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Comparative Pathogenicity, Biocontrol Efficacy, and Multilocus Sequence Typing of *Verticillium nonalfalfae* from the Invasive *Ailanthus altissima* and Other Hosts

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ABSTRACT

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Verticillium wilt, caused by *Verticillium nonalfalfae*, is currently killing tens of thousands of highly invasive *Ailanthus altissima* trees within the forests in Pennsylvania, Ohio, and Virginia and is being considered as a biological control agent of *Ailanthus*. However, little is known about the pathogenicity and virulence of *V. nonalfalfae* isolates from other hosts on *Ailanthus*, or the genetic diversity among *V. nonalfalfae* from confirmed Ailanthus wilt epicenters and from locations and hosts not associated with Ailanthus wilt. Here, we compared the pathogenicity and virulence of several *V. nonalfalfae* and *V. alfalfae* isolates, evaluated the efficacy of the virulent *V. nonalfalfae* isolate VnAa140 as a biocontrol agent of *Ailanthus* in Pennsylvania, and performed multilocus sequence typing of *V. nonalfalfae* and *V. alfalfae.* Inoculations of seven *V. nonalfalfae* and *V. alfalfae* isolates from six plant hosts on healthy *Ailanthus* seedlings revealed that *V. nonalfalfae* isolates from hosts other than *Ailanthus* were not pathogenic on *Ailanthus*. In the field, 100 canopy *Ailanthus* trees were inoculated across 12 stands with VnAa140 from 2006 to 2009. By 2011, natural spread of the fungus had resulted in the mortality of >14,000 additional canopy *Ailanthus* trees, 10,000 to 15,000 *Ailanthus* sprouts, and nearly complete eradication of *Ailanthus* from several smaller inoculated stands, with the exception of a few scattered vegetative sprouts that persisted in the understory for several years before succumbing. All *V. nonalfalfae* isolates associated with the lethal wilt of *Ailanthus*, along with 18 additional isolates from 10 hosts, shared the same multilocus sequence type (MLST), MLST 1, whereas three *V. nonalfalfae* isolates from kiwifruit shared a second sequence type, MLST 2. All *V. alfalfae* isolates included in the study shared the same MLST and included the first example of *V. alfalfae* infecting a non-lucerne host. Our results indicate that *V. nonalfalfae* is host adapted and highly efficacious against *Ailanthus* and, thus, is a strong candidate for use as a biocontrol agent.

Additional keywords: tree-of-heaven, *Verticillium albo-atrum*.

Ailanthus altissima (Mill.) Swingle, commonly known as the tree-of-heaven or *Ailanthus*, is a highly invasive tree species first introduced into Philadelphia in 1784. *Ailanthus* is now present throughout most of the contiguous United States, mainly as an urban and roadside weed. The earliest report of Verticillium wilt of *Ailanthus* in the United States is from 1915 in Beaver County, PA (The Pennsylvania State University Plant Disease Clinic, *unpublished archives*). Other early Verticillium wilt epidemics of *Ailanthus* were reported in New York, Philadelphia, and Roanoke, VA in the late 1920s and early 1930s (3,10,16). During the 1990s, extensive Verticillium wilt epidemics were observed on *Ailanthus* in New York on eastern Long Island and in the Hudson River Valley, and in Connecticut (7). Since 2003, unprecedented wilt and mortality of *Ailanthus* has nearly eliminated nearly 10,000 canopy *Ailanthus* trees in previously oak-dominated stands in south-central Pennsylvania (39). In 2010, another diseased *Ailanthus* stand was discovered in Washington County, PA, located ≈230 km west of the initial 2003 wilt epicenter and 35 km south of the 1915 location, containing several hundred infected *Ailanthus*. We determined the causal agent of these recent epidemics in Pennsylvania to be *Verticillium nonalfalfae*. This finding, along with recent reports of *V. nonalfalfae* killing *Ailanthus* in Virginia (44,45) and Ohio (36), suggests that the lethal Ailanthus wilt caused by *V. nonalfalfae* is more widespread than previously considered and may implicate *Ailanthus* as an important host of *V. nonalfalfae* in the United States.

V. nonalfalfae is a vascular wilt pathogen of hops, solanaceous crops, spinach, and forest and shade trees in Canada, Japan, Slovenia, the United Kingdom, and the United States (19,20,32,40). Recent molecular systematics work has clarified its relationship to *V. alfalfae* and *V. albo-atrum* (20), which are morphologically indistinguishable. Early reports of *Ailanthus* infections by "*V. albo-atrum*" were likely caused by *V. nonalfalfae*, especially because *V. albo-atrum* sensu stricto has been reported only from potato (20).

Spatiotemporal migration patterns of invading *Ailanthus* in Pennsylvania suggest a recent increase of this tree in forested areas (23). Likewise, disease epidemics on *Ailanthus* appear to have intensified during recent decades (7,39). Unlike Verticillium wilt epidemics in the forest setting, reports of lethal wilt epidemics in urban areas have been well documented for nearly a century. Even so, high natural mortality rates of *Ailanthus* within urban areas (31), as well as the presumed short life expectancy of *Ailanthus* (23), may have resulted in many Verticillium wilt

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epidemics being overlooked or mistaken for natural senescence or, more recently, herbicide damage along roadsides.

Even with significant mortality and rapid turnover, extant populations of *Ailanthus* persist throughout most of the conterminous United States and are especially abundant in the Mid-Atlantic region, where they have occurred for ≈200 years (1,23,27). The population structure of *Ailanthus* across the United States supports both East and West Coast introductions (1) and *Ailanthus* has been reported to be invasive in natural areas within at least 30 states (http://www.nps.gov/plants/alien/fact/aial1.htm). The development and utilization of *V. nonalfalfae* as a potential biological control of *Ailanthus* has been stymied, in part, by incomplete efficacy and host range testing (40) as well as unknown facets of host genetics (1,8). Federal and state agencies are interested in using *V. nonalfalfae* to control *Ailanthus* both in forests and along transportation corridors, where conventional control measures have proven ineffective (M.T. Kasson, *personal communications*).

The objectives of this study were to (i) conduct comparative pathogenicity testing against *A. altissima* using a panel of *V. alfalfae* and *V. nonalfalfae* isolates from diverse hosts, (ii) evaluate efficacy of *V. nonalfalfae* isolate VnAa140 on canopy *Ailanthus* trees across various sites in Pennsylvania, (iii) assess long-term effects of *V. nonalfalfae* VnAa140 on *Ailanthus* regeneration in stands following death of *Ailanthus* canopy trees, and (iv) characterize the phylogenetic diversity, using a four-gene data set, of a collection of *V. nonalfalfae* isolates from Pennsylvania, Virginia, and Ohio and other isolates previously classified as *V. albo-atrum*.

MATERIALS AND METHODS

Inoculum preparation and culture maintenance. *Verticillium* cultures were maintained on plum extract agar (PEA), a semi-selective media for *Verticillium* spp. (48), amended with streptomycin sulfate and neomycin sulfate, hereafter called PEA+SN (39), in controlled environment chambers at 23°C for 3 weeks under a 12-h photoperiod. Inoculum was prepared by adding 5 to 10 ml of sterile distilled water to 3-week-old *V. nonalfalfae* isolate VnAa140 (formerly *V. albo-atrum* PSU140) cultures on PEA+SN and scraping the surface with a sterile glass rod. The resulting spore suspension was collected, vortexed, and passed through a sterile milk filter (KenAg, Ashland, OH) to remove mycelial fragments. Conidial concentrations were determined using a hemocytometer and adjusted to 1×10^7 conidia ml^{-1}. Viability of conidia was evaluated by counting CFU from 10-fold dilutions of suspensions on PEA+SN plates. Inoculum was maintained at 4°C until viability was confirmed. Only inoculum with >80% viability was utilized.

