Ailanthus altissima Wilt and Mortality: Etiology

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ABSTRACT

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Extensive, unprecedented wilt and mortality of the highly invasive tree-of-heaven (*Ailanthus altissima*) occurred recently within mixed-hardwood forests in south-central Pennsylvania. Until this study, the cause of the epidemic was unknown. *Verticillium albo-atrum* was consistently isolated from symptomatic *Ailanthus* seedlings and trees in areas having high levels of mortality, whereas *V. dahliae* was isolated from small scattered patches of diseased *Ailanthus*. Inoculations of potted *Ailanthus* seedlings in the greenhouse, as well as canopy trees in the field, revealed that both *V. albo-atrum* and *V. dahliae* were capable of infecting *Ailanthus,* but *V. albo-atrum* was more pathogenic. All *Ailanthus* seedlings and canopy trees inoculated with *V. albo-atrum* died within 3 months. In contrast, *Ailanthus* seedlings and canopy trees inoculated with *V. dahliae* became symptomatic, but 84% were still living 1 year following inoculation. We conclude that the major cause of *Ailanthus* wilt within forests of south-central Pennsylvania is *V. albo-atrum*. Pending further studies, including risk analysis, *V. albo-atrum* may be considered as a possible biocontrol agent for invasive *Ailanthus*.

Tree-of-heaven (*Ailanthus altissima* (Miller) Swingle), henceforth referred to as *Ailanthus*, is an exotic, highly invasive, undesirable tree species introduced into Pennsylvania in 1784 that has since spread across most of the United States (19). *Ailanthus* has many characteristics that make it undesirable within both urban and forested areas. The pollen is an allergen (3), and leaves and stems may cause contact dermatitis (5). *Ailanthus* trees shed numerous leaf rachises that are a nuisance to homeowners. Practically every part of the tree, including leaves and wood, has a very disagreeable odor when crushed (10). Even flowers produce a foul odor, making *Ailanthus* unsuitable as a flowering ornamental (19). *Ailanthus* wood is light and weak, often breaking in windstorms or when weighed down with snow or ice (30). Weak, brittle wood makes *Ailanthus* trees dangerous along highways and in public areas, since the species is prone to limb fall and stem breakage.

Although *Ailanthus* is usually a short-lived tree, surviving for 30 to 50 years, it is very fast growing and can reach a maximum height of 27 m and trunk diameter of 1 m at maturity (19). Female trees are prolific seed producers, capable of producing more than 300,000 seeds/tree on trees as young as 12 years old. Seeds are produced in the middle of flat twisted samaras and are very light, making them well suited for long-range wind dispersal. Seeds can be dispersed immediately after ripening, but usually persist on trees through winter and are dispersed in the spring (7,19). Seeds do not require stratification and germinate quickly. Resultant seedlings grow vigorously, reaching a height of 1 to 2 m within the first growing season. Dense thickets of *Ailanthus* often arise beneath parent trees from seed and root sprouts.

Ailanthus produces several quassinoids including ailanthone, amarolide, acetyl amarolide, 2-dihydroailanthone, ailanthinone, chaparrin, chaparrinone, quassin, neoquassin, shinjulactone, and shinjudilactone (13). These quassinoids possess phytotoxic allelopathic properties (16) and have strong herbicidal activity on more than 35 broadleaved and 34 coniferous species (18), giving invasive *Ailanthus* plants an advantage over competing plant species. *Ailanthus* is a vigorous competitor and quickly becomes established and thrives in varied niches where it is unwanted, such as agricultural fields, forests, and roadsides. Various measures have been attempted to control *Ailanthus* in such areas, including pulling and digging; tree felling or girdling; mowing and chipping aboveground plant parts; and burning. Such control measures usually need to be accompanied by stump treatments using herbicides, or roots and stumps will sprout aggressively (27). However, use of chemical herbicides has come under increasingly rigid restrictions, and weed species such as *Ailanthus* can develop resistance to chemical controls, leading to consideration of biocontrols.

Biocontrol of undesired plant species with plant pathogens has been considered for decades (26) and may be the most promising control option for *Ailanthus*. However, biocontrol for *Ailanthus* has not progressed beyond the theoretical stage due to lack of a suitable pathogen. Farr et al. (9) and Ding et al. (6) listed 68 fungi that occur on *Ailanthus,* but many are minor pathogens or saprophytes, not suitable as potential biocontrol agents.

