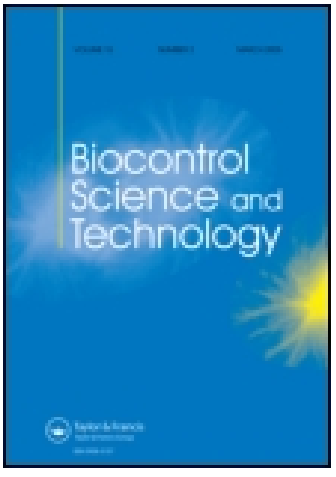

Biocontrol Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/cbst20

Biocontrol of Ailanthus altissima: inoculation protocol and risk assessment for Verticillium nonalfalfae (Plectosphaerellaceae: Phyllachorales)

E.S. O'Neal[a] & D.D. Davis[a]

[a] Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, University Park, PA, USA

Accepted author version posted online: 25 Feb 2015.Published online: 11 Apr 2015.

To cite this article: E.S. O'Neal & D.D. Davis (2015) Biocontrol of Ailanthus altissima: inoculation protocol and risk assessment for Verticillium nonalfalfae (Plectosphaerellaceae: Phyllachorales), Biocontrol Science and Technology, 25:8, 950-969, DOI: 10.1080/09583157.2015.1023258

To link to this article: http://dx.doi.org/10.1080/09583157.2015.1023258

Biocontrol Science and Technology, 2015
Vol. 25, No. 8, 950–969, http://dx.doi.org/10.1080/09583157.2015.1023258

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

Biocontrol of *Ailanthus altissima*: inoculation protocol and risk assessment for *Verticillium nonalfalfae* (Plectosphaerellaceae: Phyllachorales)

E.S. O'Neal and D.D. Davis*

Department of Plant Pathology and Environmental Microbiology, The Pennsylvania State University, University Park, PA, USA

(*Received 12 September 2014; returned 21 October 2014; accepted 18 February 2015*)

Verticillium nonalfalfae has been proposed as a biocontrol for invasive *Ailanthus altissima* (tree-of-heaven) in Ohio, Pennsylvania and Virginia. However, previous studies evaluating this potential biocontrol utilised a conidial suspension with a short shelf life as inoculum. Anticipating future expanded use of *V. nonalfalfae*, we evaluated other inoculum formulations, inoculation protocols and sensitivity of non-target (non-*Ailanthus*) plant species within Pennsylvania. The most effective inoculum formulation, with an extended shelf life, was prepared by mixing water with stored, refrigerated soil containing *V. nonalfalfae*. Less successful, but positive infections were obtained by simply using infected *Ailanthus* wood and leaves as inoculum. Monthly inoculation of *Ailanthus* trees demonstrated that the optimal time for successful inoculations was April to May, but limited infections were achieved during all months, including the winter. The health of *Ailanthus* and non-target species was evaluated within a decade-old natural Verticillium wilt epicentre, where all mature *Ailanthus* trees had been killed by *V. nonalfalfae*. Verticillium wilt was observed on a few small *Ailanthus* trees, likely newly established seedlings, whereas non-target species were asymptomatic. Our findings reveal that soil formulated and natural inocula are effective biocontrols against *Ailanthus*, and *V. nonalfalfae* appears to pose little threat to non-target plants.

Keywords: biological control; tree-of-heaven; Verticillium wilt; formulation; host range

Introduction

In Pennsylvania and other Mid-Atlantic states, few other plant species are as invasive as tree-of-heaven [*Ailanthus altissima* (Mill.) Swingle], commonly referred to as *Ailanthus*. This native of China was first introduced into Philadelphia in 1784 (Shah, 1997). Since then *Ailanthus* has become increasingly abundant in and along the edges of eastern forests, where it competes with native desirable plant species and disrupts sound forest management practices (Feret, 1985; Kasson, Davis, & Davis, 2013). *Ailanthus* also thrives within urban environments and along transportation corridors, damaging infrastructure, obscuring visibility along roads and falling onto roadways (Almeida, Mouga, & Barracosa, 1994; Hu, 1979). It also poses human health problems, causing contact dermatitis and possibly immunoallergic respiratory

*Corresponding author. Email: ddd2@psu.edu

issues and myocarditis (Bennett, Paget, & Mackenzie, 2013; Bisognano, McGrody, & Spence, 2005; Derrick & Darley, 1994). In addition, attempts to control *Ailanthus* by cutting, mowing and girdling are ineffective, and use of herbicides may require repeated treatments (Kasson et al., 2013).

In recent years, Verticillium wilt caused by *Verticillium nonalfalfae* Inderbitzin et al. (2011), previously classified as *Verticillium albo-atrum* Reinke and Berthold, has been proposed as a biological control for *Ailanthus*. This soilborne fungus was discovered in 2002–2003 naturally killing thousands of *Ailanthus* trees within a large *Ailanthus* stand in the forests of south-central Pennsylvania (Schall & Davis, 2009a, 2009b). To date, this potential mycoherbicide has been isolated from infected *Ailanthus* in Pennsylvania, Virginia and Ohio and may be native to eastern forests (Kasson, Short, et al., 2014; Rebbeck et al., 2013; Schall & Davis, 2009a; Snyder et al., 2013). Aetiology and efficacy studies, using *V. nonalfalfae* isolate VnAa140 (previously PSU 140), have shown that *V. nonalfalfae* is extremely virulent on *Ailanthus* (Kasson, Short, et al., 2014) and rapidly transmitted from diseased to healthy *Ailanthus* trees by intraspecific root grafts (O'Neal & Davis, 2015). Not only does *V. nonalfalfae* effectively kill the above-ground stem, it also kills the roots and subsequent root suckers. Inoculation of the parent stem of a clonal group of *Ailanthus* stems can control most, if not all, associated root sprouts (O'Neal & Davis, 2015). The effectiveness of this mycoherbicide was demonstrated when Kasson, Short, et al. (2014) inoculated 100 canopy *Ailanthus* trees from 2006 to 2009 across 12 *Ailanthus* stands in south-central Pennsylvania. By late 2011, the natural spread of *V. nonalfalfae* had caused infection and mortality of >14,000 *Ailanthus* trees. Importantly, *V. nonalfalfae* isolate VnAa140 appears to be host adapted to *Ailanthus* and causes little or no adverse effects on non-target forest species (Kasson, O'Neal, & Davis, 2015; Kasson, Short, et al., 2014).

For biological control of *Ailanthus* trees, we routinely inject 3 ml of laboratory prepared conidial suspension (10^7 conidia ml^{-1}) into trees using a hypo-hatchet (OEM Fabricators Inc., Woodville, WI, USA; Schall & Davis, 2009a). However, if *V. nonalfalfae* is to be widely used in forests and along transportation corridors as a mycoherbicide, more practical and stable formulations need to be developed that can be readily utilised by land managers. The methods and materials used to formulate a biological control agent are largely dependent on its biological characteristics (Fravel, 2005). *V. nonalfalfae* forms melanised resting mycelia that enable it to survive in soil in the absence of a host (Inderbitzin et al., 2011). We observed that viable *V. nonalfalfae* could be recovered from a refrigerated soil mixture after >4 years storage at 4°C. Thus, the soil mixture currently used to store *Verticillium* strains may serve as a carrier, requiring only the development, optimisation and testing of an appropriate delivery system. In addition to developing a formulation, natural inoculum (e.g., infected leaves, wood and infested soil) could be easily used to initiate new infections in healthy *Ailanthus* stands. Such methods have been used in biological control of common persimmon (*Diospyros virginiana* L.) in Oklahoma using *Acremonium diospyri* (Crand.) W. Gams (Wilson, 1969).

In this study, *V. nonalfalfae* inoculation protocols, formulation and optimisation are explored. Also, an additional more realistic risk assessment of potential non-target hosts is conducted in the field to ensure that *V. nonalfalfae* presents no risks to non-*Ailanthus* forest species. The four main objectives of this study were to: (1) develop an effective and efficient formulation and delivery system for *V. nonalfalfae*,

(2) determine the optimal season for inoculation of *Ailanthus*, (3) determine if naturally infected plant and infested soil material can serve as inoculum and (4) assess current plant species composition and health in a Verticillium wilt epicentre where all mature canopy *Ailanthus* trees were killed during the last decade.

