal and Davis 2015).

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Management Implications

Ailanthus altissima (tree-of-heaven) is a cosmopolitan, highly invasive tree species. Because it aggressively suckers, attempts to remove it mechanically can exacerbate an invasion, while chemical treatments often require repeated applications to be effective. Fortunately, *A. altissima* is susceptible to a native fungal pathogen, *Verticillium nonalfalfae*, which causes a vascular wilt disease that can spread to neighboring stems through shared roots and prevent regeneration. *Verticillium nonalfalfae* has been petitioned for federal approval as a bioherbicide in the United States, but its efficacy in restoration efforts can be limited, because stands of *A. altissima* tend to be composed of several non-native plant species. In these cases, removal of the dominant tree is not sufficient to allow for the natural succession of a native plant community. More active measures should be incorporated into restoration of these invaded sites, including mitigation of other invasives, planting native species, and recurring follow-up to ensure success.

Here, we test the outcomes of natural regeneration following inoculation of *A. altissima* with *V. nonalfalfae* using a 5-yr controlled experiment. Our approach is distinguished by the variety of sites over a large geographic area and an analysis of viable propagules. Specifically, we quantified the standing vegetation and the seedbank of areas across Virginia that remain dominated by *A. altissima* and plots that were previously treated with the bioherbicide. We expected to find no differences in vegetative and propagule communities between treatments, except for the dramatic reduction in the single targeted tree species. Put another way, we predicted these heavily invaded sites would maintain a large component of non-native species despite the removal of *A. altissima*.

Materials and Methods

Vegetative Surveys

This study evaluates plant and seedbank communities at six sites around Virginia that partially comprised a prior biocontrol study: in 2017, Brooks *et al.* (2020b) established 0.04-ha plots in stands across Pennsylvania and Virginia that were dominated by *A. altissima*. They inoculated treatment trees with *V. nonalfalfae*, which resulted in extensive mortality and near eradication of *A. altissima* from the treated areas while it continued to dominate control plots. We revisited the six Virginia sites in 2022 (Figures 1 and 2). All six *V. nonalfalfae* treatment plots were available for surveying, but we could only use three of the six control plots (two were infected with *V. nonalfalfae* at an unknown time, and one was destroyed in a windstorm). The six Virginia sites are equally distributed across the mountains and Piedmont, whereas the three sites with paired control and treatment plots include two in the mountains and one in the Piedmont.

We established a 41-m² (6.4 by 6.4 m) quadrat at the center of each plot and identified all vascular plants within it to the lowest taxonomic level possible by first subdividing into four equal sub-quadrats, inventorying each in immediate succession, and then combining cover data. We considered foliage and plant canopies (or portions thereof) under 2 m in height as part of the understory, including trees and shrubs <2.5-cm diameter at breast height, and visually estimated the percent cover of each taxon. This cutoff was

established to ensure we captured the entire understory while maintaining precise cover estimates.

Seedbank

We used a seedling emergence method to study the viable propagules in the seedbank (Brooks *et al.* 2021; Brown 1992) by using a trowel to excavate 0.33 L of soil (8 by 8 by 5 cm) across three random locations within each quadrat. This was done on two occasions, one in July 2022 and the second in March 2023 to reflect both the transient and persistent seedbanks, respectively (Csontos 2007; Mahé *et al.* 2021), and pooled them together. We stored seedbank samples at 4 C for up to 2 wk and mixed them with 2 L of potting soil (0.14-0.11-0.88 N-P-K, Sta-Green Moisture Max® Potting Mix) (Sta-Green Lawn & Garden, Mooresville, NC) and 60 ml of activated charcoal (Soil D-Tox™) (Charcoal House, Crawford, NE) to mitigate potential allelopathy (Inderjit, 2003) from residual ailanthone (Heisey 1996). We spread the mixture evenly in 25 by 51 cm trays, monitored them for 24 wk in a greenhouse with daily irrigation, then identified and removed seedlings as early as possible to prevent competition. We confirmed identification for both the seedbank samples and field surveys using the Flora of Virginia (Weakley *et al.* 2020).