In 2002 to 2003, we observed unprecedented, extensive *Ailanthus* wilt and mortality within oak-dominated, mixed-hardwood forests in south-central Pennsylvania. As of fall 2008, more than 8,000 canopy *Ailanthus* trees had died within the region from an unknown cause. The objective of this study was to determine the etiology of *Ailanthus* wilt and mortality. Forthcoming papers will discuss host range, potential agents of dissemination, isolate variability, and potential for biocontrol.

MATERIALS AND METHODS

Study area. The study area, located in Franklin and Perry counties within the Tuscarora State Forest in south-central Pennsylvania (Fig. 1), is an oak-dominated, mixed-hardwood forest that contains numerous wilting and dying *Ailanthus* seedlings, root sprouts, saplings, and canopy trees. Associated cohort tree species within affected forest stands include American basswood (*Tilia americana* L.), black locust (*Robinia pseudoacacia* L.), black oak (*Quercus velutina* Lam.), chestnut oak (*Quercus montana* Willd.), northern red oak (*Quercus rubra* L.), red maple (*Acer rubrum* L.), shellbark hickory (*Carya laciniosa* Michx. f.), striped maple (*Acer pensylvanicum* L.), sugar maple (*Acer saccharum* Marsh.), sweet birch (*Betula lenta* L.), white ash (*Fraxinus americana* L.), and yellow-poplar (*Liriodendron tulipifera* L.). The understory of affected stands consists primarily of *Ailanthus* seedlings and root sprouts, a small component of sweet birch and red maple seedlings, and minor amounts of grasses, forbs, and ferns in open areas. The study area receives approximately 100 cm annual precipitation, has an average annual temperature of 18°C and an average growing season (1 April to

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31 August) temperature of 26°C (31). Soils are Dystrochrepts bouldery, Meckesville very stony silt loam, Hazelton extremely stony sandy loam, Buchanan very stony loam, and Weikert shaly silt loam (17,31).

The 56-ha forest stand selected for this study contained numerous wilting and dying *Ailanthus* trees. Oak trees formerly dominated the stand canopy, but had been defoliated by gypsy moth (*Lymantria dispar* L.) in the early 1980s. Following oak defoliation and mortality, the stand was subsequently salvage-harvested in 1984, resulting in open areas ideal for *Ailanthus* invasion. Approximate year of *Ailanthus* establishment was determined by felling 50 of the largest *Ailanthus* trees in the stand and counting annual growth rings. Ring counts revealed that most *Ailanthus* had invaded the stand during 1984 to 1985.

Fungal isolation. Leaves, stems, and wood chips from 36 symptomatic *Ailanthus* seedlings and trees throughout the study area were collected during summers of 2005 and 2006. Samples were returned within 6 h to the laboratory, flame-sterilized, and cultured onto potato dextrose agar (PDA). Cultures were grown in the dark within a controlled environment chamber at 21°C for 3 weeks, at which time culture morphology was examined using light and scanning electron microscopy. *Verticillium* was consistently isolated. Therefore, all additional samples were cultured on plum extract agar (PEA; 900 ml distilled water, 20 g agar, 100 ml concentrated plum extract, 1 g yeast, 5 g lactose, pH 5.6 to 6.0), a semiselective medium for *Verticillium* (2), amended with streptomycin and neomycin. All previously collected *Verticillium* samples were transferred from PDA to PEA and maintained at 22°C in the dark.

Molecular identification. A total of 48 *Verticillium* isolates were collected from symptomatic *Ailanthus* and grown on PEA. Mycelium from 19 isolates was transferred to sterile culture tubes containing potato dextrose broth (EM Science, Gibbstown, NJ) and incubated at room temperature for 14 to 21 days without shaking. Mycelium was recovered from the broth by filtration, lyophilized (24), DNA extracted using a DNeasy Plant Minikit (Qiagen Inc., Hilden, Germany), and concentration determined by electrophoresis in a 1% agarose gel. A standard polymerase chain reaction (PCR) protocol was used to amplify the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA gene repeat. Forward primer ITS5 and reverse primer ITS4 (28) were used in the PCR reaction to amplify a 566-bp product of the ITS region, which was used in identification (8). The PCR reaction mixture (50 µl) included 0.2 mM dNTP, 2.0 mM $MgCl_2$, 0.2 mM primer ITS5, 0.2 mM primer ITS4, 1 unit GoTaq DNA polymerase (Promega Corp, Madison, WI), and 1 µl of undiluted DNA template. PCR was performed in a PTC-100 Programmable Thermal Cycler (MJ Research Inc., Waltham, MA); parameters were 2 min at 95°C, 35 cycles of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C; and a 5-min extension at 72°C. Products were separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and visualized under UV light. PCR products were purified using an EXOSAP-IT PCR Purification Kit (USB Corporation, Cleveland, OH). Purified amplification products were sequenced at the Pennsylvania State University Nucleic Acid Facility on an ABI 3730XL automated DNA sequencer, using both ITS5 and ITS4 primers. Consensus sequence data were assembled and edited using Sequencher 3.1 (Gene Codes, Ann Arbor, MI) from both forward and reverse DNA sequences. Consensus sequences were analyzed using NCBI's BLASTN (National Center for Biotechnology Information, Bethesda, MD) for species identification.