For long-term maintenance of *Verticillium* strains, 10-ml scintillation vials containing a 1:1:1 sterile mix of potting soil/peat moss/vermiculite were flooded with conidia harvested from 4-week-old cultures. After a 2-week incubation period, vials were stored at 4°C. Preliminary laboratory studies had previously confirmed viability of isolates >3 years following inoculation and subsequent storage at 4°C (M. T. Kasson, *unpublished data*). Active cultures of VnAa140, the primary strain used in efficacy field studies, as well as other isolates were obtained by transferring inoculated soil from stored scintillation vials onto PEA+SN. To maintain pathogenicity of VnAa140 and other isolates, conidial suspensions were inoculated annually or biannually into several 1-month-old potted *Ailanthus* seedlings. Following symptom onset, cross-sections from symptomatic stems were plated on PEA+SN, and *Verticillium* spp. were reisolated and subcultured for use as inoculum.

Comparative pathogenicity testing of representative isolates of *V. alfalfae* and *V. nonalfalfae* on *Ailanthus*. Six isolates of *V. nonalfalfae* and one isolate of *V. alfalfae* were chosen for comparative pathogenicity testing on *Ailanthus* (Table 1). Inoculations of *Ailanthus* seedlings were conducted using a root-dip method (34). Roots of 3-week-old seedlings were rinsed, dipped into a 1×10^7 ml^{-1} conidial suspension or water, and individually transplanted into tapered plastic containers (3.8 cm top diameter by 20.3 cm deep) containing ≈140 g of premoistened Sunshine mix (Sun Gro Horticulture Canada Ltd., Vancouver, BC, Canada) and supplemented with Osmocote plant fertilizer (12:12:12, $N\text{-}P_2O_3\text{-}K_2O$ slow-release; Scotts Miracle-Gro, Marysville, OH). After transplanting, 1 ml of additional inoculum was pipetted onto the base of each stem at the soil line. Disease progression was recorded weekly for 8 weeks, after which the experiment was terminated and indices developed by Qin et al. (34) utilized to quantify disease on a scale from 0 to 6, where 0 = no symptoms, 1 = 1 to 25% vascular discoloration (VD), 2 = 26 to 50% VD, 3 = 51 to 75% VD, 4 = 76 to 100% VD, 5 = 4 + wilt, and 6 = 5 + mortality. Mean ratings were calculated based on average ratings of 10 plants per treatment, including the negative control. Isolates yielding a mean disease severity ≥2 were considered pathogenic (34). All plants were destructively sampled and tissue samples exhibiting VD collected for reisolation.

***Verticillium* isolation and identifiers.** Shoot tissues exhibiting VD, or rachises or petioles from newly wilted leaves, were excised, surface disinfested in 95% ethanol, flamed, sectioned, and plated on PEA+SN. The plates were incubated on laboratory benches for 10 days and developing *Verticillium* colonies were plated onto fresh PEA+SN plates to determine species identities. Isolate identifiers were standardized as proposed by Bhat and Subbarao (4), where isolate identification is based on the *Verticillium* sp. (Vn = *V. nonalfalfae*), binomial nomenclature of the infected host (Aa = *A. altissima*), and a unique numerical identifier (e.g., VnAa140) (Table 2).

Field study locations. The field study area spanned nine counties in south-central Pennsylvania, encompassing 14 forested stands on three state forests, one state game lands, one state park, and Army Corps of Engineering (ACOE) lands (Supplemental Figure 1). Tree species composition was typical of oak-dominated, mixed hardwood forests in southern Pennsylvania, including *Ailanthus* and numerous cohort species (39). In the northern region of the study area, additional cohort species included American elm, black cherry, and black walnut. Specific study plot locations are described in Supplemental Table 1. Stand histories for forests and individual locations were described by Kasson et al. (23).

***Ailanthus* canopy tree inoculations.** *Ailanthus* was inoculated with *V. nonalfalfae* at 12 sites proximal to the area where Verticillium wilt of *Ailanthus* in Pennsylvania forests was first discovered (39). Correlations between Verticillium wilt disease development and geography, stem density, stem diameter, stand size, and spatial distribution of inoculated trees relative to each

TABLE 1. Pathogenicity ratings of different *Verticillium* isolates from different hosts on *Ailanthus*

Isolates	Origin	Disease rating[z]
VnAa100	*Ailanthus altissima*	5.3
VnAa140	*A. altissima*	5.1
VaMs102	*Medicago sativa*	3.6
VnCi21	*Ceanothus integerrimus*	1.0
VnAc3	*Actinidia chinensis*	0.8
VaSm621	*Solanum melongena*	0.4
VnSt462	*S. tuberosum*	0.3
Control	…	0.0

[z] Mean vascular discoloration of 10 tested plants of *Ailanthus altissima* seed source HSAa23 from Penn State University campus. A 0 to 6 scale was adopted to assess disease severity, in which 0 = no vascular discoloration, 1 = 1 to 25% vascular area discolored (vad), 2 = 26 to 50% vad, 3 = 51 to 75% vad, 4 = 75 to 100% vad, 5 = 4 + wilting, and 6 = 5 + associated mortality. Isolates causing a mean disease severity vascular area discolored with of ≥2 on a certain host were considered pathogenic.

other were assessed. Healthy *Ailanthus* stands near naturally infected stands were chosen for artificial inoculation to increase the likelihood of capturing similar host plant genotypes with comparable susceptibility to *V. nonalfalfae* infection.

In May 2006, a forest stand containing healthy *Ailanthus* trees atop Blue Mountain (BM) in Tuscarora State Forest was selected for inoculation. Twenty healthy canopy *Ailanthus* trees were clustered across four different sites separated by 0.3 to 2.0 km along a forest road. Five *Ailanthus* trees at each site were inoculated with isolate VnAa140 using a hypo-hatchet (OEM Fabricators Inc., Woodville, WI) that delivered 1 ml of inoculum (10^7 conidia ml^{-1} in sterile distilled water) per injection at three points on the stem base. A separate stand, located within the same forest, served as a control site, where trees were wounded with a surface-sterilized hatchet at three points on the stem base and treated with sterile distilled water (39). In May 2008, 10 additional stands, located across two state forests on state game lands and ACOE lands, were selected for inoculation studies. Five canopy *Ailanthus* trees were randomly selected for inoculation in five smaller stands and three larger stands, and 10 trees were selected in two additional larger stands. Trees were inoculated with VnAa140 as above. At two additional sites, five healthy canopy *Ailanthus* trees were selected as controls and wounded and treated as described above. In June 2009, a forested stand within the Canoe Creek State Park (CCSP) was selected to serve as a public demonstration site to illustrate the effectiveness of VnAa140 as a biocontrol agent of *Ailanthus*, wherein 20 healthy canopy *Ailanthus* trees were randomly selected and stem inoculated with VnAa140 using a hypo-hatchet.

Inoculated and control stands were evaluated biweekly, beginning 4 weeks after treatment (approximately early June) until late September to early October, except for CCSP, where evaluations began in late June. Evaluations were resumed the following spring and continued through the end of the second field season, except that stands BM and CCSP were evaluated biweekly for only one field season, and two of the 2008 stands (SGL1 and BSF2) were evaluated biweekly from May to October during a third field season. Of the 12 inoculated stands, five (BM, BSF2, CCSP, RLK1, and SGL1) were selected for longer-term studies and were evaluated annually until 2011, except for BM, which was evaluated every other year through 2011.