Materials and methods

Study sites

Four field study sites were established in south-central Pennsylvania within oak dominated, mixed hardwood forests that had been invaded by *Ailanthus*. Study site SGL 1 was located within PA State Game Lands 211 (Dauphin County), as was described by Kasson, Short, et al. (2014). This site consisted of an *Ailanthus* stand that had been artificially inoculated with *V. nonalfalfae* isolate VnAa140 in 2008 (Kasson, Short, et al., 2014) and contained dead and dying *Ailanthus* trees infected with *V. nonalfalfae*. Study site SGL 281, located within PA State Game Lands 281 (Perry County), contained a large stand of healthy *Ailanthus*, as described by O'Neal and Davis (2015). Study area RLK, located near Raystown Lake (Huntingdon County), consisted of scattered uninoculated patches of healthy *Ailanthus* trees. A few wilting and dying *Ailanthus* trees near RLK had acquired *V. nonalfalfae* isolate VnAa140 from nearby artificial inoculations in 2008 (Kasson, Short, et al., 2014). The County-Line study site was within the PA Tuscarora State Forest on the Perry/Franklin County border, as described by Schall and Davis (2009a, 2009b). The County-Line site had contained thousands of *Ailanthus* that were wilting and dying from natural *V. nonalfalfae* infections during 2002–2003. *V. nonalfalfae* VnAa140 was originally isolated from this location and used in subsequent studies (O'Neal & Davis, 2015; Schall & Davis, 2009a, 2009b). At the time of this study, the County-Line site was dominated by a red maple (*Acer rubrum* L.) overstory and a striped maple–black birch (*Acer pensylvanicum* L.–*Betula lenta* L.) understory. Previous canopy *Ailanthus* trees had been killed by natural *V. nonalfalfae* infections during the preceding 10 years.

Culture maintenance, inoculum preparation and isolation

Protocols for *V. nonalfalfae* culture maintenance and inoculum preparation followed those of Kasson, Short, et al. (2014) and Schall and Davis (2009a). *V. nonalfalfae* isolates were maintained on Petri dishes containing plum extract agar (PEA; Talboys, 1960) amended with streptomycin sulphate and neomycin sulphate (PEA + SN; Kasson, Short, et al., 2014; Schall & Davis, 2009a). Petri dishes with actively growing *V. nonalfalfae* were incubated at 23°C with a 12-h photoperiod. After 4 weeks, the dishes were flooded with 10 ml sterile distilled water. Mycelia and conidia were loosened with a sterile spatula and the resultant suspension pipetted in 3-ml aliquots into each of three 10-ml scintillation vials containing a 1:1:1 mixture (by volume) of sterile potting soil:perlite:peat moss. Vials were loosely capped and maintained at room temperature for 2 weeks to allow colonisation. After 2 weeks, vials were tightly capped and stored at 4°C. To recover *V. nonalfalfae*, soil particles were removed from the vials and placed onto fresh PEA + SN Petri dishes. Within 3–5 days, emerging colonies were transferred to new PEA + SN dishes and allowed to grow for 3 weeks.

To prepare inoculum, the 3-week-old cultures were flooded with sterile distilled water and mycelia and conidia loosened with a spatula. The resultant suspension was vortexed and passed through a milk filter (KenAg, Ashland, OH, USA) to remove mycelial fragments. Conidial concentration was adjusted to 10^7 conidia ml^{-1} and viability determined by making 10-fold serial dilutions and counting colony forming units on PEA + SN. Inoculum having <80% conidial viability was discarded. Inoculum was stored at 4°C and viability determined again immediately before field use. Inoculum pathogenicity was maintained by inoculating *Ailanthus* seedlings in the greenhouse and recovering fresh VnAa140 colonies, which were maintained in pure culture. Additional *V. nonalfalfae* isolates were recovered from symptomatic *Ailanthus* trees in the field, where bark was removed and wood chips collected from discoloured vascular tissue. The sample chips were disinfested with 70% ethanol and cut into progressively smaller sub-samples, which were plated onto fresh PEA + SN Petri dishes. After 10 days, emerging *Verticillium* colonies were transferred onto new PEA + SN dishes. This method eliminated most contamination and consistently yielded pure *V. nonalfalfae* colonies.

DNA extraction and molecular identification of isolates

Genomic DNA was extracted from air-dried mycelial plugs harvested from potato dextrose broth (BD, Franklin Lakes, NJ, USA) using a Wizard genomic DNA purification protocol (Promega Corp., Madison, WI, USA), following procedures used by Kasson, Short, et al. (2014). For isolate identification, the PCR protocols of Inderbitzin et al. (2011), a Nyx Technik ATC 201 Programmable Thermal Cycler (Nyx Technik, Inc., San Diego, CA, USA), GoTaq PCR kits (Promega Corp., Madison, WI, USA) and Platinum Taq (Invitrogen, Carlsbad, CA, USA) were used to amplify three loci: the protein-coding genes elongation factor 1-α, glyceraldehyde-3-phosphate dehydrogenase and tryptophan synthase. Amplified PCR products were electrophoresed in 2% agarose gels and stained with EZ-Vision Three DNA Dye & Buffer 6X (AMRESCO, Solon, OH, USA) to visualise bands. PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA, USA). Sanger sequencing was conducted on an ABI 3730 XL automated DNA sequencer (Applied Biosystems, Carlsbad, CA, USA) at the Penn State Genomics Core Facility, University Park, PA. Sequencher 4.8 (Gene Codes, Ann Arbor, MI, USA) was used to revise the raw sequence data and assemble consensus sequences, which were used as BLAST queries searched against the National Center for Biotechnology Information GenBank database, to identify isolates.

Preliminary V. nonalfalfae formulation *field testing*

To determine if the soil mixture used for long-term storage of *Verticillium* spp. could serve as a successful carrier for *V. nonalfalfae* inoculum, a 10-ml scintillation vial containing 4 g *V. nonalfalfae* isolate VnAa140-infested soil was removed after >2 years of storage at 4°C in July 2012. The 4-g sample was mixed with 100 ml sterile distilled water and filtered once through cheesecloth. At study site RLK, five healthy canopy *Ailanthus* trees were wounded three times at the stem base with a hatchet and 1 ml inoculum filtrate applied to each wound. One additional wounded stem, treated with sterile distilled water, served as a control. Percentages of foliar wilt and defoliation on the five inoculated *Ailanthus* trees were recorded monthly until the

trees died. In January 2013, wood chips were removed from the xylem of the five inoculated trees and cultured to reisolate and identify *V. nonalfalfae* isolate VnAa140.

V. nonalfalfae *isolate VnAa140 formulation and delivery*

V. nonalfalfae isolate VnAa140 was subcultured by placing a colonised, inverted 0.5 cm diameter agar plug in the centre of a PEA + SN Petri dish and incubated for 4 weeks, at which time conidia and mycelia were loosened, vortexed and filtered as described previously. Approximately 20 ml of the resulting conidial suspension was added to a sterile jar containing 30 g of 1:1:1 sterile potting soil:perlite:peat moss. Each jar was shaken and maintained on the laboratory bench at room temperature to allow *V. nonalfalfae* colonisation. After 2 weeks, the colonised soil was divided into three sterile 50-ml centrifuge tubes (10 g colonised soil/tube) with tightly secured lids and stored at 4°C. As needed, tubes were removed from storage and the contents emptied onto a sterile 20-cm^2 square of cheesecloth, which was wrapped around the soil and secured with a twist tie to form a pouch. To prepare deliverable inoculum, one or more pouches were placed in 500 ml distilled water in a wash bottle and shaken for ca. 30 s to release small colonised soil particles, resting mycelia fragments and conidia into the water.

