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Restoring with a bioherbicide

The bioherbicide *Verticillium nonalfalfae* effectively removes *Ailanthus altissima* but leaves many other nonnative plants

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Abstract

Ailanthus altissima (Mill.) Swingle readily exploits disturbances, grows quickly into dense monocultures, and suppresses native plant species. The vascular wilt pathogen, *Verticillium nonalfalfae* Inderb., native to the eastern U.S., 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 five years after inoculation with *V. nonalfalfae* to understand which species are present or being naturally recruited. *A. altissima* remained dominant in untreated areas but was nearly eradicated from the treatment plots. Other nonnative 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.

Keywords: biocontrol, invasion biology, invasive species management, restoration ecology, tree-of-heaven, *Verticillium* wilt

Management Implications

Ailanthus altissima (Mill.) Swingle, or 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* Inderb., which causes a vascular wilt disease that can spread to neighboring stems through shared roots and prevent regeneration. *V. nonalfalfae* has been petitioned for federal approval as a bioherbicide in the U.S., but its efficacy in restoration efforts can be limited because stands of *A. altissima* tend to be composed of several nonnative 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.

Introduction

Several recent studies have demonstrated the safety and efficacy of using *Verticillium nonalfalfae* Inderb. (*Plectosphaerellaceae*), as a bioherbicide to treat and kill *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 two to four months, 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 (Rebbeck et al. 2013; Schall and Davis 2009a; Snyder et al., 2013) and two European nations (Maschek and Halmsclager 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 (Schall and Davis 2009b; Kasson et al. 2015).

A. altissima is typically only one of several nonnative plants in heavily invaded sites characterized by fewer plant species (Constán-Nava 2015; Motard et al. 2011) and lower native plant diversity (Vilà et al. 2006). Invasion by *A. altissima* is also associated with increased nonnative plant diversity and decreased ratios of native to nonnative plant cover and species richness, effects which 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 post-inoculation. In a shorter-term study, Burch and Zedaker (2003) treated *A. altissima* with a chemical herbicide and observed an understory shift towards 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

(Kowarik 1995; Kasson et al. 2014) despite an herbaceous component typically rife with invasive species (O'Neal and Davis 2015).

Here, we test the outcomes of natural regeneration following inoculation of *A. altissima* with *V. nonalfalae* using a five-year, 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 target tree species. Put another way, we predicted these heavily invaded sites to maintain a large component of nonnative species despite the removal of *A. altissima*.

Materials and Methods

Vegetative Surveys

This study evaluates plant and seedbank communities at six sites around Virginia which 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. nonalfalae*, 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. nonalfalae* treatment plots were available for surveying, but we could only use three of the six control plots (two were infected with *V. nonalfalae* 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 (6.4×6.4 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, 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 (DBH), 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 (Brown 1992; Brooks et al. 2021) by using a trowel to excavate 0.33 L of soil (8×8×5 cm) across three random locations within each quadrat. This was done on two occasions, one in July 2022 and the second March 2023 to reflect both the transient and persistent seed banks, respectively (Csontos 2007; Mahé et al. 2021), and pooled them together. We stored seedbank samples at 4°C for up to two weeks and mixed them with 2 L of potting soil (0.14-0.11-0.88 N-P-K, Sta-Green Moisture Max® Potting Mix) and 60 mL of activated charcoal (Soil D-Tox™) to mitigate potential allelopathy (Inderjit and Callaway 2003) from residual aitanthone (Heisy 1996). We spread the mixture evenly in 25×51-cm trays, monitored them for 24 weeks 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 version 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. nonnative), and lifeform (woody vs. herbaceous), their

interaction, and site as a random factor; the second model excluded lifeform 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 vs. 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. nonalfalvae* plots from statistical analyses, leaving only three control and three *V. nonalfalvae* plots that were paired by site. We used permutational analysis of variance (PERMANOVA) to compare the three excluded *V. nonalfalvae* plant communities to the three we tested to investigate whether they were similar enough that management implications derived from our analysis may be considered relevant to the untested group.