Disease severity was assessed using an ordinal rating system, where 1 = asymptomatic foliage, 2 = general marginal leaf chlorosis or necrosis, 3 = presence of wilt, and 4 = death (40). The area under the disease progress curve (AUDPC) was calculated for each plot to compare disease progression among species

TABLE 2. *Verticillium* isolates used for molecular studies

Taxa, strain[y]	Host, substrate	GenBank accession numbers[z]				
		MAT	IGS	EF1-α	GPD	TS
Verticillium albo-atrum						
VaSt112	Soil	…	…	JN188273	JN188209	JN188081
V. alfalfae						
VaCb241	*Catalpa bignonioides*	…	…	…	…	…
VaMs102	*Medicago sativa*	…	…	HQ414636	HQ414731	HQ414921
VaMs449	*M. sativa*	…	…	…	…	…
VaMs629	*M. sativa*	…	…	…	…	…
V. dahliae						
VdLs17	*Lactuca sativa*	…	…	HQ414624	HQ414719	HQ414909
V. nonalfalfae						
VnAp142	*Acer pensylvanicum*	…	…	…	…	…
VnAp143	*A. pensylvanicum*	…	…	…	…	…
VnAp144	*A. pensylvanicum*	…	…	…	…	…
VnAc1	*Actinidia chinensis*	…	…	…	…	…
VnAc3	*A. chinensis*	**KF802863**	**KF802861**	**KF802866**	**KF802864**	**KF802865**
VnAc4	*A. chinensis*	…	…	…	…	…
VnAa1	*Ailanthus altissima*	…	…	KC307761	KC307762	KC307763
VnAa140	*A. altissima*	**KF802862**	**KF802860**	KC307764	KC307766	KC307768
VnAa171	*A. altissima*	…	…	…	…	…
VnAa172	*A. altissima*	…	…	…	…	…
VnAa100	*A. altissima*	…	…	…	…	…
VnAa200	*A. altissima*	…	…	KC307758	KC307759	KC307760
VnAa48	*A. altissima*	…	…	…	…	…
VnAa72	*A. altissima*	…	…	…	…	…
VnAa30	*A. altissima*	…	…	…	…	…
VnAa32	*A. altissima*	…	…	…	…	…
VnAs145	*Aralia spinosa*	…	…	…	…	…
VnAs146	*A. spinosa*	…	…	…	…	…
VnAs147	*A. spinosa*	…	…	…	…	…
VnAs148	*A. spinosa*	…	…	…	…	…
VnCi21	*Ceanothus integerrimus*	…	…	…	…	…
VnHl1	*Humulus lupulus*	…	…	…	…	…
VnHl2	*H. lupulus*	…	…	…	…	…
VnHl3	*H. lupulus*	…	…	…	…	…
VnHl48	*H. lupulus*	…	…	…	…	…
VnRm141	*Rosa multiflora*	…	…	…	…	…
VnSt462	*Solanum tuberosum*	…	…	…	…	…
VnSt1856	*S. tuberosum*	…	…	JN188227	JN188163	JN188035
VnSl179	*S. lycopersicum*	…	…	…	…	…
VnSm621	*S. melongena*	…	…	…	…	…
VnSo1855	*Spinacia oleracea*	…	…	…	…	…

[y] Taxa and strain identifiers used in this study. Strain identifiers in bold indicate isolates previously confirmed with DNA sequencing. See corresponding GenBank accession numbers in three right-most columns.

[z] IGS = intergenic spacer, EF-1α = elongation factor 1α, GPD = glyceraldehyde-3-phosphate dehydrogenase, and TS = tryptophan synthase. GenBank accession numbers in bold denote representative sequences for *V. nonalfalfae* deposited as part of this study.

and treatments (39). Tree diameters of inoculated and control *Ailanthus* were averaged across each stand as well as placed into 5-cm increment designations, regardless of stand location (data not shown), to determine the relationships between stem diameter and disease progression.

Long-term effects of *V. nonalfalfae* VnAa140 on *Ailanthus* regeneration survival and establishment. In 2011, five stands (BM, BSF2, CCSP, RLK1, and SGL1) that had been previously inoculated with *V. nonalfalfae* VnAa140 and contained numerous dead and dying *Ailanthus* canopy trees were selected for regeneration studies. Stand BM had been inoculated in 2006 and the remaining stands were inoculated in 2008 to 2009. One east-west transect was established within the infected areas of stands in BSF2, CCSP, RLK1, and SGL1. Two east-west transects were established in stand BM, because diseased *Ailanthus* trees were more widely spaced, with four separate disease foci. Because most *Ailanthus* stands had an east-west orientation and were typically located on south-facing slopes, east-west transects encompassed the widest area of the diseased stands and therefore included the maximum number of *Ailanthus* stems.

Within each stand, the transect line was established through the main Verticillium wilt infection center and extended 30 m beyond the outer edge of the infection center (Fig. 1). Six 4.05-m^2 circular regeneration subplots were established in three pairs in opposing directions, including two subplots in the approximate center of each stand, where the *Ailanthus* stems had been originally inoculated; two subplots 10 m inside the advancing edge of disease epicenter; and two subplots 30 m beyond the advancing edge (outside) of the diseased stand (into the asymptomatic portion of the stand) (Fig. 1). Outside subplots served as pretreatment controls, because these areas remained undisturbed at the time of observations and were basically unaffected by canopy openings 30 m distant. Numbers, heights, and disease ratings (0 to 2) of *Ailanthus* regeneration were recorded using an ordinal rating system, as described by Schall and Davis (40). *Ailanthus* roots were excavated to determine whether regeneration had occurred from seed or root sprout. Likewise, numbers and disease ratings (if applicable) of native woody species were also recorded for each sampled subplot.

Statistical analyses of field data. Analysis of variance (ANOVA) and Tukey's mean comparisons were used to evaluate significant differences among AUDPC data, mean diameters of *Ailanthus* among inoculation sites, and seedling counts and heights in regeneration subplots. All analyses were conducted using Minitab 16.1.0 (Minitab Inc., State College, PA).

DNA extraction and molecular characterizations. Total genomic DNA was extracted from lyophilized mycelial plugs harvested from potato dextrose broth (BD, Franklin Lakes, NJ) using a Wizard genomic DNA purification protocol (Promega Corp., Madison, WI). For multilocus sequence typing and phylogenetic analyses, previously published protocols were used (21, 34) to amplify the following loci: the protein-coding genes elongation factor 1α (EF-1α), glyceraldehyde-3-phosphate dehydrogenase (GPD), and tryptophan synthase (TS); and a portion of the intergenic spacer (IGS) region of the ribosomal DNA (rDNA) repeat. Polymerase chain reaction (PCR) assays targeting *MAT1-1-1* and *MAT1-2-1* were also used to determine mating types of 36 isolates, as previously described (21).

Sanger sequencing using the forward and reverse PCR primers was performed at the Penn State Genomics Core Facility, University Park, PA, on an ABI 3730 XL automated DNA sequencer (Applied Biosystems, Carlsbad, CA). Sequencher (version 5.1; GeneCodes Corporation, Ann Arbor, MI) was used to edit the raw chromatograms. Edited sequences were used as BLAST queries against the National Center for Biotechnology Information (NCBI) GenBank database for preliminary identification.

A concatenated four-locus alignment was generated using CLUSTAL-W (http://www.genome.jp/tools/clustalw) followed by manual improvement. Separate partitions were created for each gene to permit analyses for both individual genes and the combined dataset. Maximum likelihood (ML) analyses were conducted using MEGA 5.1 (49). ML bootstrap analyses of the individual and combined dataset were run on the CIPRES Science Gateway (http://www.phylo.org/portal2/login) using GARLI ver. 1.0 (54) on the TeraGrid. Sequence data from NCBI GenBank for *V. dahliae* and *V. albo-atrum* were used as outgroups.

To verify species identity, DNA from *Verticillium* isolates from *Actinidia* were used as templates in a *V. albo-atrum–V. alfalfae–V. nonalfalfae* multiplex PCR assay and a *V. nonalfalfae* simplex PCR, as previously described (22). For the multiplex and simplex PCRs, the reference isolates PD 338 (*V. alfalfae*), PD 745 (*V. nonalfalfae*), and PD 592 (*V. nonalfalfae*) were used as positive controls.