Field testing soil-formulated V. nonalfalfae isolate VnAa140 inoculum

The soil-formulated inoculum was stored for 1 month at 4°C until May 2013, at which time healthy canopy *Ailanthus* trees within study site SGL 281 were inoculated with one of six treatments. In all treatments, each stem was wounded three times at the base with a hatchet and each wound received 1 ml inoculum via pipette. Treatments 1, 2 and 3 each consisted of 10 *Ailanthus* trees each. Inoculum used for Treatments 1, 2 and 3 was prepared in the field by mixing 20, 10 or 5 g colonised soil with 500 ml distilled water, respectively, as previously described. Treatment 4, the positive control, consisted of 10 *Ailanthus* trees inoculated with our standard conidial suspension (10^7 conidia ml^{-1}). Treatment 5, a negative control, consisted of four *Ailanthus* trees inoculated with one of two control sub-treatments. Treatment 5a and 5b each consisted of two trees treated with inoculum prepared by mixing 20 or 10 g uninfested soil mixture with 500 ml distilled water, respectively. Treatment 6, a second negative control, consisted of two trees treated with distilled water only. Treatment descriptions are described in Table 1. Disease severity was quantified biweekly for all trees using a scale of 1–4 [1 = healthy foliage, 2 = chlorosis and/or necrotic margins on leaves, 3 = wilt (≥15% wilting foliage) and 4 = dead] through September 2013, at which time one randomly selected symptomatic tree from Treatments 1 to 4 was sampled to isolate *V. nonalfalfae*.

2012–2013 monthly canopy Ailanthus *tree inoculations*

At study site RLK, 17 separate groups of *Ailanthus* trees, each consisting of five healthy canopy *Ailanthus* trees, were selected for inoculation. During the first week of every month from April 2012 to August 2013, one group of trees was inoculated. Three wounds were made at the base of each stem with a hypo-hatchet, which injected 1 ml conidial suspension (10^7 conidia ml^{-1}) per hit. The 17 groups of trees were geographically separated to minimise cross-contamination. An additional

Table 1. Mean AUDPC values, at 16 weeks after inoculation, for canopy *Ailanthus* trees inoculated in May 2013 with different formulation or control treatments.[a]

Treatment	Inoculum type	*n*	AUDPC	
1	20 g infested soil/500 ml DI water	10	39.4	a
2	10 g infested soil/500 ml DI water	10	34.4	ab
3	5 g infested soil/500 ml DI water	10	29.7	b
4	Spore suspension (10^7 conidia ml^{-1}) in sterile DI water	10	38.2	a
5	10 or 20 g uninfested soil/500 ml DI water	4	19.0	c
6	DI water only	2	16.0	c

[a]Mean AUDPC values within a given column that do not share a letter are significantly different according to Tukey's mean comparisons ($P = 0.05$). Treatment 4 served as a positive control treatment. Treatments 5, consisting of two sub-treatments, and 6 served as the negative control treatments. All trees were inoculated with a total of 3 ml appropriate inoculum applied via a pipette (1 ml/wound) to three basal wounds made with a hatchet.

group of *Ailanthus* trees served as a control group, in which one tree was treated every month with sterile distilled water.

Disease severity on inoculated trees and controls was quantified monthly from June 2012 through July 2014 using a scale of 0–8, where 0 = healthy foliage, 1 = chlorosis and/or necrotic margins on leaves, 2 = slight wilt (<15% wilting foliage) with no or slight defoliation (<15%), 3 = moderate wilt (15% to <50% wilting foliage) with no or slight defoliation (<15%), 4 = severe wilt (50–100% wilting foliage) with no or slight defoliation (<15%), 5 = moderate defoliation (15% to <50%), 6 = severe defoliation (50% to <90%), 7 = very severe defoliation (90–100%) with epicormic sprouting and 8 = dead.

Control of Ailanthus *using natural* V. nonalfalfae *inoculum*

Natural *V. nonalfalfae* inoculum (infested soil, infected wood and infected leaves) was collected from study site SGL 1 and used to determine if natural inoculum could be used to infect healthy *Ailanthus* trees (Figure 1).

Infested soil as inoculum

To prepare soil-based inoculum, a 7.5-l container of putatively *V. nonalfalfae*-infested soil was collected from a depth of 0–5 cm around the base of an *Ailanthus* tree recently killed by *V. nonalfalfae*. For use as a control, a 2-l sample of putatively uninfested soil was collected from the base of a healthy *Ailanthus* tree in the same stand.

Twelve healthy canopy *Ailanthus* trees in study site SGL 281 were each wounded three times at the stem base with a hatchet. Wounds were approximately 60 cm^2 and exposed the xylem. Ten of the 12 trees had ca. 235 ml putatively infested soil applied directly to each wound (Figure 1A). The remaining two healthy *Ailanthus* trees served as controls and had uninfested soil applied to the wounds.

To prepare injectable inoculum, the above soil samples were mixed with sterile distilled water and 50 ml placed onto a 20-cm^2 square of cheesecloth that was folded, forming a soil-filled pouch secured with a twist tie. The pouch was added to a container having 500 ml distilled water and shaken. In study site SGL 281, 10 healthy *Ailanthus* trees were wounded with a hatchet three times at the stem base,

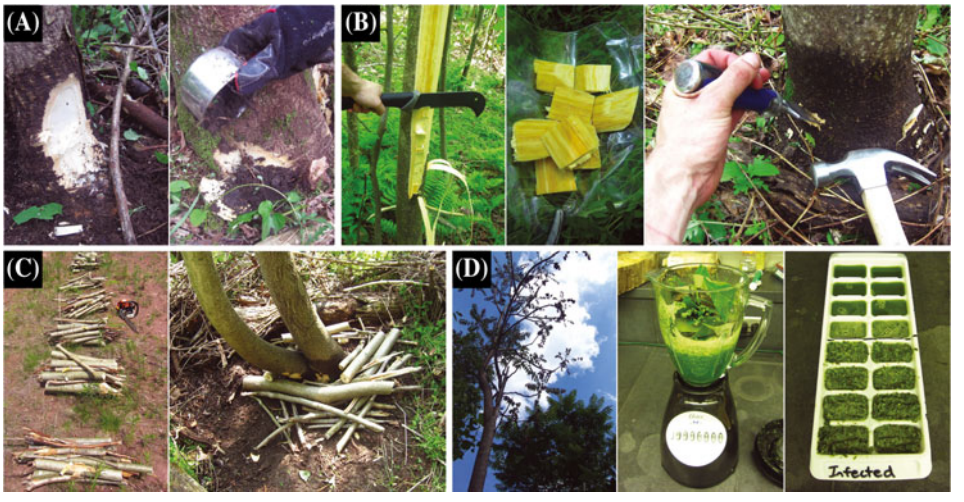

Figure 1. (Colour online) Collection, processing and inoculation methods using natural *V. nonalfalfae* inoculum sources, showing (A) wounding of a healthy *Ailanthus* tree and application of putatively infested soil, (B) excision, clipping and insertion of yellow discoloured, infected wood chips into the base of a healthy *Ailanthus* tree, (C) collection and placement of infected wood debris around the base of a healthy *Ailanthus* tree and (D) collection and processing of infected leaves into leaf filtrate and paste.

and each wound received 1 ml inoculum mixture via a pipette. Two healthy *Ailanthus* trees were treated with an uninfested soil and distilled water mixture to serve as controls.

Infected wood chips as inoculum

In study site SGL 1, one canopy *Ailanthus* tree that was heavily colonised with *V. nonalfalfae* and nearly 100% defoliated was selected. Bark was removed and wood chip samples were removed from dark yellow-discoloured vascular wood. Samples were trimmed to ca. 2.54 cm^2 and 2–3 mm thick (Figure 1B). Using the same methodology, wood chips from a healthy *Ailanthus* tree were also collected. Samples were placed within clean plastic bags in a cooler. Ten healthy *Ailanthus* trees were wounded three times at the stem base using a 2.54-cm wide wood chisel. An infected wood chip was inserted and tapped into each wound (Figure 1B). Similar wounds, made on two additional *Ailanthus* trees, had wood chips excised from a healthy tree inserted into their wounds, serving as controls.