Results and Discussion

Results

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 nonnative 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 lifeform 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. 63 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 five years post-inoculation (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. nonalfalvae*-inoculated plots included in the analysis compared to the remaining three treatment plots that were left out.

Discussion

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 seed banks 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 nonnative 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 clearcuts, 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 found the shift in herbaceous species to be independent of their treatment. This may partially be an artifact of phenology because their pre- and post-treatment 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* Thunberg, *Microstegium vimenium* (Trinius) A. Camus, *Phytolacca americana* Linnaeus, *Rosa multiflora* Thunberg ex Murray, *Rubus phoenicolasius* Maximowicz, and *Verbesina occidentalis* (Linnaeus) Walter. Two of these (*P. americana* and *V. occidentalis*) are common native herbaceous species, three are

considered invasive by the USDA (*E. umbellata*, *M. vimenium*, 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 nonnative 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 invasive plant mitigation across these areas would accordingly have to vary. 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 re-establishment by *A. altissima*, inoculation has the potential to create the veritable “weed-shaped hole” (Buckley et al. 2007) into which other nonnative species establish, often from nearby. *A. altissima*-invaded sites are not unusual strictly in terms of the presence of other nonnative 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.

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Competing Interests

The authors declare none.

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Figures



Figure 1. A stand of *Ailanthus altissima* five years after inoculation with *Verticillium nonalfalae*. 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* and the nonnative *Paulownia tomentosa*.

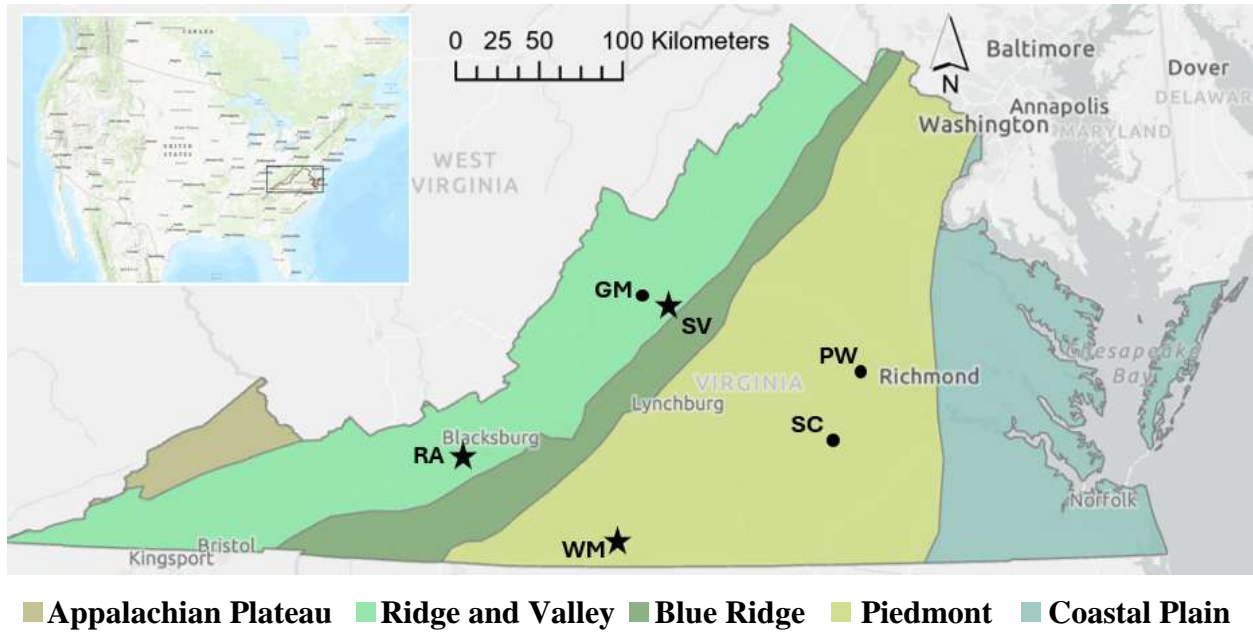


Figure 2. Study site locations across physiographic regions of Virginia (Esri 2023; Hitt 2023). Site codes correspond to Brooks et al. (2020b). Sites marked with a star had paired control and *Verticillium nonalfalfae*-inoculated plots. Sites marked with a circle had no available control plots and were excluded from formal analysis, but their inoculated plots were inventoried and compared to 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 United States Army installation; and SV is a Virginia Tech Agricultural and Research Extension Center.