Species Resolution

We were able to identify most plants to species. However, sedges (*Carex* sp.) were an exception, because none were reproductive during surveys. Their origin is thus unclear, and we excluded them from analysis. All sedges that germinated from seedbank samples did reach reproductive maturity, so we identified them to species and incorporated them into statistical analyses. Similarly, we identified *Viola* spp. and *Sanicula* spp. only to genus in the field due to a lack of distinctive features, but we were able to further identify *Viola* spp. in the greenhouse to species (no *Sanicula* spp. emerged from seedbank samples). We included both genera in all analyses, because we could determine their nativity.

Statistical Analyses

To analyze the vegetative and seedbank communities, we constructed generalized linear mixed models using the GLMMTMB package (Brooks *et al.* 2017) in RStudio v. 4.3.2 (Posit Team 2023). We transformed percent cover and seedling count data using the Hellinger method (Legendre and Gallagher 2001) and used a gamma distribution for analysis. Richness data were analyzed with a negative binomial distribution. We compared two models for each of the four response variables using Akaike information criterion corrected for small sample sizes (AICc) and evidence ratios (ER): one model included the fixed effects of inoculation (control vs. *V. nonalfalfae*), origin (native vs. non-native), and life-form (woody vs. herbaceous), their interaction, and site as a random factor; the second model excluded life-form and contained all other factors. We then evaluated selected models using the DHARMA package (Hartig and Lohse 2022), which includes tests for overdispersion, outliers, residual versus predicted, and Kolmogorov-Smirnov goodness of fit. We used a priori contrasts to evaluate the interactive effects of interest, with a multivariate *t*-distribution adjustment for multiple comparisons. We excluded the three unpaired *V. nonalfalfae* plots from statistical analyses, leaving only three control and three *V. nonalfalfae* plots that were paired by site. We used permutational analysis of variance (PERMANOVA) to compare the three excluded *V. nonalfalfae* plant communities to the three we tested to investigate whether they were



Figure 1. A stand of *Ailanthus altissima* 5 yr after inoculation with *Verticillium nonalfalfae*. Visible beneath an opening canopy is a dense understory, including several invasive species. Mixed among the dead and diseased *A. altissima* stems in the foreground are *Liriodendron tulipifera* L. (tuliptree) and the non-native *Paulownia tomentosa* (Thunb.) Siebold & Zucc. ex Steud (royal paulownia).

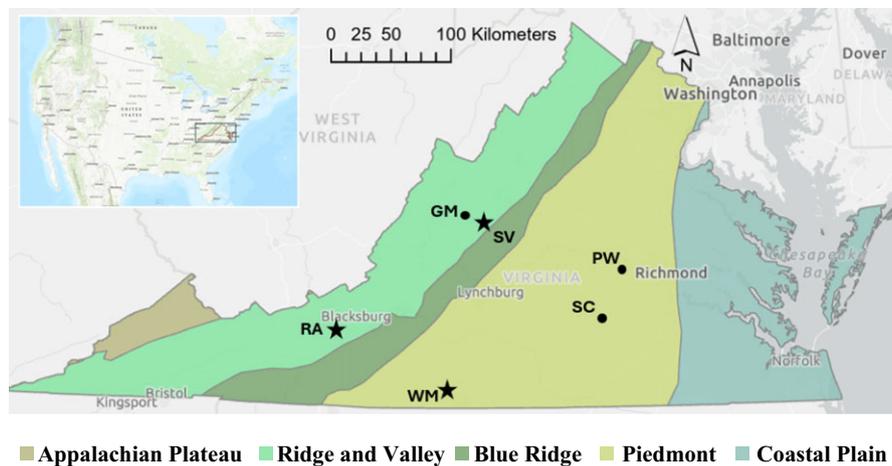


Figure 2. Study site locations across physiographic regions of Virginia (Esri 2023; Hitt 2023). Site codes correspond to Brooks et al. (2020b). Sites marked with a star had paired control and *Verticillium nonalfalfae*-inoculated plots. Sites marked with a circle had no available control plots and were excluded from formal analysis, but their inoculated plots were inventoried and compared with those of the starred sites. GM and WM are located in wildlife management areas; PW and SC are located in state parks; RA is on a U.S. Army installation; and SV is a Virginia Tech Agricultural and Research Extension Center.

similar enough that management implications derived from our analysis may be considered relevant to the untested group.