Infected wood debris as inoculum

In mid-May 2013, five *V. nonalfalfae*-infected *Ailanthus* trees in study site SGL 1 that exhibited nearly 100% defoliation and dark yellow vascular discolouration were felled and cut into ca. 45-cm long sections (Figure 1C) to serve as inoculum. One healthy *Ailanthus* tree of comparable size within the same study site was also felled and sectioned to serve as control inoculum.

In study site SGL 281, forest floor litter was removed and bare soil exposed around the bases of six healthy *Ailanthus* trees. Exposed roots and tree bases were

wounded with a machete. Branches and stem sections were transported from study site SGL 1 to SGL 281. One pile of infected wood was placed against each base of five wounded, but otherwise healthy, trees (Figure 1C). The pile of healthy wood was placed against the base of the sixth healthy *Ailanthus* tree to serve as a control.

Infected leaves as inoculum

The collection of *V. nonalfalfae*-infected leaves could not take place until late May 2013, when foliar Verticillium wilt symptoms in the tree crowns became apparent following leaf emergence. Two *Ailanthus* trees that exhibited 100% wilt, but only 5 to 10% defoliation, were selected (Figure 1D), felled and wilted leaves collected. Leaves from a healthy control *Ailanthus* tree were collected similarly.

Infected leaves (100 g) were blended with 500 ml sterile distilled water (Figure 1D), filtered through cheesecloth and frozen in ice cube trays (Figure 1D). Healthy *Ailanthus* leaves were processed similarly to serve as control inoculum. During the first week of June 2013, 20 healthy canopy *Ailanthus* trees in stand SGL 281 were wounded three times at the stem base with a hatchet. Ten trees were treated with 1 ml thawed infected leaf filtrate per wound. The wounds of the remaining 10 trees were filled with the infected leaf paste. Four additional *Ailanthus* trees served as controls; wounds on two control trees were treated with healthy leaf paste and wounds on two control trees received healthy leaf filtrate.

After all trees had been inoculated with the various forms of natural *V. nonalfalfae* inoculum, the disease incidence in the treated *Ailanthus* trees was quantified biweekly through July 2014. Trees exhibiting typical Verticillium wilt symptoms, wilt and/or vascular discolouration, were tallied as diseased. All symptomatic trees were sampled for *V. nonalfalfae* to confirm infections.

Forest composition and crown health after a decade of Ailanthus *mortality*

Twelve circular plots were established within the County-Line study site, near those plots established in 2007 by Schall (2008). Plots were 0.081 ha with 16.06 m radii. The diameter at breast height (DBH 1.37 m) of all plot stems ≥2.54 cm DBH was measured and their crowns rated using a 0–2 scale (0 = healthy, 1 = wilting and 2 = dead). The crown class (dominant, codominant, intermediate or suppressed) of each stem was recorded using established criteria (http://www.fs.fed.us/database/feis/glossary2.html). To evaluate the forest understory/regeneration, a nested 0.0004 ha understory circular subplot with a radius of 1.1 m was established at each plot centre. Number of tree seedlings/saplings with DBH <2.54 cm was tallied and per cent cover of other understory plant species was recorded. In addition, foliage was rated using the 0–2 scale described earlier.

Statistical analyses

Progression of Verticillium wilt within *Ailanthus* trees that had been inoculated using different formulations was evaluated using the area under the disease progress curve (AUDPC; Shaner & Finney, 1977). Significant differences among mean AUDPC values and among mean DBH values for the six treatments were evaluated with analysis of variance (ANOVA) and Tukey's mean comparisons (P = 0.05) using Minitab 16.1.0 (Minitab Inc., State College, PA, USA). Mean DBH was analysed as a covariate to determine if tree DBH prior to inoculation influenced AUDPC values.

To evaluate the effectiveness of the monthly 2012–2013 inoculations, disease severity ratings could only be taken from June to September, since they had to be recorded when the *Ailanthus* trees had foliage. For example, for trees inoculated in August 2012, the 'first rating' was in September 2012, but the 'fourth rating' did not occur until August 2013 when foliage was present. Also, for all late, dormant, or early season inoculations during September to May, the 'first rating' was in June 2013, again when foliage was present. We chose to evaluate the results of the monthly inoculations during the first, fourth and sixth ratings. Thus, this rating system eliminated the monthly offset between times of inoculation and host dormancy and allowed a valid statistical comparison between disease severity and month of inoculation. Ratings of all control trees were combined into one group for statistical analyses. Significant differences among mean disease severity ratings and among mean DBH values of the monthly inoculations were evaluated at the time of first, fourth and sixth ratings using ANOVA and Tukey's mean comparisons ($P = 0.05$) using Minitab 16.1.0.

Results

Preliminary V. nonalfalfae *formulation field testing (data not shown)*

The five canopy *Ailanthus* trees inoculated in July 2012 with a mixture of *V. nonalfalfae*-colonised soil and sterile distilled water developed Verticillium wilt symptoms between 1 and 2 months post-inoculation (MPI). At 2 MPI, September 2012, all five trees displayed severe wilt (>50%) and defoliation. During January 2013, *V. nonalfalfae* was successfully isolated from all five inoculated dormant trees. In June 2013 (11 MPI), all five trees were 99–100% defoliated. In addition, *V. nonalfalfae* spread naturally to adjacent, previously healthy, non-inoculated trees presumably through root grafts. By August 2013 (13 MPI), all five inoculated trees were dead. The control tree remained asymptomatic throughout the experiment.

Field testing of soil-formulated V. nonalfalfae *inoculum*

The disease progress on individual trees varied with individual treatments, from time of inoculation to the end of the experiment. Two of 10 trees in Treatment 1 and 5 of 10 trees in Treatment 4 exhibited the first typical Verticillium wilt symptoms at 4 weeks post-inoculation (WPI, data not shown). Inoculated trees in other treatments did not show typical wilt symptoms at that time. By 6 WPI, 8 of 10 trees in Treatment 1 and 9 of 10 trees in Treatment 4 exhibited substantial wilt and defoliation; at that time, initial wilt symptoms began to appear in a few trees inoculated with Treatments 2 and 3. At 8 WPI, all 30 trees in Treatments 1, 2 and 4 exhibited severe wilt and defoliation. Trees in Treatments 1 and 4 had only moderate defoliation at that time. Six trees in Treatment 3 were wilting at 8 WPI but exhibited only slight defoliation. By September 2013 (16 WPI), all 30 trees in Treatments 1, 2 and 4 were severely wilted and defoliated. Eight of 10 trees in Treatment 3 were also severely wilted at that time. Trees in the negative control Treatments 5a, 5b and 6 remained asymptomatic for the duration of the experiment, except one tree in Treatment 5b that became symptomatic from apparent acquisition of *V. nonalfalfae* via root grafts from a nearby infected tree. *V. nonalfalfae* was successfully isolated from all four randomly sampled trees in Treatments 1–4 (1 sampled tree/treatment).

As shown in Table 1, the final mean AUDPC values at 16 WPI for Treatments 1–4 (39.4, 34.4, 29.7 and 38.2, respectively) were all significantly different from Treatment 5 (19.0) and Treatment 6 (16.0), the two negative control treatments. Mean AUDPC values from Treatments 1, 2 and 4 were statically similar. Treatment 3 had a significantly lower mean AUDPC value than Treatments 1 and 4 but was not significantly different from Treatment 2. Covariate analysis (data not shown) revealed that DBH was not a significant ($P = 0.1463$) covariate influencing AUDPC.

2012–2013 monthly canopy **Ailanthus** *tree inoculations*

Values discussed in the following section are shown in Table 2. Trees inoculated from early spring to early autumn (April 2012 to October 2012; April 2013 to August 2013), excluding trees inoculated in June 2012, July 2012 and 2013, exhibited moderate to severe Verticillium wilt symptoms at time of the first rating. These trees had the highest mean disease severity ratings (range: 3.4–5.6), which were significantly greater than the control rating (0.5). In contrast, trees inoculated in mid-summer (June 2012, July 2012 and July 2013) or winter (November 2012 to March 2013) were asymptomatic or exhibited only minimal wilt symptoms at the first rating and had lower mean disease severity ratings (range: 0.0–3.0), which were not significantly different from the control value (0.5).