RESULTS

Comparative pathogenicity testing. Comparative pathogenicity testing revealed differential responses of the *Verticillium* spp. and isolates on *Ailanthus*. Of the seven isolates tested, two isolates of *V. nonalfalfae* from *Ailanthus* and one isolate of *V. alfalfae* from alfalfa were pathogenic (average disease score ≥2) on *Ailanthus*. *V. alfalfae* isolate VaMs102 resulted only in VD but the two *V. nonalfalfae* isolates from *Ailanthus* resulted in acute wilt in all plants and some mortality by 8 weeks post-inoculation (Table 1). *V. nonalfalfae* isolates from deerbrush, eggplant, kiwifruit, and potato were not pathogenic on *Ailanthus*, although low levels (<25%) of VD were observed in some individuals from each isolate (Table 1).

***Ailanthus* canopy tree inoculations.** Twenty canopy *Ailanthus* trees within stand BM inoculated with VnAa140 developed wilt symptoms and yellow to yellowish-brown VD within 3 to 4 weeks, followed by occasional necrosis of previously wilted leaves prior to senescence, premature defoliation, and mortality (Fig. 2), similar to symptoms observed in naturally infected *Ailanthus*

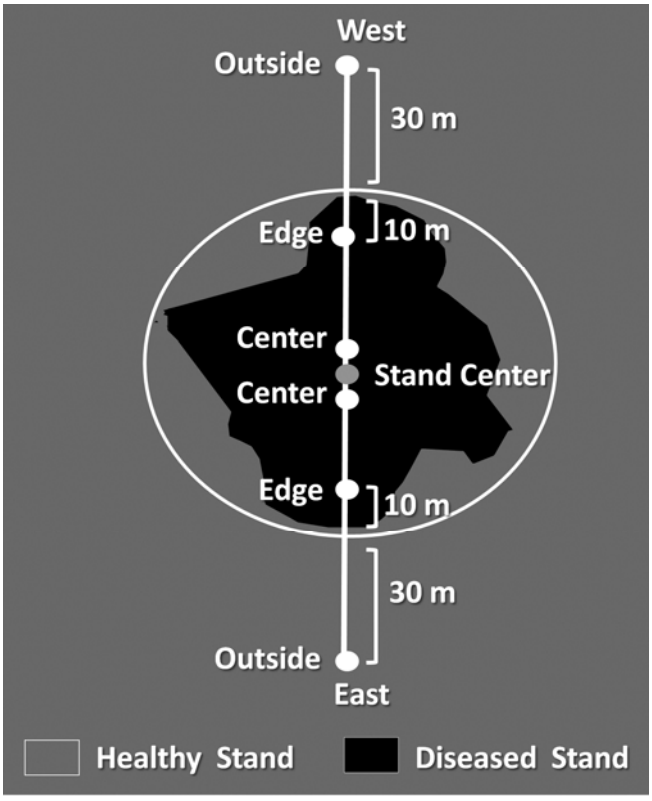

Fig. 1. Schematic diagram illustrating the location of regeneration subplots within diseased *Ailanthus* stands.

(39). By 12 months post-inoculation (MPI), all inoculated trees were dead. The pathogen spread from the original inoculation point, causing symptoms on 800 formerly healthy *Ailanthus* trees (Figs. 3 and 4), a majority of which eventually died, except for a few infected trees that sprouted along the main stem (Figs. 2D and 4C) that also ultimately wilted and died. By 36 MPI, there were 2,663 dead and dying *Ailanthus* stems surrounding the point of inoculation (Fig. 3). By the final survey, conducted 63 MPI in August 2011, a total of 8,897 canopy *Ailanthus* trees had succumbed as a result of natural spread of this pathogen from the 20 inoculated trees (Fig. 3).

The 10 *Ailanthus* stands that had been inoculated with VnAa140 in 2008 were of various sizes, ages, and stem densities (23). Inoculated stands exhibited first disease symptoms ≈3 weeks post-inoculation (WPI), with the exception of site RLK3, which exhibited wilt symptoms at 5 WPI. The control plots (BSF3 and MSF2) remained asymptomatic for the duration of the experiment.

Mean AUDPC values were significantly greater for inoculated plots than noninoculated controls (P = 0.000) (Table 3). Mean AUDPC values also differed significantly among the 10 inoculated sites, indicating that disease progression differed among sites (Table 3). Stands BSF1 and SGL1 had the highest mean AUDPC (247.0 and 242.0, respectively) at 69 WPI. These two stands had significantly greater AUDPC values than RLK3 (207.8) and MSF1 (201.2) (Table 3). Mean AUDPC for BSF2 (236.8) and MSF5 (239.4) were also significantly different from the AUDPC values for MSF1 (201.2) (P = 0.024 and 0.049, respectively) (Table 3).

Significant differences in mean stem diameters also occurred among stands. Mean stem diameter at RLK3 (31.8 cm) was significantly greater than at SGL1 (11.6 cm, P = 0.000), MSF3 (13.2 cm, P = 0.006), MSF4 (15.5 cm, P = 0.027), and RLK2

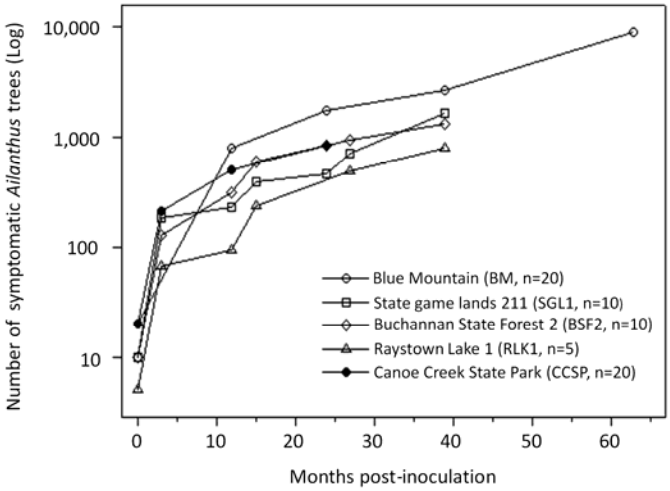

Fig. 3. Disease progression of *Verticillium nonalfalfae* isolate VnAa140 across five artificially inoculated *Ailanthus* stands in south-central Pennsylvania during 2006 to 2011. Inoculation dates were: BM (May 2006); SGL1, BSF2, and RLK1 (May 2008); and CCSP (June 2009). Observations were made in early growing season (May), late growing season (July to August), or both and include 3, 12, 15, 24, 27, 36, and 63 months post-inoculation.

Fig. 2. Symptoms of Ailanthus wilt following artificial inoculation with *Verticillium nonalfalfae* isolate VnAaP140, showing **A,** debarked *Ailanthus* with yellow vascular discoloration and streaking, characteristic of *Verticillium* infection; **B,** acute wilt and defoliation in crowns of infected *Ailanthus* trees; **C,** vegetative *Ailanthus* spouts exhibiting acute wilting, following inoculation of adjacent canopy *Ailanthus* tree; and **D,** wilting epicormic sprouts that emerged following wilt and defoliation of the main crown.

(16.4 cm, $P = 0.048$) (Table 3). In general, stands with smaller-diameter *Ailanthus* trees had greater AUDPC values (Table 3). For example, trees in the 15-cm class across all locations had an average AUDPC value of 248.7, which is significantly greater ($P < 0.001$) than the AUDPC values for trees in the 45-cm diameter class (195.6) However, tree diameter was not significantly related to the AUDPC values at MSF1 (Table 3).