Seedling inoculations. *Ailanthus* seeds were collected during March 2005 from a tree on the Pennsylvania State University campus, University Park, and placed in greenhouse flats containing a blend of 20% Hagerstown silt loam topsoil and 80% Metro Mix 200 (The Scotts Company, Marysville, OH). Resultant seedlings were transplanted to 6 × 25 cm tubes containing the same potting medium and maintained in a greenhouse. Seedlings were fertilized once with 12:12:12 ($N:P_2O_3:K_2O$) Osmocote (Scotts Miracle-Gro, Marysville, OH).

Isolate PSU140 of *V. albo-atrum* (GenBank accession no. FJ424082) and isolate PSU154 of *V. dahliae* (GenBank accession no. FJ424083) were randomly selected from a suite of 19 isolates that had been identified molecularly. To ensure isolates had not lost pathogenicity in culture, *Ailanthus* seedlings were inoculated with both *Verticillium* isolates in January 2006 and respective *Verticillium* species reisolated. Reisolated *Verticillium* cultures were used in all following studies. Conidia were harvested from PEA culture plates and spore concentrations adjusted to 10^7 conidia ml^{-1} in sterile distilled water (23). Inoculum viability, indicated by at least 75% spore germination, was confirmed immediately prior to inoculation. Twenty-five seedlings were inoculated on 3 March 2006 with either *V. albo-atrum* or *V. dahliae* isolate at two points on the stem (4), using a 1-ml syringe containing 0.1 ml of 1 × 10^7 spores ml^{-1}. At time of inoculation, seedlings averaged 30 cm tall, 0.5 cm diameter, and had 4 to 8 leaves. Twenty-five control plants were stem-injected with sterile distilled water. Each *Verticillium*-inoculated treatment was replicated three times ($n = 3 \times 25$). Controls were not replicated ($n = 25$). Treatments were applied in a completely randomized design.

Seedling height (cm) and disease severity were measured weekly for 15 weeks after inoculation. Total cumulative height was measured from the soil line to the plant apex. Disease severity was rated using a 0 to 4 scale related to symptom progression: 0 = healthy leaves, 1 = chlorotic leaves, 2 = necrotic leaf margins, 3 = wilting leaves, and 4 = dead or defoliated seedlings (2). Line graphs were drawn to illustrate temporal trends in disease severity and cumulative height for inoculated and control *Ailanthus* seedlings. At the end of each experiment, leaf tissue was sampled and cultured onto PEA.

At the end of summer 2006, all seedlings inoculated with *V. albo-atrum* were dead, but 84% of *Ailanthus* seedlings inoculated with *V. dahliae* were still alive. The latter seedlings, as well as noninoculated controls, were placed in a cold environment to undergo dormancy for the winter. Seedlings were returned to the greenhouse on 14 March 2007 to break dormancy. Budbreak and leaf emergence, as an indicator of seedling viability, was measured 3 weeks later. Each inoculated and control seedling was rated on a 0 to 3 scale, based on characteristics of the apical bud: 0 = bud dormant, 1 = bud swollen, 2 = bud swollen but leaves unexpanded, and 3 = bud broken and leaves expanded. Since some overwintered seedlings had dead stem tips, length (cm) of tip necrosis on

Fig. 1. Location of the field study area (closed circle) within oak-dominated, mixed hardwood forests of Franklin and Perry counties in south-central Pennsylvania.

the terminal stem was measured on all seedlings.