Table 2. Mean disease severity ratings during the first, fourth and sixth ratings for 17 groups of canopy *Ailanthus* trees inoculated from April 2012 to August 2013.[a]

		Mean disease severity ratings					
Month of inoculation	*n*	First rating		Fourth rating		Sixth rating	
April 2012	5	5.2	ab	7.2	ab	8.0	a
May 2012	5	4.4	abc	7.2	ab	8.0	a
June 2012	5	2.6	abcdef	7.6	a	8.0	a
July 2012	5	0.2	f	7.2	ab	7.8	a
August 2012	5	4.2	abc	7.4	a	8.0	a
September 2012	5	5.0	abc	7.0	ab	8.0	a
October 2012	5	3.4	abcde	7.0	ab	8.0	a
November 2012	5	3.0	abcdef	6.8	abc	8.0	a
December 2012	5	0.8	def	2.8	cd	5.6	ab
January 2013	5	0.4	ef	3.2	bcd	4.4	ab
February 2013	5	0.0	f	2.0	d	6.8	a
March 2013	5	2.0	cdef	7.0	ab	7.4	a
April 2013	5	3.8	abcd	7.2	ab	8.0	a
May 2013	5	4.0	abc	7.0	ab	8.0	a
June 2013	5	5.0	abc	8.0	a	–	
July 2013	5	2.2	bcdef	7.0	ab	–	
August 2013	5	5.6	a	–		–	
Controls	17	0.5	f	1.4	d	2.6	b

[a]Mean disease severity ratings followed by the same letter within a given column are not significantly different according to Tukey's mean comparisons ($P = 0.05$). Disease severity was rated using a 0–8 scale related to symptom progression. '–' indicates data were not collected.

By the fourth rating, *Ailanthus* trees that had been inoculated from April 2012 to November 2012 and from March 2013 to July 2013 were severely wilted and many were >90% defoliated (data were not collected at the fourth rating for the August 2013 inoculations). Some trees that had been inoculated in April and May 2012, as well as in June 2013, began to exhibit mortality. All trees inoculated from April 2012 to November 2012 and in March 2013 to July 2013 had significantly greater mean disease severity ratings (range: 6.8–8.0) than the controls (1.4). *Ailanthus* trees inoculated from December 2012 to February 2013 had lower mean disease severity ratings (range: 2.0–3.2), generally not significantly different from the controls (1.4). However, a few individual trees inoculated in December 2012 and January 2013 began to develop moderate Verticillium wilt symptoms, increasing the mean disease severity ratings to levels not significantly different from those trees inoculated in November 2012 and prior.

At the sixth rating, most inoculated *Ailanthus* trees were dead, with mean disease severity rating of 8.0, which was significantly different from the controls (1.4). Trees inoculated in July and December 2012, as well as January to March 2013, exhibited limited instances of mortality and extreme defoliation, while some trees remained mostly asymptomatic. The mean disease severity ratings for these trees ranged from 4.4 to 7.8. The ratings of trees inoculated in December 2012 and January 2013 were not significantly different from the control trees (2.6) (disease severity ratings were not collected at the time of the sixth rating for trees inoculated from June to August 2013).

Control of Ailanthus *using natural* V. nonalfalfae *inoculum*

In May 2013, infested soil, as well as infected wood chips, leaves and wood debris collected from a *V. nonalfalfae*-infected *Ailanthus* stand were used to inoculate healthy *Ailanthus* trees in a nearby stand. From 0 to 7 WPI, treated *Ailanthus* trees did not exhibit Verticillium wilt symptoms. At 8 WPI, one tree inoculated with infected wood chips exhibited initial wilt symptoms. That tree remained the only symptomatic tree until 16 WPI (September 2013), at which time 3 additional trees inoculated with wood chips displayed slight wilt symptoms and 1 tree treated with infected wood debris placed at its base developed wilt symptoms. At the final rating, 56 WPI, 3 more *Ailanthus* trees treated with infected wood debris, as well as 2 trees inoculated with infected leaf filtrate, exhibited Verticillium wilt symptoms.

Streaks of yellow vascular discolouration, typical of Verticillium wilt, were observed in all 10 symptomatic *Ailanthus* trees. *V. nonalfalfae* was subsequently isolated from the discoloured tissue. All control *Ailanthus* trees, as well as trees inoculated with putatively infested soil and infected leaf paste, remained asymptomatic throughout the study.

Forest composition and health after a decade of Ailanthus *mortality*

Most if not all mature overstory *Ailanthus* trees at the County-Line site had been killed during 2002–2012 by natural *V. nonalfalfae* infections. By July 2013, the site was dominated by native non-*Ailanthus* species, many of which had apparently established immediately following mortality of the previous *Ailanthus* overstory (Supplemental Figure 1). Seventy dead *Ailanthus* trees/ha, which had been killed

during the original natural *V. nonalfalfae* epidemic, were still standing at the site in 2013.

Striped maple had the most live stems within the plots in 2013 (257 stems/ha), followed by black birch (229 stems/ha) and red maple (*A. rubrum* L.; 108 stems/ha). Young *Ailanthus* saplings, which likely arose from airborne seed, were present (75 stems/ha; Supplemental Figure 1). Red maple had the greatest basal area (13.62 m^2/ha), followed by black birch (2.83 m^2/ha), yellow-poplar (*Liriodendron tulipifera* L.; 1.24 m^2/ha) and white ash (*Fraxinus americana* L.; 1.07 m^2/ha). Living re-established *Ailanthus* saplings only had a basal area of 0.07 m^2/ha, while standing dead *Ailanthus* trees had a basal area of 7.97 m^2/ha (Supplemental Figure 2).

The dominant and codominant crown classes in 2013 were comprised mainly of red maple, black birch and black locust (*Robinia pseudoacacia* L.) trees (Supplemental Figure 3). The intermediate and suppressed crown classes contained striped maple, black birch, witch hazel and *Ailanthus* saplings. The re-established *Ailanthus* saplings were confined to the intermediate or suppressed crown classes (Supplemental Figure 3).

Regarding overstory tree health on the plots, four living *Ailanthus* saplings exhibited severe wilt, defoliation and vascular discolouration, characteristics of Verticillium wilt. Twenty-nine per cent of the living striped maples had symptoms indicative of Botryosphaeria canker or anthracnose, while 70% of the black locust trees were infested with locust leafminers (*Odontota dorsalis* Thunb.), resulting in necrotic leaves and defoliation. The dead black locust trees had been killed by severe locust borer (*Megacyllene robiniae* Forster) infestations. Of the 118 dead trees tallied, 59% were *Ailanthus* trees that likely died from natural *V. nonalfalfae* infections. The remaining dead trees were black locust (23%), striped maple (10%) and a few hardwood trees in the lower crown classes (8%; Supplemental Figure 1). Verticillium wilt symptoms were not observed on any non-*Ailanthus* species.

Similar to the overstory, the vegetation in the understory plots was dominated by living non-*Ailanthus* hardwood species regeneration, including red maple (1236 seedlings/ha), black birch (824 seedlings/ha), black locust (206 seedlings/ha), striped maple (206 seedlings/ha) and white ash (206 seedlings/ha; Table 3). *Ailanthus* regeneration was not present within the understory plots. The understory plots contained both invasive and native understory plant species, including hay-scented fern [*Dennstaedtia punctilobula* (Michx.) Moore; 31.7% cover], Japanese stiltgrass [*Microstegium vimineum* (Trin.) Camus; 30.1% cover], Virginia creeper [*Parthenocissus quinquefolia* (L.) Planch.; 5.2% cover] and garlic mustard [*Alliaria petiolata* (Bieb.) Cavara and Grande; 2.5% cover; Table 3].

Discussion

The overall objectives of this *V. nonalfalfae–A. altissima* biocontrol study were to evaluate: (1) formulation and delivery systems for *V. nonalfalfae*, (2) most effective months for *Ailanthus* inoculation, (3) ability of natural inoculum to initiate infections and (4) forest plant health and composition a decade after a natural Verticillium wilt epidemic killed an entire *Ailanthus* stand.