In all four inoculated stands <2 ha in size and in one of two stands that were 2 to 8 ha and inoculated with VnAa140 in 2008, 100% mortality of overstory *Ailanthus* occurred. Disease or mortality of overstory *Ailanthus* trees did not occur in the water-treated control stands. Therefore, long-term stand data were collected only from the three larger remaining stands inoculated in 2008 (BSF2, RLK1, and SGL1). In stand BSF2, 10 trees were originally inoculated with VnAa140; at 12 MPI, 309 *Ailanthus* trees were symptomatic; at 39 MPI, ≈1,300 *Ailanthus* stems were symptomatic (Fig. 3). In stand RLK1, 5 trees were inoculated with VnAa140; at 12 WPI, 93 *Ailanthus* stems were symptomatic; at 39 MPI, >790 *Ailanthus* trees had Verticillium wilt at this site and were either dead or dying (Fig. 3). At SGL1, 10 trees were inoculated with VnAa140; at 12 MPI, 231 stems were symptomatic; at 39 MPI, 1,646 stems were symptomatic. However, unlike BSF2 and RLK1, the number of symptomatic *Ailanthus* stems in SGL1 increased nearly fourfold between 24 to 39 MPI, from 464 to 1,646 stems (Fig. 3).

In June 2009, 20 canopy *Ailanthus* trees were inoculated at CCSP in Blair County. The initial disease symptoms appeared <2 weeks after inoculation, and were similar to those observed in other inoculated stands (Fig. 2). At 12 MPI, there were 503

TABLE 3. Mean area under the disease progress curve (AUDPC) values 69 weeks post-inoculation and mean diameter at breast height (DBH) for 2008 field-inoculated *Ailanthus*[z]

Plot	AUDPC	DBH
BSF1	247.0 a	19.7 abcd
SGL1	242.0 a	11.6 d
MSF5	239.4 ab	21.8 abcd
BSF2	236.8 ab	20.1 abcd
RLK2	234.8 abc	16.4 bcd
MSF4	230.6 abc	15.5 bcd
RLK1	229.6 abc	26.4 abc
MSF3	214.6 abc	13.2 cd
RLK3	207.8 bc	31.8 a
MSF1	201.2 c	20.8 abcd
BSF3-Control	69.0 d	30.5 ab
MSF2-Control	69.0 d	21.4 abcd

[z] AUDPC and DBH values followed by the same letter within a given column are not significantly different according to Tukey's pairwise comparisons ($P = 0.001$). Controls include *Ailanthus* injected with sterile distilled water at two separate locations.

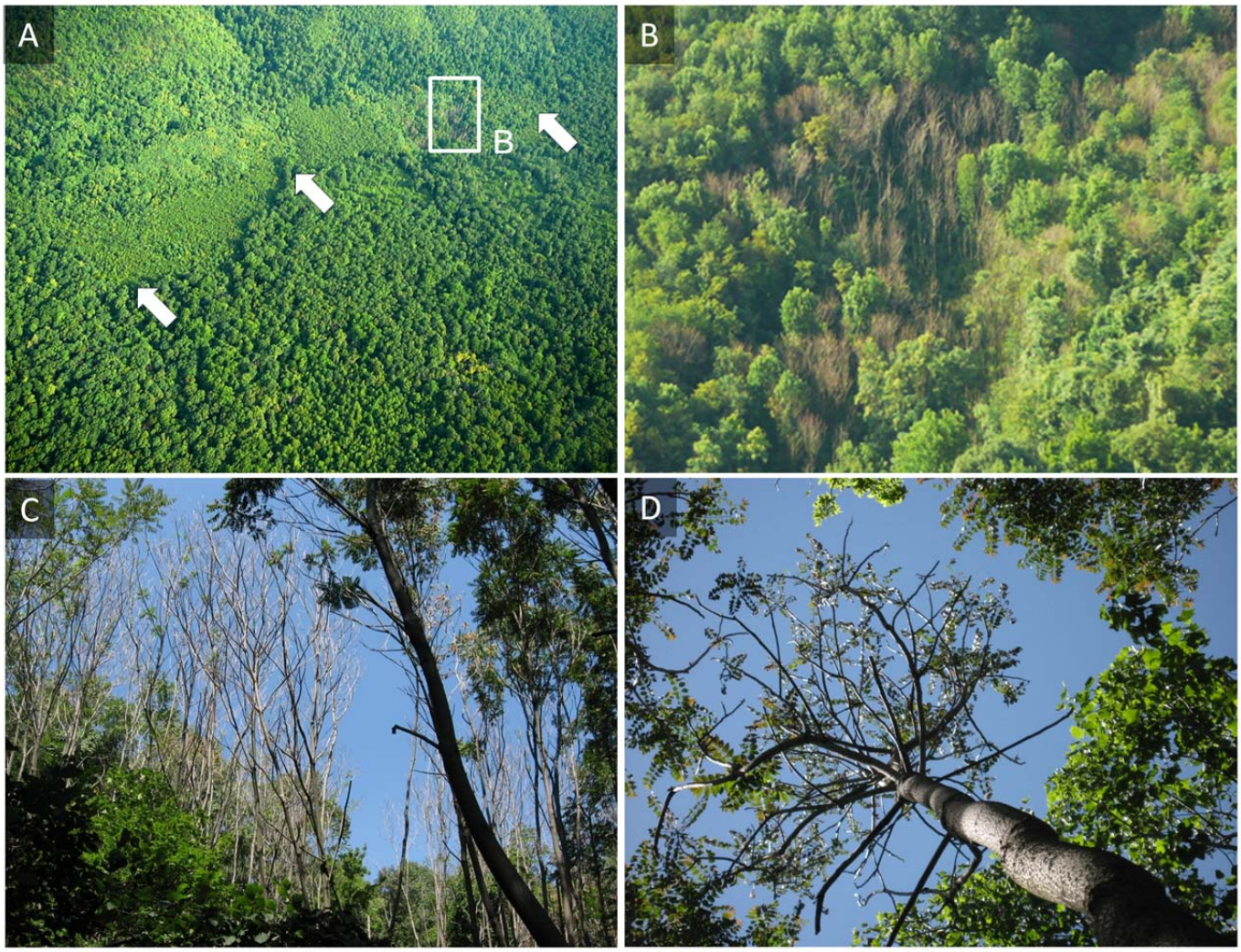

Fig. 4. Stand symptoms following artificial inoculation of *Ailanthus* with *Verticillium nonalfalfae* isolate VnAa140. Photos include an aerial view of stand showing **A,** boundaries of old clear-cut (white arrows) that incited invasion by *Ailanthus*; **B,** close-up of *Ailanthus* mortality with crowns of non-*Ailanthus* still occupying the sites >3 years post-inoculation; **C,** view from below of dead and dying *Ailanthus* trees with epicormic sprouting along the upper limbs of trees in the back left; and **D,** close-up of wilting *Ailanthus* tree surrounded by asymptomatic *Ailanthus*.

diseased stems at this site; at 24 MPI, 841 stems were symptomatic (Fig. 3).

From the initial focus of 100 *Ailanthus* trees inoculated with VnAa140 across 12 stands in south-central Pennsylvania from 2006 to 2009, natural spread of the pathogen resulted in mortality of 14,162 canopy stems (Fig. 3).

Effect of VnAa140 on site recolonization by *Ailanthus*. After inoculation of *Ailanthus* canopy trees with VnAa140, some *Ailanthus* seedlings or sprouts persisted within all inoculated stands for several years but only vegetative root sprouts were found within the regeneration plots. *Ailanthus* regeneration occurred in 20 of 36 4-m^2 test subplots across five forested stands (Fig. 5A). On average, 12 *Ailanthus* sprouts were found across the 20 positive subplots (range = 1 to 54). However, differences were observed in the number of *Ailanthus* observed among the three subplot locations within the larger stands. *Ailanthus* was observed in only 4 of 12 of center subplots, whereas *Ailanthus* was observed in 9 of 12 of edge subplots and 7 of 12 of outside subplots (Fig. 5A). In terms of total number of trees, 16 *Ailanthus* occurred within center subplots, whereas 163 and 74 trees were observed in edge and outside subplots, respectively (Fig. 5C).