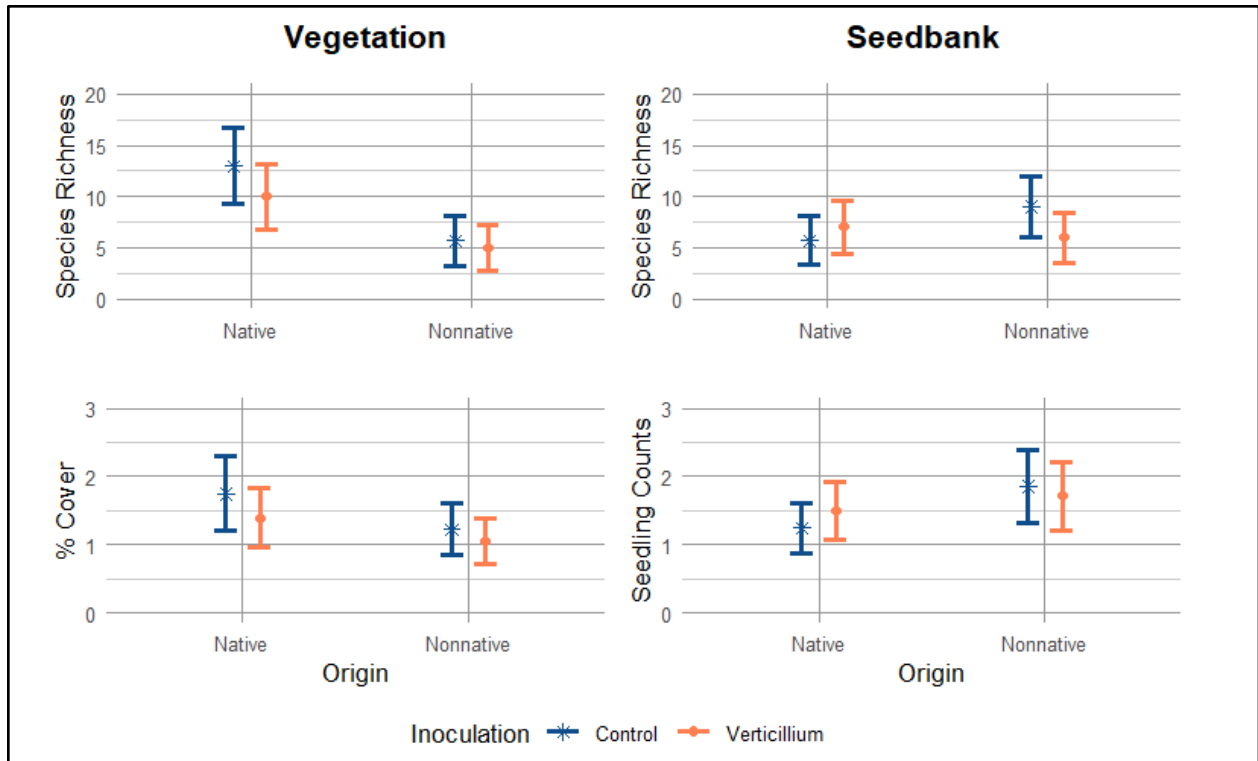


Figure 3. Native and nonnative species richness, vegetative cover, and seedlings germinated for control and *Verticillium nonalfalfae*-inoculated plots. Vegetation includes observations in the field while the seedbank consists of greenhouse-germinated samples. Raw percent cover and seedling counts were Hellinger-transformed. Confidence intervals represent predicted means +/- one standard deviation.