Table 3. Understory tree seedlings and plant composition within the County-Line site, a natural Verticillium wilt epicentre in Tuscarora State Forest.[a]

Tree seedlings/saplings	No. seedlings and saplings/ha
Red maple (*A. rubrum*)	1236
Black birch (*B. lenta*)	824
Striped maple (*A. pensylvanicum*)	206
White ash (*F. americana*)	206
Black locust (*R. pseudoacacia*)	206
Understory plant species	% coverage/ha
Hay-scented fern (*D. punctilobula*)	31.7
Japanese stiltgrass (*M. vimineum*)	30.1
Virginia creeper (*P. quinquefolia*)	5.2
Garlic mustard (*A. petiolata*)	2.5
Mile-a-minute (*Persicaria perfoliata*)	2.5
Common blackberry (*Rubus allegheniesis*)	1.9
Nettle (*Urtica* spp.)	0.5
Pennsylvania smartweed (*Polygonum pensylvanicum*)	0.3
Jewelweed (*Impatiens capensis*)	0.1
Empty space or large rocks	25.3

[a]Understory/regeneration 0.0004 hectare, 1.1 m radius plots were nested within overstory permanent plots. The number of tree seedlings/saplings <2.54 cm in DBH and per cent cover all understory plant species were recorded per plot.

Formulation and delivery of V. nonalfalfae

The feasibility of simply using colonised soil mixed with water as inoculum was demonstrated in the 2012 preliminary *Ailanthus* tree inoculations. The inoculations also revealed that pathogenicity of *V. nonalfalfae* is maintained during long-term storage, water is a sufficient liquid carrier, and that resting mycelia can germinate within the liquid carrier as well as within the host. Similarly, Isaac and MacGarvie (1966) reported that water was the main requirement for germination of *V. albo-atrum* resting mycelia and that small mycelial fragments could germinate and produce conidia within 6 h. These preliminary inoculations formed the basis for the formulation of our soil-based inoculum, which is similar to the common protocol for the storing of *Verticillium* spp. under refrigeration. However, our method produces larger quantities of colonised soil that can be stored, transported and easily mixed with water in the field to prepare inoculum for land managers using the common 'hack-and-squirt' method (DiTomaso & Kyser, 2007).

We have observed that storing *Verticillium* in refrigerated soil (our routine storage method) can maintain viable propagules for >4 years, extending the usable shelf life of *V. nonalfalfae* inoculum as compared to a water-based conidial suspension. The maximum shelf life of *V. nonalfalfae* stored by these storage and formulation protocols remains unknown, but Wilhelm (1955) reported *V. dahliae* Klebahn microsclerotia can survive 10–15 years in soil. Therefore, soil-formulated *V. nonalfalfae* inoculum stored at 4°C may remain viable for at least a decade, whereas the conidial suspension, which we routinely used in the past, is likely viable for only weeks. In addition, the soil formulation, as opposed to the conidial suspension, is more stable in transit, such as during transport to a field site, since the

resting mycelia of *V. nonalfalfae* contains protecting melanin (Brandt & Reese, 1964). However, *Verticillium* may be killed at high summer temperatures approaching 33°C (Chaudhuri, 1923) and thus the formulated inoculum should be kept in a cooler prior to use to ensure inoculum viability.

Soil-formulated V. nonalfalfae *as an* Ailanthus *biocontrol*

The soil-formulated, water-delivered *V. nonalfalfae* inoculum was field tested by inoculating canopy *Ailanthus* trees in May 2013. Verticillium wilt infections were induced in all inoculated trees, regardless of treatment method (Table 1). However, the volume of colonised soil in the inoculum slightly influenced the amount of disease at the end of the experiment, which occurred 16 weeks after inoculation, as measured by the final AUDPC (Table 1). The highest level of disease at 16 weeks was greatest in Treatment 1 (20 g infested soil/500 ml water), slightly less in Treatment 2 (10 g soil) and least following Treatment 3 (5 g soil). To ensure potency similar to our standard conidial suspension, 10–20 g *V. nonalfalfae*-colonised soil should be used in the biocontrol treatment. However, 10 g colonised soil appears to be sufficient, since inoculum prepared with 20 g colonised soil did not result in significantly increased effectiveness compared to the 10 g treatment (Table 1). However, it may be efficient to use only 5 g soil-formulated *V. nonalfalfae* when treating small *Ailanthus* trees or saplings.

Although we did not determine the minimum concentration of soil-formulated *V. nonalfalfae* inoculum that would kill *Ailanthus* trees, soil formulations may be more efficient to prepare as compared to our standard conidial suspension (10^7 conidia ml^{-1} water). We observed that one Petri dish of *V. nonalfalfae* generally produces ca. 200–300 ml of a standard conidial suspension. This amount of conidial inoculum will effectively treat up to 100 canopy *Ailanthus* (Kasson, Short, et al., 2014; Schall & Davis, 2009a). However, using the soil-based formulation protocol described above, the same Petri dish can be used to make three 50-ml centrifuge tubes filled with 10 g *V. nonalfalfae*-colonised soil, which can each be mixed with 500 ml distilled water. In this manner, the same Petri dish can produce 1.5 L soil-formulated *V. nonalfalfae* inoculum, which could effectively treat 500 *Ailanthus* trees. Thus, the soil formulation allows for inoculum to be produced in larger quantities, a characteristic of a successful formulation (Stubbs & Kennedy, 2012) and will treat up to 10 times more *Ailanthus* trees than the water-based conidial suspension.

After field testing, the soil-based formulation protocol was slightly revised to simplify preparation and minimise contamination. Inoculum produced by this improved formulation was not field tested in this study but is presented in the Supplemental Text. We consider this improvement to be important, since it minimises the incorporation of contaminants that could render the inoculum useless. Furthermore, a less contaminated soil-formulated inoculum may retain a longer shelf life and increased efficacy. Therefore, it is recommended that the revised protocol be used for future production of soil-formulated *V. nonalfalfae* inoculum for biocontrol of *Ailanthus* trees.

Optimal timing of Ailanthus *tree inoculations*

To further improve and expand utilisation of this biological control agent, we evaluated optimal times of year to inoculate *Ailanthus* trees with *V. nonalfalfae* using

the standard conidial suspension (10^7 conidia ml^{-1}) as inoculum. Separate groups of canopy *Ailanthus* trees were inoculated every month from April 2012 to August 2013. Although temperature strongly influences Verticillium wilt disease development and progression (Pegg & Brady, 2002), our results revealed that inoculation of *Ailanthus* trees during most months, including the winter, could control this invasive tree species.

All inoculations made from March to November resulted in severe Verticillium wilt symptoms, either during the same growing season or in the following season. Inoculations made from March to November usually achieved similar levels of *Ailanthus* control by the fourth rating (Table 2). However, month of inoculation did influence the initial rate of disease progression. The greatest differences among mean disease severity ratings for all months of inoculation occurred at the first rating, when some trees inoculated in June and July developed wilt symptoms more slowly than trees inoculated in April to May or August to September. These results suggest that high temperatures slow *V. nonalfalfae* colonisation within *Ailanthus* trees. This suggestion is supported by reports that the growth rate of *Verticillium* in culture declines as temperatures increase from the optimal growth temperature of 22.5°C to >33°C, at which point the fungus is reduced to a yeast-like budding stage and may be killed (Chaudhuri, 1923; Pegg & Brady, 2002).

Surprisingly, dormant *Ailanthus* trees inoculated during November 2012 exhibited Verticillium wilt symptoms the following June (Table 2). This finding suggests that fall inoculations may result in germination of conidia that establish an initial infection, followed by formation of resting mycelia that remain stable during the winter. The resting mycelia would then expand colonisation during the following spring and summer, when environmental conditions are ideal for disease development. In support of this idea, the growth rate of *Verticillium* in culture is greatly reduced but can still occur at 12°C (Chaudhuri, 1923).