On average, three diseased or dead sprouts per plot were found across 4 of 12 edge plots but in none of the center and outside subplots. In terms of total number of *Ailanthus*, 13 of 163 *Ailanthus* were symptomatic or dead within edge subplots (Fig. 5C). Total estimated density for diseased sprouts across all stands was ≈440 diseased sprouts/ha across all edge plots. In total, some 10,000 to 15,000 diseased *Ailanthus* sprouts were estimated to occur across all plots.

To examine the effect of VnAa140 on *Ailanthus* reestablishment, mean heights of *Ailanthus* in regeneration plots were compared in the BM regeneration plots, due to the presence of asymptomatic *Ailanthus* trees (172 of 253) in 9 of the 12 subplots. Average heights of *Ailanthus* were significantly greater in the center subplots (21.3 cm) than in the edge (7.5 cm) and outside subplots (8.2 cm) ($P = 0.000$) (Fig. 5D).

Total numbers of native perennial woody plant species were also recorded across the five long-term study areas in all regeneration subplots. In total, 183 non-*Ailanthus* native seedlings and saplings were observed in 23 of 36 subplots (Fig. 5A). On average, 5 plants/plot (range = 1 to 40) were observed within the subplots containing native species (Fig. 5). The most abundant

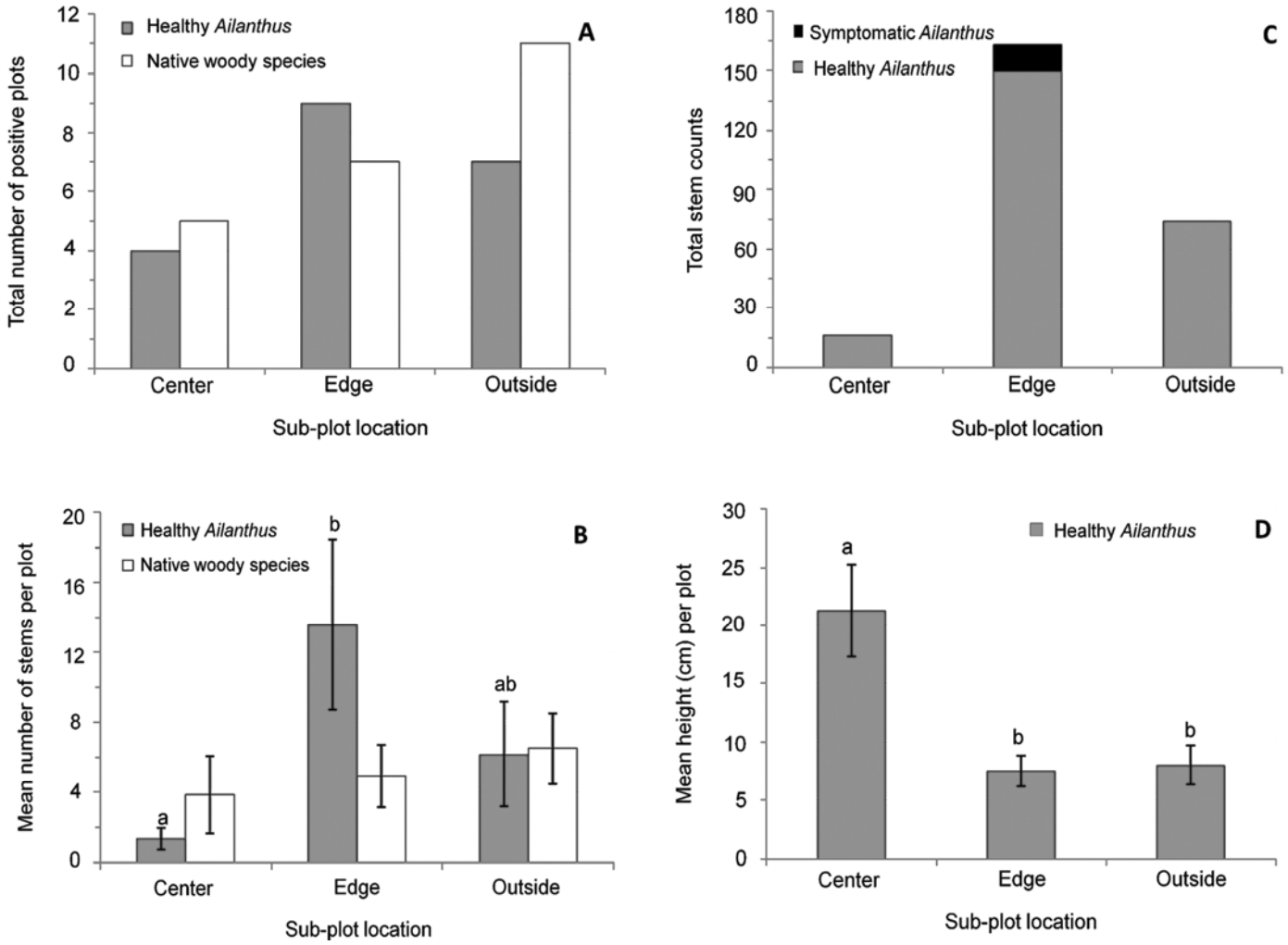

Fig. 5. Incidence of asymptomatic and symptomatic *Ailanthus* and native woody species and mean height of *Ailanthus* in regeneration subplots following wilt and mortality of canopy *Ailanthus* trees induced by *Verticillium nonalfalfae* isolate VnAa140 in south-central Pennsylvania. Subplots were established in pairs along east-west transects in opposing directions from a defined inoculation point in five *Ailanthus* stands inoculated between 2006 and 2009 and included the following locations: center of stand near initial inoculations; edge of diseased stand near most recent symptomatic stems; and outside, 30 m outside of the diseased stand, which served as a "pretreatment" control. **A to B,** Data collected from 36 4-m^2 circular subplots spanning five *Ailanthus* stands in summer 2011. Sample size is $n = 12$ for each of the three subplot types. **C,** Distribution of *Ailanthus* stems by subplot location for 20 positive subplots. **D,** Data collected from 12 4-m^2 circular subplots along two east-west transects in summer 2011 at Blue Mountain, 5 years post-inoculation. Sample size is $n = 12$ for each of the three subplot types and numbers of stems measured per subplot type are as follows: center ($n = 8$); edge ($n = 164$); and outside ($n = 70$). **B and D,** Significantly different means are indicated with different letters. Levels of significance are $P < 0.010$ and 0.050 for A and B, respectively. Bars indicate standard errors of the means.

tree species were American elm (*Ulmus americana* L.), red maple (*Acer rubrum* L.), sugar maple (*A. saccharum* Marsh.), and black cherry (*Prunus serotina* Ehrh.). Poison-ivy seedlings (*Toxicodendron radicans* (L.) Kuntze) were also abundant but were restricted to location CCSP. ANOVA revealed no significant differences in the mean number of native plants among center (4 plants/subplot), edge (5 plants/subplot), and outside (7 plants/subplot) subplots ($P = 0.644$) (Fig. 5B). Verticillium wilt symptoms were not observed in native regeneration, although plants were not destructively sampled to evaluate VD.