2006 Canopy tree inoculations. Ten stands, which contained numerous canopy *Ailanthus* trees and lack of *Verticillium* disease symptoms, were randomly selected for field inoculations in 2006. Stands ranged from 0.3 to 1.0 ha and were spaced approximately 1 km apart. Within each stand, five co-dominant *Ailanthus* trees (average height = 20 m, trunk diameter = 16.5 cm) were randomly selected and inoculated on 21 April 2006. *Ailanthus* trees in four stands were inoculated with *V. albo-atrum* isolate PSU140 (20 trees), trees in five stands were inoculated with *V. dahliae* isolate PSU154 (25 trees), and one stand served as a control (5 trees). Each group of 5 trees/stand was the experimental unit and served as a replicate.

The base of each trunk was surface-sterilized with 70% ethanol, which was allowed to evaporate. Three wounds, 7 to 10 cm long, were made on each trunk with a sterilized ax and 1.5 ml of 1×10^7 conidia ml^{-1} sprayed into each wound. Inoculum viability, indicated by at least 75% spore germination, was confirmed immediately prior to inoculation. Five trees in the control stand received 1.5 ml of sterile distilled water in each sterile wound. Disease severity was rated periodically from time of inoculation until 10 September 2006 using the previously described 0 to 4 scale.

2007 Canopy tree inoculations. The 2006 canopy tree inoculations with *V. albo-atrum* had induced disease symptoms more rapidly than anticipated. During our first evaluation in week 7 of 2006, severe symptoms had already developed on inoculated trees. Therefore, additional *Ailanthus* trees were inoculated with *V. albo-atrum* (only) in 2007 and ratings made at 2, 4, and 6 weeks after inoculation. Inoculations were made on 5 May 2007 with *V. albo-atrum* isolate PSU140 using a Hypohatchet (OEM Fabricators Inc., Woodville, WI) that delivered 1 ml of inoculum per tree each time a tree was struck. Preliminary studies indicated a Hypohatchet was an efficient method for delivering inoculum. Five trees within each of five stands (replicates) were randomly selected and inoculated (25 trees) at each of three areas on the trunk base with 1 ml of 1×10^7 spores ml^{-1} in sterile distilled water. Inoculum viability was indicated by at least 75% spore germination immediately prior to inoculation. Five trees within one control stand (replicate) were randomly selected and wounded with a sterile ax and each wound treated with sterile distilled water. Control treatments were not replicated. Inoculated and control trees were observed biweekly and rated using the previously described 0 to 4 scale.

Statistical analyses. ANOVA revealed that canopy tree disease severity ratings from 2006 and 2007 were not significantly different, and they were pooled for analyses. The temporal trend in seedling disease severity, seedling height growth, and canopy tree disease severity (Figs. 2 to 4) were evaluated using area under disease progress curve (AUDPC; 15):

$$[(y_i + y_{i+1})/2)][t_{i+1} - t_i]$$

where y_i is the disease severity rating or height, t_i is the time of the ith rating, and $i = 1, 2, 3 \ldots n - 1$ (5,11). Analysis of variance (ANOVA) and Tukey's mean comparisons (20) were used to detect significant differences ($P < 0.001$) among AUDPC data. ANOVA and Tukey's comparisons tests were used to evaluate the effect of *V. dahliae* on budbreak and length of terminal dieback on overwintered seedlings.

RESULTS AND DISCUSSION

Culture morphology on PEA. Light and electron microscopy revealed that fungi consistently isolated from symptomatic *Ailanthus* seedlings and trees produced white hyphal colonies and verticillate conidiophores, indicative of *Verticillium*. Most cultures formed heavily melanized hyphae but no microsclerotia, and were putatively identified as *V. albo-atrum*. Occasional cultures produced abundant microsclerotia and were putatively identified as *V. dahliae*, awaiting molecular confirmation.