However, cold tolerance likely varies among *Verticillium* spp. and isolates from different geographic locations, and *Verticillium* growth at temperatures as low as ca. 6°C has been reported (Pegg & Brady, 2002). Thus, the lower limit of cold tolerance of *V. nonalfalfae* isolate VnAa140 may be as low as 6°C. In addition, inoculation during the dormant winter season did occasionally result in limited Verticillium wilt symptoms (Table 2), suggesting that low winter temperatures may reduce conidia viability, as reported by Galanopoulos and Tribe (1974). However, *Ailanthus* trees inoculated in March eventually developed severe Verticillium wilt symptoms, suggesting that some conidia might survive until arrival of warm spring temperatures conducive for colonisation.

While inoculations in all months resulted in Verticillium wilt, inoculations during April and May were most successful. *Ailanthus* trees that were inoculated during these months were often dead by September. High or low temperatures in central Pennsylvania during these spring months are seldom extreme enough to inhibit *V. nonalfalfae* colonisation or render inoculum nonviable. In addition, the cool to mild temperatures in April and May are generally conducive for rapid *Verticillium* colonisation of *Ailanthus*. The success of April and May inoculations confirms our previous results that revealed the best biocontrol of *Ailanthus* with *Verticillium* is achieved when inoculations are conducted in the spring (Kasson, Short, et al., 2014; Schall & Davis, 2009a).

Natural V. nonalfalfae *inoculum as an* Ailanthus *biocontrol*

Although the *V. nonalfalfae* conidial suspension and soil formulations successfully initiated wilt symptoms in *Ailanthus*, land managers have expressed interest in moving *V. nonalfalfae* from infected to healthy *Ailanthus* stands, to avoid having to obtain laboratory-prepared inoculum. Landowners in Oklahoma successfully used a similar practical application of 'natural' (non-laboratory produced) inoculum during the late 1940s. They felled common persimmon trees infected with persimmon wilt, caused by *A. diospyri* and placed them near healthy invasive persimmon trees in pastures, resulting in successful biocontrol (Wilson, 1969). We evaluated several forms of natural, field inoculum that could be used by land managers in a practical manner to establish new *V. nonalfalfae* infections within healthy stands of invasive *Ailanthus*.

We evaluated inserting *V. nonalfalfae*-infected wood chips, as evidenced by yellow vascular discolouration, into healthy *Ailanthus* trees in May. We also evaluated placing infected wood debris around at the base of artificially wounded, but otherwise healthy, *Ailanthus* trees in May. Both methods resulted in Verticillium wilt within formerly healthy *Ailanthus* trees by September of the same year. The results suggest that these natural forms of inoculum contain adequate amounts of *V. nonalfalfae* to induce disease. Similarly, Foreman, Rouse, and Hudelson (2002) reported that non-composted *V. dahliae*-infected wood chips, when used as mulch, can start new Verticillium wilt infections if placed around healthy suspects. Therefore, the use of infected wood, as indicated by our results, may be a good source of natural inoculum for initiating *V. nonalfalfae* infections in healthy *Ailanthus* stands.

In contrast, use of putatively infested soil or infected leaves as natural *V. nonalfalfae* inoculum were not efficient or practical. Our soil samples likely contained low populations of soilborne *V. nonalfalfae*, possibly due to the acidic forest soils of Pennsylvania (Schall, 2008). It has been reported that aluminium and/or low soil-pH adversely affects *Verticillium* spp. resting structure formation and survival (Baard & Pauer, 1982; Orellana, Foy, & Fleming, 1975). Likewise, the *V. nonalfalfae* inoculum density within infected *Ailanthus* leaves may have been low, since *Verticillium*-induced plugging of xylem vessels likely limits distal translocation of *Verticillium* propagules into the foliage, as reported for xylem-colonising pathogens (Yadeta & Thomma, 2013).

In addition to inserting infected wood chips into, or placing infected wood around, healthy *Ailanthus* trees, additional field biocontrol techniques using wood should be considered and explored. Vegetation management controls along highways, where *Ailanthus* is often a major problem, often involve felling and mechanically chipping whole *Ailanthus* trees. If *V. nonalfalfae*-infected *Ailanthus* were felled and chipped, the resultant chips could then be placed within nearby healthy *Ailanthus* stands to initiate new *V. nonalfalfae* infections. A quick, efficient method of wounding the healthy trees (e.g., as simple as scraping the root flares with a rake) might ensure success. This process could be repeated for many miles along infested highway corridors, likely resulting in sustainable *Ailanthus* biocontrol.

The effects of V. nonalfalfae *on forest composition and non-target plant species*

With expanded use of *V. nonalfalfae* as a biocontrol agent for *Ailanthus* comes the potential for increased risk to non-target hosts. Therefore, in addition to our

previous host-range studies (Kasson, O'Neal, et al., 2015; Kasson, Short, et al., 2014; Schall & Davis, 2009b), we decided that it was important to continue assessing potential adverse effects of *V. nonalfalfae* on non-target plant species. As a part of this continuing assessment, in 2013 we evaluated the condition of forest plants within a large natural Verticillium wilt epicentre in south-central Pennsylvania (Schall & Davis, 2009a). This epicentre had experienced more than a decade of *Ailanthus* mortality caused by *V. nonalfalfae*. The original canopy of the epicentre consisted mainly of *Ailanthus* trees, which were all killed by *V. nonalfalfae* infections. Our 2013 assessment revealed that the current (2013) forest overstory within the epicentre was dominated by native non-target tree species, including black birch, black locust, red maple and striped maple (Supplemental Figures 1–3). We did record low numbers of young *Ailanthus* saplings within the epicentre, probably originating as airborne seeds, which we hypothesise will become infected by residual soilborne *V. nonalfalfae* and die. Fortunately, the re-established *Ailanthus* saplings were small and their growth was inhibited by a lack of sunlight, due to the rapid growth of released native tree species.

Relatedly, a small number of wilting *Ailanthus* saplings were observed within and adjacent to the permanent plots, which upon sampling, yielded *V. nonalfalfae*. This finding suggests that *V. nonalfalfae* is persisting within the infested soil and likely within the infected *Ailanthus* saplings. Systematic soil sampling may reveal that *V. nonalfalfae* is common, but likely scattered, within eastern forests. Verticillium wilt is likely reported only when the fungus encounters an extremely susceptible host, such as *Ailanthus*. In support of this hypothesis, *V. nonalfalfae* has been reported from Ohio, Pennsylvania and Virginia, but only from readily visible dying *Ailanthus* trees (Kasson, Short, et al., 2014; Rebbeck et al., 2013; Snyder et al., 2013).

Although Verticillium wilt symptoms were observed on a few re-established *Ailanthus* saplings within the old epicentre, wilt was not observed on non-target plant species. Striped maple trees in the plots did not exhibit Verticillium wilt symptoms, even though the species is listed as susceptible to *V. nonalfalfae* (Kasson, O'Neal, et al., 2015). Red elderberry (*Sambucus racemosa* L.) is also listed as susceptible to *V. nonalfalfae* (Kasson, O'Neal, et al., 2015). However, elderberry plants growing near the permanent plots were healthy and asymptomatic (elderberry did not occur within the permanent plots). The fact that these susceptible non-target plant species did not exhibit Verticillium wilt infections may indicate low soil inoculum densities (Schall & Davis, 2009a), lack of vectors such as ambrosia beetles (Kasson, O'Neal, et al., 2015), or unknown factors.

As the canopy *Ailanthus* trees were killed by *V. nonalfalfae*, understory species like striped maple would have been exposed to intense sunlight and possible moisture stress. Sudden exposure to full sunlight has been reported to cause stress and wilt on striped maples (Wilson & Fischer, 1977). Such stress may then predispose striped maple to opportunistic fungal pathogens such as *Botryosphaeria dothidea* Moug. ex Fr. Mortality of black locust was also common within the permanent plots but was related to severe locust borer infestations, as reported previously (Schall & Davis, 2009b).