Multilocus sequence typing and species and mating type identification using PCR. Sequence data for 37 taxa were obtained from portions of three nuclear protein coding genes (EF-1α, GPD, and TS) as well as a portion of the IGS rDNA region in the study. Representative nucleotide sequences for previously uncharacterized lineages and gene regions, denoted in Table 2, were deposited in GenBank (accession numbers KF802860 to KF802866). Ten isolates from *A. altissima* and 18 other non-lucerne isolates previously identified as "*V. albo-atrum*" resolved to *V. nonalfalfae* and one previously designated "*V. albo-atrum*" isolate VaCb241, from *Catalpa bignonioides*, aligned with three *V. alfalfae* strains from alfalfa (Fig. 6). The 28 *V. nonalfalfae* isolates formed a clonal group along with holotype *V. nonalfalfae* isolate PD 592 from potato and are hereafter designated collectively as multilocus sequence type (MLST) 1 (Fig. 6). Isolates from gold kiwifruit (*Actinidia chinensis*; 2) were identified as *V. nonalfalfae* in a multiplex PCR but formed a distinct intraspecific group and are hereafter designated as MLST 2 (Fig. 6). The *V. albo-atrum–V. alfalfae–V. nonalfalfae* multiplex PCR and *V. nonalfalfae* simplex PCR (22) identified all isolates from the genus *Actinidia* as members of *V. nonalfalfae* (data not shown).

Thirty-six isolates were screened for mating type. *V. nonalfalfae* isolates, which included both MLST 1 and 2, were exclusively *MAT1-2-1*, whereas *V. alfalfae* isolates were exclusively *MAT1-1-1* (Supplemental Figure 2). All amplicons were sequenced and identified by a BLASTn search using GenBank. All *MAT1-1-1* amplicons showed 94% identity with GenBank accession AB505215 from *V. dahliae* whereas all *MAT1-2-1* amplicons showed 96% identity with GenBank accession AB505214 from *V. dahliae*.

DISCUSSION

The objectives of the current study were to compare the pathogenicity of *Verticillium* isolates from six plant species against *Ailanthus altissima*, evaluate the biocontrol efficacy of *V. nonalfalfae* isolate VnAa140 on *Ailanthus* canopy trees and shoot regeneration under various field conditions, and investigate the multilocus phylogenetic diversity of *V. nonalfalfae* isolates from lethal Ailanthus wilt epicenters in three states and from environments not associated with Ailanthus wilt.

Comparative pathogenicity testing revealed varying responses of six *V. nonalfalfae* and one *V. alfalfae* against a susceptible *Ailanthus* seed source. Isolates of *V. nonalfalfae* from deerbrush, eggplant, kiwifruit, and potato were nonpathogenic on *Ailanthus*, suggesting possible host adaptation in VnAa140 and VnAa100, both of which showed high virulence against *Ailanthus*. These results were independently confirmed for all *Ailanthus* isolates from Virginia and VnAa140 (45). Even though significant VD was observed on *Ailanthus* plants inoculated with *V. alfalfae* isolate VnMs102, the isolate did not induce wilting or mortality. VD alone may not be harmful to the host (37), because xylem occlusion can occur without inducing wilt or mortality, as observed previously for *V. dahliae* in *Ailanthus* (23). These effects appear to be transitory, following which the symptomatic plants may resume normal growth.

In south-central Pennsylvania and central Virginia, lethal wilt of *Ailanthus* has reached epidemic proportions (45), while few native flora have been affected. Naturally occurring *V. nonalfalfae* infections in Pennsylvania, along with artificial inoculations conducted from 2006 to 2009, have resulted in >22,000 dead canopy *Ailanthus* trees. Within one regions of Pennsylvania, injection of *V. nonalfalfae* inoculum into 100 trees eventually resulted in >14,000 dead *Ailanthus* as the pathogen spread from the inoculated trees to adjacent healthy *Ailanthus* trees during 2006 to 2011. Root sprouts, established after the death of overstory canopy *Ailanthus*, were also infected by *V. nonalfalfae* and accounted for an additional 10,000 to 15,000 dead *Ailanthus.* Furthermore, *V. nonalfalfae* was isolated from several naturally infected symptomatic striped maple (*Acer pensylvanicum* L.), devil's walkingstick (*Aralia spinosa* L.), and one mutliflora rose plant (*Rosa multiflora* Thunb.) but represented only a few individuals as compared to a few thousand otherwise healthy stems. Thus, VnAa140 appears to be host-adapted to *Ailanthus.*

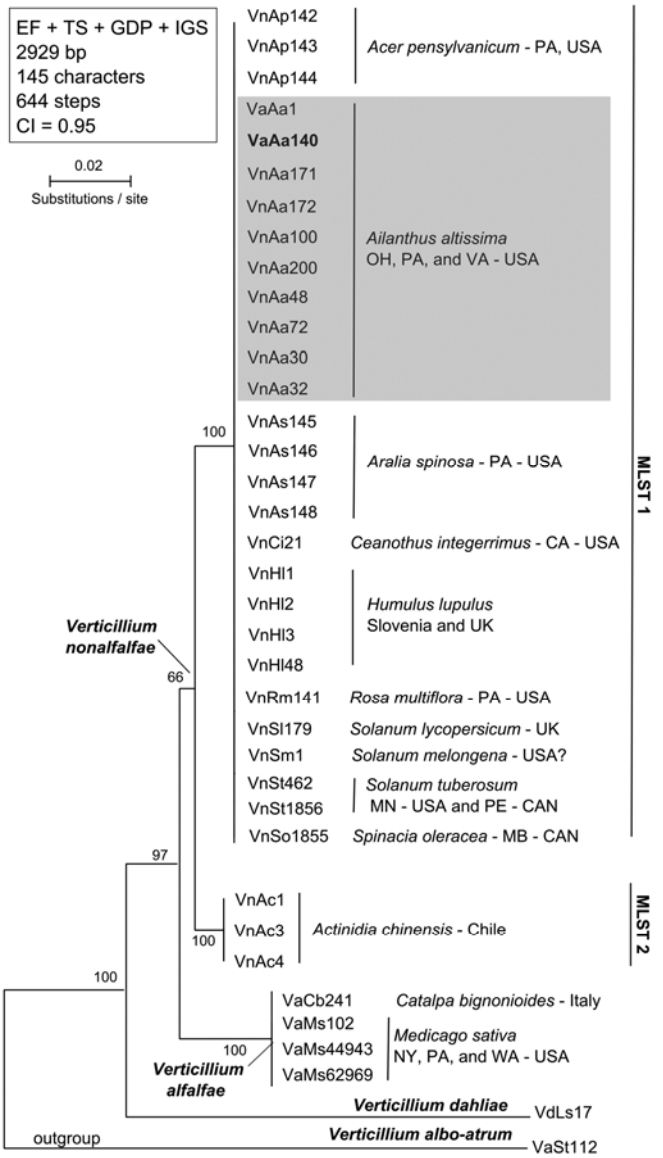

Fig. 6. Molecular phylogeny of phytopathogenic *Verticillium* sp. from *Ailanthus* (gray box) and other perennial woody and crop hosts inferred from partial elongation factor 1-α (EF), glyceraldehyde-3-phosphate dehydrogenase (GPD), tryptophan synthase (TS), and intergenic spacer (IGS) sequences. *Verticillium albo-atrum* isolate VaSt112 was used to root the tree. The maximum likelihood (ML) tree was inferred from 2,929 nucleotide characters, of which 145 were shared derived characters. ML bootstrap values are indicated at nodes based on a 1,000 pseudoreplicates of the data. MLST = multilocus sequence type.

V. nonalfalfae has been reported to cause disease in only two tree species in the forest setting—a *Ceanothus* sp. in California (13) and yellow-poplar in Delaware (28)—although infections of various woody hosts in urban environments by "*V. albo-atrum*" likely caused by *V. nonalfalfae* have been reported (10,18,19,32). Additional studies are needed to validate these earlier reports because more recent investigations by Schall and Davis (40) invalidated yellow-poplar and other previously designated "susceptible" species as hosts for *V. nonalfalfae*. Our recent discovery of *V. nonalfalfae* in a forest setting <12 km from an earlier reported infection suggests that *V. nonalfalfae* is pervasive on *Ailanthus* in western Pennsylvania. In addition, because *V. nonalfalfae* has now been confirmed killing *Ailanthus* in at least nine locations spanning three states (36,39,44,45), *Ailanthus* and *V. nonalfalfae* may now be occupying the same ecological niches and Verticillium wilt of *Ailanthus* may become more common as environmental conditions favor infection.