Results and Discussion

We find no differences in origin or treatment for both the percent cover of standing vegetation and count of germinated propagules in the seedbank assay (Figure 3). Species richness for the seedbank is also unchanged. However, there is some evidence that native species richness is higher than that of non-native species in the control plots ($P = 0.015$), and this distinction is not shared by plots

inoculated with the bioherbicide. For all four response variables, AIC and ER indicated that a simpler model without life-form better fits the data (Supplementary Table 1).

Overall, we included 123 unique taxa in our analysis, only 13 of which were shared between standing vegetation and the seedbank. We analyzed 73 taxa from the field, with 71 identified to species and 2 to genus. Sixty-three total taxa germinated from the seedbank, 100% of which were identified to species level (Supplementary Tables 2a, 2b, and 3). Notably, while some understory *A. altissima* was found in treatment plots, no overstory stems remain, and recruitment appears to be suppressed for at least 5 yr postinoculation

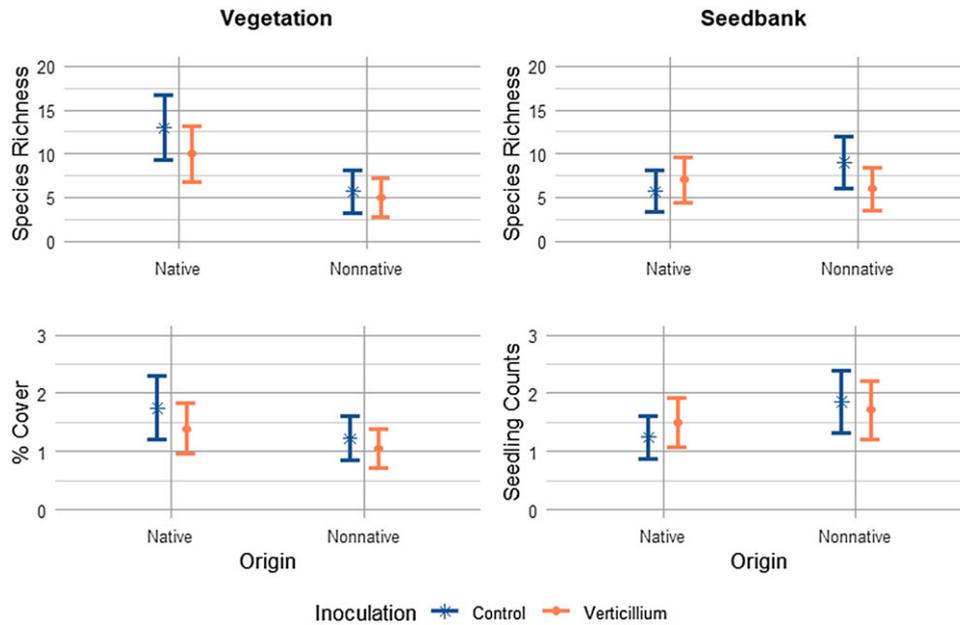


Figure 3. Native and non-native species richness, vegetative cover, and seedlings germinated for control and *Verticillium nonalfalfae*-inoculated plots. Vegetation includes observations in the field, while the seedbank consists of greenhouse-germinated samples. Raw percent cover and seedling counts were Hellinger transformed. Confidence intervals represent predicted means \pm 1 SD.

(Supplementary Figure 1). Our PERMANOVA results indicate that there is no difference between the plant communities ($P = 0.800$) and seedbanks ($P = 0.600$) of the three *V. nonalfalfae*-inoculated plots included in the analysis compared with the remaining three treatment plots that were left out.