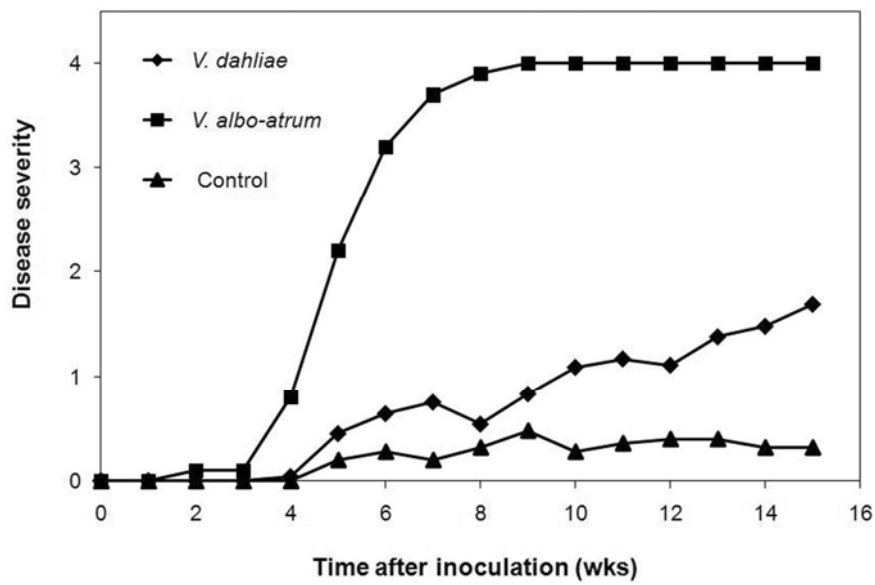

Fig. 2. Disease severity of potted *Ailanthus* seedlings at various times following stem injection with 0.1 ml of 1×10^7 spores ml^{-1} *Verticillium albo-atrum* or *V. dahliae* conidia. Control plants were injected with sterile distilled water. Disease severity was rated using a 0 to 4 scale related to symptom progression: 0 = healthy leaves, 1 = chlorotic leaves, 2 = necrotic leaf margins, 3 = wilting leaves, and 4 = dead or defoliated seedlings.

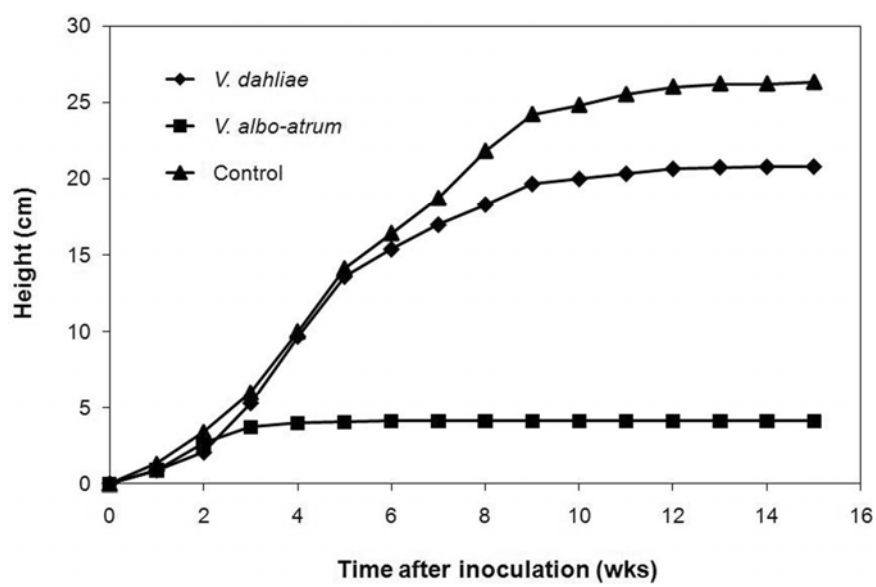

Fig. 3. Cumulative height (cm) of potted *Ailanthus* seedlings at various times following stem injection with 0.1 ml of 1×10^7 spores ml^{-1} *Verticillium albo-atrum* or *V. dahliae* conidia. Height was measured from pot soil line to plant apex. Control plants were injected with sterile distilled water.

Molecular identification. Results of the BLAST search from putative *V. albo-atrum* PSU140 most closely matched the ITS sequence of *V. albo-atrum* GenBank accession no. AB458830 (strain no. VA001, e-value = 0.0) with only one nucleotide difference in 512 bp. The 531-bp ITS sequence of putative *V. dahliae* PSU154 was an exact match with *V. dahliae* GenBank accession no. AF104926 (strain cotton001, e-value = 0.0). The BLAST results confirmed all putative species identifications based on culture morphology. Fifteen of the 19 isolates proved to be *V. albo-atrum* and four were *V. dahliae*.

V. albo-atrum isolates were collected from areas of *Ailanthus* wilt that had a high incidence of mortality. In contrast, *V. dahliae* isolates were collected from small, scattered patches of wilting *Ailanthus* that contained few dead trees.