Few potential *Ailanthus* biocontrol candidates have been previously characterized (6). In addition to *V. nonalfalfae* (39,40), the nonindigenous weevil *Eucryptorrhynchus brandti* (Coleoptera: Curculionidea) (17,46) and *Fusarium oxysporum* f. sp. *perniciosum* (Hepting) Toole (47) have also been proposed for control of *Ailanthus* in the eastern United States, though there are no data currently available regarding their field efficacy, nor has *F. oxysporum* f. sp. *perniciosum* been etiologically validated as a pathogen of *Ailanthus*.

Unintended geographic dispersal of potential biological controls beyond their intended range is of concern (33). The risk of unintentional spread of a biocontrol weevil, *Oxyops vitiosa* Pascoe, released to combat the paperbark tree (*Melaleuca quinquenervia* (Cav.) S. T. Blake) in Florida (33), appears to have resulted in its spread to New Providence Island, the Bahamas, Puerto Rico, and California, where it is thriving on landscape plantings of Melaleuca trees (33). Ultimately, reluctance toward using nonindigenous biocontrol agents is largely driven by economics. The need for effective biocontrol agents is often overshadowed by the significant investment of effort, time, and capital required to produce, register, and market the agents (11). Therefore, there is a strong impetus for development and utilization of controls that fall outside the registered pesticide model, including indigenous biocontrol agents (11) such as *V. nonalfalfae* on *Ailanthus*.

Indigenous fungal biocontrols have been used sporadically in the United States for nearly 50 years to manage native and exotic tree species. In Arkansas and south-central Oklahoma, persimmon wilt, caused by *Acremonium diospyri* (Crand.) W. Gams, has been used to control unwanted persimmon trees (*Diospyros virginiana* L.) in pastures (51). Similarly, in central Minnesota, oak wilt, caused by *Ceratocystis fagacearum* (Bretz) Hunt, has been previously tested as a silvicide for low-value oak trees (*Quercus* spp.) competing on sites better suited for pine (9). These two biocontrols have proven very effective at the local level and *A. diospyri* is still used to control persimmon (50). In New Brunswick, Canada, *Chondrostereum purpureum* (Fr.) Pouzar has also been used to combat "weed" red alder (*Alnus rubra* Bong.) and aspen (*Populus* spp. L.) (12). *Neofusicoccum batangarum* Begoude et al. was recently described to cause germination failure, wilting, dieback, and sapling mortality of the invasive Brazilian peppertree (*Schinus terebinthifolius* Raddi) in Florida (42). *F. oxysporum* f. sp. *perniciosum* (47) has proven lethal to exotic silk trees throughout the eastern United States (15) and could serve as an effective biocontrol of silk trees along highway corridors. More recently, a nonpathogenic strain of "*V. albo-atrum*" being sold commercially under the name "Dutch Trig," has been used to potentially protect elm trees against Dutch elm disease (41).

The efficacy of *V. nonalfalfae* against *Ailanthus* compares favorably with other successful biocontrol programs. In South Africa, the nonindigenous, gall-forming rust fungus *Uromycladium tepperianum* (Sacc.) McAlpine has been used successfully since 1987 to combat the invasive *Acacia saligna* (Labill.) H. L. Wendl. (29,52,53). A 15-year study revealed an 87 to 98% reduction in *Acacia* density, from ≈50,000 to ≈2,400 stems/ha in five sites following inoculation of 50 trees/site (53). In the Florida Everglades, where the invasive Melaleuca tree has had catastrophic effects on freshwater habitats, implementation of insect biocontrols has resulted in a 70% reduction in mean stem densities from ≈27,500 to 8,000 stems/ha during 1997 to 2005 (35). Similarly, *V. nonalfalfae* decreased *Ailanthus* stem densities from 1,500 canopy stems/ha to nearly zero at the leading edge of the epidemic within just 3 years. Ultimately, all inoculated *Ailanthus* overstory trees and adjacent noninoculated *Ailanthus* trees died from Verticillium wilt, and the pathogen continues to spread 7 to 10 years after inoculation.

Ailanthus sprouts generally survived for short periods within inoculated stands. The leading edge of diseased *Ailanthus* stands supported >10 times the number of *Ailanthus* sprouts compared with the center of the diseased stand and twice the number of *Ailanthus* outside the diseased area. These results indicate that vegetative sprouting may be the final reproductive strategy of *Ailanthus* in response to Verticillium wilt (39). Indeed, wilt symptoms on *Ailanthus* sprouts occurred only along the edge of the diseased area, where canopy symptoms had recently developed. Losses of second-generation spouts following mortality of *Ailanthus* parent trees likely accounts for an additional 10,000 to 15,000 diseased and dying *Ailanthus*. Our field observations indicate that surviving *Ailanthus* sprouts may live for several years, perhaps reaching 3 m in height, but eventually succumb to Verticillium wilt.

The incidence of native woody perennials in the regeneration subplots was not significantly different among subplot locations, suggesting that some native species persist among dying *Ailanthus* despite competition from other encroaching invasive species. This observation has been independently reported (14). Regardless of these findings, species richness is significantly reduced compared with sites without *Ailanthus* (30). We speculate that native species will flourish in the absence of *Ailanthus* as the stands grow and competition for resources and light intensifies.

All *V. nonalfalfae* isolates from *Ailanthus* and 18 additional isolates from nine other host species were members of the same MLST, MLST 1. Interestingly, variation in pathogenicity was observed within this single sequence type, which had not been observed in previous cross-pathogenicity testing among "*V. albo-atrum*" isolates (5), thereby further validating host-adaption in *Ailanthus* isolates. Molecular characterization confirmed reclassification of numerous isolates from the morphologically identical *V. albo-atrum* to *V. nonalfalfae,* and supports the importance of clonal reproduction in *V. nonalfalfae* strains from North America and Europe. This study also documented previously unsampled diversity within *V. nonalfalfae*, because three isolates associated with lethal wilt of *Actinidia chinensis* in Chile appeared to be divergent from the known diversity of *V. nonalfalfae* (2) and represent a second MLST within *V. nonalfalfae* (MLST 2) (Fig. 6). Phylogenetic analysis also revealed that VaCb241, which was isolated from *Catalpa bignonioides* in Italy, appears to be the first report of *V. alfalfae* recovered from a presumably naturally infected non-lucerne host.

The use of *V. nonalfalfae* VnAa140 appears to be a highly effective tool to reduce problematic *Ailanthus* populations. *Ailanthus* was first introduced in Philadelphia nearly 230 years ago (23). Although best known as "a tree that grows in Brooklyn" (43), *Ailanthus* is common in major cities in at least 40 states throughout the United States (1,23,27). In recent decades, *Ailanthus* has invaded many forests throughout the eastern United States, where it poses serious threats to native plant diversity. *V. nonalfalfae* causes mortality in *Ailanthus* and disseminates rapidly within *Ailanthus* stands of various ages and densities,

presumably through infection from the rhizosphere (25) and via root grafts (26). In addition, integration of VnAa140 with the biocontrol weevil *E. brandti* may facilitate long-range dispersal of *V. nonalfalfae* (46). Similarly, the exotic ambrosia beetle *Euwallacea validus* (Coleoptera: Scolytinae) has been implicated in the passive dissemination of *Verticillium* (24,38). On the basis of the results of the current study, as well as concurrent host range testing, root-graft transmission studies, and inoculation formulation studies, VnAa140 should be considered a viable biocontrol of *Ailanthus altissima*.

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