This study demonstrates that natural regeneration alone does not effectively restore native plant communities in sites invaded by *A. altissima* and treated with *V. nonalfalfae*. Five years after bioherbicide application, standing vegetation and seedbanks remain relatively unchanged between treatment and control plots. It is well established that these sites have large contingents of invasive species (Brooks *et al.* 2021; Motard *et al.* 2011; Vilà *et al.* 2006); therefore, a limited treatment such as a host-specific bioherbicide is unlikely to facilitate the recovery of diverse, native plant communities. Moreover, taxa in the seedbank overlapped by a mere 10.6% with standing vegetation, which is lower than what has previously been found (Brooks *et al.* 2021), and contained many non-native species. We believe the seedbanks of our study areas are markedly reflective of site history and dispersal into *A. altissima* stands. All six sites are either within or bordering heavily disturbed areas, such as forest clear-cuts, agricultural fields, and other anthropogenic activities. Many ruderal species may have established immediately following a past disturbance or could be continuing to disperse into the study areas from nearby.

Our findings support several other studies conducted in *V. nonalfalfae*-treated areas (Harris *et al.* 2013; Kasson *et al.* 2014; Kowarik 1995; Pile Knapp *et al.* 2022), and similar to O'Neal and Davis (2015), there is a substantial presence of invasive herbaceous species across our treatments. Unlike Burch and Zedaker (2003), however, there is no increased cover of native species in our study areas. It is important to note that Burch and Zedaker (2003) found the shift in herbaceous species to be independent of their treatment. This may partially be an artifact of phenology, because their pre- and posttreatment surveys were not conducted during the same season. Both Harris *et al.* (2013) and Pile Knapp *et al.* (2022) documented slower than expected rates of

disease progression and variable rates of pathogen spread in their studies. They surmised that shifts in resource availability due to the death of *A. altissima* proceeded gradually enough that changes in the understory community were minimal.

It is likely that site-specific context is pertinent to employing the bioherbicide and initiating restoration projects as a general rule. For example, we observed six species that cover 20% or more of at least one inventoried quadrat: *Elaeagnus umbellata* Thunb. (autumn olive), *Microstegium vimineum* [(Trin.) A. Camus] (Japanese stiltgrass), *Phytolacca americana* L. (common poke-weed), *Rosa multiflora* Thunb. (multiflora rose), *Rubus phoenicolasius* Maxim. (wineberry), and *Verbesina occidentalis* [(L.) Walter] (yellow crownbeard). Two of these (*P. americana* and *V. occidentalis*) are common native herbaceous species, three are considered invasive by the USDA (*E. umbellata*, *M. vimineum*, and *R. multiflora*) (USDA n.d.), and *R. phoenicolasius* is an introduced species considered to be a noxious weed in multiple states (USDA-NRCS n.d.). Interestingly, the four non-native species listed rarely co-occur in large quantities at our study sites, so control techniques for each site would likewise differ. One study site is particularly illustrative of this fact: *E. umbellata* dominates both plots, measuring 100% cover in each quadrat. However, the shrub was not identified at any other location, so an adaptive strategy must be applied to mitigating even the most problematic species. As a caveat, although our survey quadrats are relatively large, they are spatially limited. Some amount of species' co-occurrence may be missed.

While *V. nonalfalfae* promises to be a powerful tool for land managers and restoration practitioners, it is not a silver bullet to restore most *A. altissima*-dominated areas. Although it prevents reestablishment by *A. altissima*, inoculation has the potential to create the veritable "weed-shaped hole" (Buckley *et al.* 2007) into which other non-native species establish, often from nearby. *Ailanthus altissima*-invaded sites are not unusual strictly in terms of the presence of other non-native plants, so similar factors for invasive plant mitigation and suppression relevant to other areas

must also be considered. Selective methods for removing *A. altissima* are desirable to achieve certain aims, but a more integrated approach may be required for successful restoration. While a limited sample size may make it difficult to reveal strong patterns in this study, high levels of observed variability mean that land managers will need to comprehensively assess the local plant community and incorporate sound restoration techniques to help reassemble diverse, resilient native plant communities. Even in sites with a light disturbance history and ample native plant sources, restoration initiated by bioherbicide application may require an assisted natural regeneration approach with adaptive management to control co-invasions and facilitate native community recovery.

Supplementary material. To view supplementary material for this article, please visit <https://doi.org/10.1017/inp.2024.27>

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