Seedling inoculations. ANOVA performed on the overall AUDPC data at the end of the experiment revealed significant differences among treatments for both disease severity ratings and height growth. Based on Tukey's mean comparisons test, *Ailanthus* seedlings inoculated with *V. albo-atrum* had significantly greater disease severity and significantly less height growth than seedlings inoculated with *V. dahliae* or the noninoculated controls (Fig. 2). Disease severity values for seedlings inoculated with *V. dahliae* were significantly greater than controls. Heights of seedlings inoculated with *V. albo-atrum* were significantly less than those inoculated with *V. dahliae*, as well as controls. Heights of seedlings inoculated with *V. dahliae* were not significantly different from noninoculated controls (Fig. 3).

The speed at which symptoms developed was clearly illustrated by the seedling inoculation results. *Ailanthus* seedlings inoculated with *V. albo-atrum* exhibited a rapid increase in disease severity between weeks 4 and 7 (Fig. 2). By week 5, inoculated seedlings had chlorotic leaves with necrotic leaflet tips and began to wilt. Defoliation, which involved loss of the entire compound leaf with leaflets still attached to the rachis, developed rapidly in week 6. Height growth of seedlings inoculated with *V. albo-atrum* essentially ceased by week 3 (Fig. 3), and all inoculated seedlings were dead by week 10.

In contrast, seedlings inoculated with *V. dahliae* exhibited a very slow, gradual increase in disease severity from time of inoculation until the end of the growing season at week 15 (Fig. 2). Inoculated seedlings initially exhibited chlorosis, starting with the oldest leaflets at the base of the rachis. Chlorotic leaflets progressively wilted and defoliated slowly in a systematic manner from the base of the rachis to the leaf apex. After all leaflets had fallen, the bare rachis abscised.

Most seedlings inoculated with *V. dahliae* were still alive at the end of the experiment (week 16), and approximately 84% were still alive during the spring following dormancy. However, overwintered seedlings had significantly greater terminal dieback (mean = 55.7%) than noninoculated controls (mean = 1.3%) and exhibited significantly delayed budbreak (3 weeks) compared with control seedlings. In addition, only 52.6% of the inoculated, overwintered seedlings broke bud. Noninoculated control seedlings exhibited normal timing and incidence of budbreak.

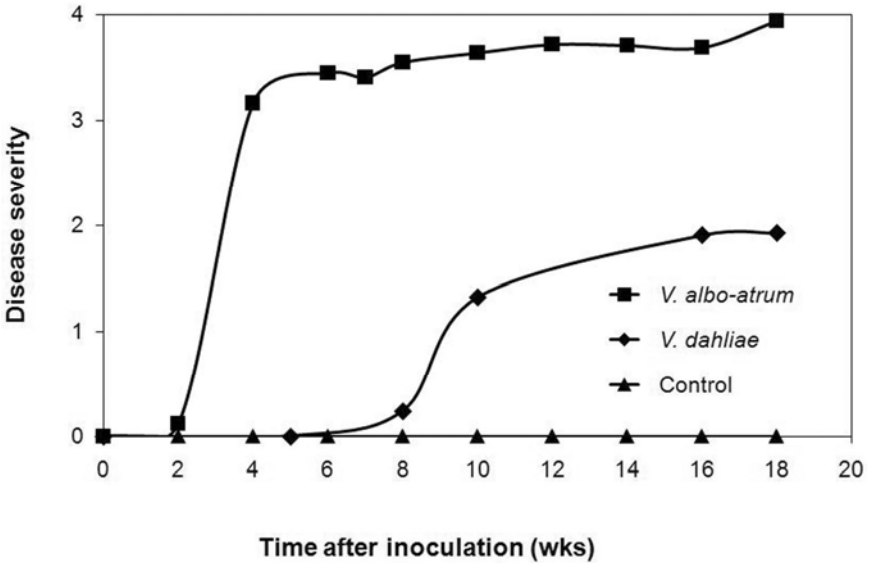

Fig. 4. Disease severity of *Ailanthus* canopy trees following three stem inoculations per tree with *Verticillium albo-atrum* or *V. dahliae* conidia at concentrations of 1.5 ml of 1×10^7 spores ml^{-1} in 2006 and 1 ml of 1×10^7 spores ml^{-1} in 2007. Control trees were treated with sterile distilled water. Disease severity was rated using a 0 to 4 scale related to symptom progression: 0 = healthy leaves, 1 = chlorotic leaves, 2 = necrotic leaf margins, 3 = wilting leaves, and 4 = dead or defoliated seedlings. The 2006 and 2007 disease severity values were not significantly different and were pooled.

Reisolation of the two *Verticillium* species fulfilled Koch's postulates (1), proving that both *V. albo-atrum* and *V. dahliae* were pathogenic to *Ailanthus* seedlings and capable of inducing *Ailanthus* wilt and mortality. However, *V. albo-atrum* was much more pathogenic than *V. dahliae*. Greater pathogenicity of *V. albo-atrum*, as compared to *V. dahliae* on the same host species, has been reported for yellow-poplar trees, as well as crops including hop (*Humulus lupulus* L.), cotton (*Gossypium* spp.), lucerne (*Medicago sativa* L.), potato (*Solanum tuberosum* L.), strawberry (*Fragaria* spp.), sainfoin (*Onobrychis* spp.), clover (*Trifolium* spp.), tomato (*Lycopersicon lycopersicum* L.), and cucumber (*Cucumis sativus* L.) (14,21).

Canopy tree inoculations. As with seedlings, *Ailanthus* trees inoculated with *V. albo-atrum* exhibited significantly greater disease levels than *Ailanthus* trees inoculated with *V. dahliae*. Control trees remained asymptomatic. ANOVA revealed that disease severity values for 2006 and 2007 inoculations with *V. albo-atrum* were not significantly different and datasets were pooled. Tukey's mean comparisons test revealed that *Ailanthus* trees inoculated with either *V. albo-atrum* or *V. dahliae* had significantly greater disease AUDPC than noninoculated controls. Trees inoculated with *V. albo-atrum* had significantly greater AUDPC than those inoculated with *V. dahliae*. It was very apparent that *V. albo-atrum* was much more pathogenic overall than *V. dahliae* to both seedlings and canopy trees of *Ailanthus* (Figs. 2 and 4).

Ailanthus trees inoculated with *V. albo-atrum* exhibited rapid increase in disease severity by week 4 (Fig. 4), at which time symptoms appeared rather uniformly throughout the entire crown, and many trees were 50% defoliated. Symptoms included sudden wilting of entire compound leaves, with leaflets and rachis intact, followed by defoliation. By week 8, many trees were dying and disease severity remained level. By week 18, all canopy trees inoculated with *V. albo-atrum* were dead (Fig. 4).

As with seedlings, canopy *Ailanthus* trees inoculated with *V. dahliae* exhibited slow developing, less severe, and rather erratic symptoms compared to trees inoculated with *V. albo-atrum*. Trees inoculated with *V. dahliae* exhibited only a slight increase in disease severity by week 8 (Fig. 4), but disease severity then increased steadily through week 16 (Fig. 4), at which time the trend in severity values became level. *Ailanthus* trees inoculated with *V. dahliae* wilted irregularly in the canopy, with symptoms often appearing on branches on opposite sides of the crown. Occasionally, the upper canopy remained asymptomatic, while lower branches exhibited defoliation. At other times, the entire canopy of inoculated trees exhibited

only slight wilting. All trees inoculated with *V. dahliae* were still alive the following spring after overwintering.

V. dahliae is often associated with wilting woody plants in North America, but usually within nurseries, orchards, and landscape settings (25). There are only two reports, in addition to this paper, of *Verticillium* associated with wilting trees within a forest setting in North America: *V. albo-atrum* from wilting *Liriodendron* seedlings in Delaware (22) and wilting *Ceanothus* in California (12).

V. albo-atrum has potential to serve as an effective biocontrol agent for the highly invasive *Ailanthus.* Use of fungal pathogens as biological silvicides has been explored in pathosystems involving trees. Persimmon wilt, caused by *Cephalosporium diospyri* Crandall, has been used to control unwanted persimmon trees within pastures in Arkansas for many years (29). Oak wilt, caused by *Ceratocystis fagacearum* (Bretz) Hunt, was suggested as a control for low-value oak trees on sites better suited for pine production in Minnesota (11). However, before *V. albo-atrum* can be considered as a biological control agent for *Ailanthus*, additional determinations must be made, including host range, molecular characteristics of additional isolates, means of dissemination, and risk analyses.

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