Wilt symptoms were not observed on understory plants within the nested plots. Understory plots were dominated by invasive species, including hay-scented fern, Japanese stiltgrass, garlic mustard and mile-a-minute weed. Many of these invasive weeds were observed throughout the epicentre and will be of concern regarding site

restoration (Table 3). These results also indicate that opportunistic weeds may dominate the understory following the removal of the *Ailanthus* overstory using *V. nonalfalfae* as a biocontrol agent. However, as Harris, Cannon, Smith, and Muth (2013) reported, removal of overstory *Ailanthus* usually does not cause increased immigration of new weeds to the epicentres but instead encourages the proliferation of previously established weeds. Therefore, the proliferation of invasive weeds within epicentres would occur if the *Ailanthus* trees had been removed by timber harvesting, mining, or any site disturbance. Nearby seed sources of invasive weeds should be controlled prior to creating large canopy gaps by using *V. nonalfalfae* to control *Ailanthus*.

In summary, this study has presented a protocol for the preparation of an efficient, stable form of *V. nonalfalfae* inoculum to control *Ailanthus* trees. Once *V. nonalfalfae* infections are established in healthy *Ailanthus* stands, new infections in other healthy stands can be initiated by using the already infected trees as natural inoculum. Our data indicate that *V. nonalfalfae* poses minimal hazards to non-target plant species likely has become host adapted to *Ailanthus* and is a desirable biological control agent against the highly invasive *A. altissima*.

Acknowledgements

We thank K. Wickert, R. Long and R. Harvey for their assistance, as well as the Pennsylvania Game Commission, Pennsylvania Department of Conservation and Natural Resources and the United States Army Corps of Engineers for allowing use of their forestland as study sites.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was made possible by funding from the US Department of Agriculture Forest Service, Forest Health Technology Enterprise Team, Morgantown, WV, USA (Cooperative Agreement 11-CA-11420004-320).

Supplemental data

Supplemental data for this article can be accessed here: http://dx.doi.org/10.1080/09583157.2015.1023258.

References

Almeida, M. T., Mouga, T., & Barracosa, P. (1994). The weathering ability of higher plants. The case of *Ailanthus altissima* (Miller) Swingle. *International Biodeterioration & Biodegradation*, *33*, 333–343. doi:10.1016/0964-8305(94)90011-6

Baard, S. W., & Pauer, G. D. C. (1982). Effect of acid soils on survival of *Verticillium dahliae*. *Transactions of the British Mycological Society*, *79*, 513–517. doi:10.1016/S0007-1536(82)80044-2

Bennett, W. O., Paget, J. T., & Mackenzie, D. (2013). Surgery for a tree surgeon? Acute presentation of contact dermatitis due to *Ailanthus altissima*. *Journal of Plastic, Reconstructive & Aesthetic Surgery*, *66*, e79–e80. doi:10.1016/j.bjps.2012.11.040

Bisognano, J. D., McGrody, K. S., & Spence, A. M. (2005). Myocarditis from the Chinese sumac tree. *Annals of Internal Medicine*, *143*, 159–160. doi:10.7326/0003-4819-143-2-200507190-00031

Brandt, W. H., & Reese, J. E. (1964). Morphogenesis in *Verticillium*: A self-produced, diffusible morphogenetic factor. *American Journal of Botany*, *51*, 922–927. doi:10.2307/2440241

Chaudhuri, H. (1923). A study of the growth in culture of *Verticillium albo-atrum*, B. et Br. *Annals of Botany*, *37*, 519–539.

Derrick, E. K., & Darley, C. R. (1994). Contact reaction to the tree of heaven. *Contact Dermatitis*, *30*, 178–178. doi:10.1111/j.1600-0536.1994.tb00706.x

DiTomaso, J. M., & Kyser, G. B. (2007). Control of *Ailanthus altissima* using stem herbicide application techniques. *Arboriculture & Urban Forestry*, *33*, 55–63.

Feret, P. P. (1985). *Ailanthus*: Variation, cultivation, and frustration. *Journal of Arboriculture*, *11*, 361–368.

Foreman, G. L., Rouse, D. I., & Hudelson, B. D. (2002). Wood chip mulch as a source of *Verticillium dahliae* (abstract). *Phytopathology*, *92*, S26.

Fravel, D. R. (2005). Commercialization and implementation of biocontrol. *Annual Review of Phytopathology*, *43*, 337–359. doi:10.1146/annurev.phyto.43.032904.092924

Galanopoulos, N., & Tribe, H. T. (1974). Conidial survival in *Verticillium dahliae*. *Transactions of the British Mycological Society*, *63*, 85–91. doi:10.1016/S0007-1536(74)80138-5

Harris, P. T., Cannon, G. H., Smith, N. E., & Muth, N. Z. (2013). Assessment of plant community restoration following tree-of-heaven (*Ailanthus altissima*) control by *Verticillium albo-atrum*. *Biological Invasions*, *15*, 1887–1893. doi:10.1007/s10530-013-0430-2

Hu, S. Y. (1979). *Ailanthus*. *Arnoldia*, *39*, 29–50.

Inderbitzin, P., Bostock, R. M., Davis, R. M., Usami, T., Platt, H. W., & Subbarao, K. V. (2011). Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. *PLoS ONE*, *6*, e28341. doi:10.1371/journal.pone.0018260

Isaac, I., & MacGarvie, Q. (1966). Dormancy and germination of resting structures of *Verticillium* spp. *Transactions of the British Mycological Society*, *49*, 669–678.

Kasson, M. T., Davis, M. D., & Davis, D. D. (2013). The invasive *Ailanthus altissima* in Pennsylvania: A case study elucidating species introduction, migration, invasion, and growth patterns in the Northeastern US. *Northeastern Naturalist*, *20*, 1–60.

Kasson, M. T., O'Neal, E. S., & Davis, D. D. (2015). Expanded host range testing for *Verticillium nonalfalfae*: Potential biocontrol agent against the invasive *Ailanthus altissima*. *Plant Disease*. doi:10.1094/PDIS-04-14-0391-RE

Kasson, M. T., Short, D. P. G., O'Neal, E. S., Subbarao, K. V., & Davis, D. D. (2014). Comparative pathogenicity, biocontrol efficacy, and multilocus sequence typing of *Verticillium nonalfalfae* from the invasive *Ailanthus altissima* and other hosts. *Phytopathology*, *104*, 282–292. doi:10.1094/PHYTO-06-13-0148-R

O'Neal, E. S., & Davis, D. D. (2015). Intraspecific root grafts and clonal growth within *Ailanthus altissima* stands influence *Verticillium nonalfalfae* transmission. *Plant Disease*. doi:10.1094/PDIS-07-14-0722-RE

Orellana, R. G., Foy, C. D., & Fleming, A. L. (1975). Effect of soluble aluminum on growth and pathogenicity of *Verticillium albo-atrum* and *Whetzelinia sclerotiorum* from sunflower. *Phytopathology*, *65*, 202–205.

Pegg, G. F., & Brady, B. L. (2002). *Verticillium wilts*. Wallingford: CAB International.

Rebbeck, J., Malone, M. A., Short, D. P. G., Kasson, M. T., O'Neal, E. S., & Davis, D. D. (2013). First report of Verticillium wilt caused by *Verticillium nonalfalfae* on tree-of-heaven (*Ailanthus altissima*) in Ohio. *Plant Disease*, *97*, 999. doi:10.1094/PDIS-01-13-0062-PDN

Schall, M. J. (2008). *Verticillium wilt of Ailanthus altissima*. (Unpublished doctoral dissertation). The Pennsylvania State University, University Park.

Schall, M. J., & Davis, D. D. (2009a). *Ailanthus altissima* wilt and mortality: Etiology. *Plant Disease*, *93*, 747–751. doi:10.1094/PDIS-93-7-0747

Schall, M. J., & Davis, D. D. (2009b). Verticillium wilt of *Ailanthus altissima*: Susceptibility of associated tree species. *Plant Disease*, *93*, 1158–1162. doi:10.1094/PDIS-93-11-1158

Shah, B. (1997). The checkered career of *Ailanthus altissima*. *Arnoldia*, *57*, 21–27.

Shaner, G., & Finney, R. E. (1977). The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. *Phytopathology*, *77*, 1051–1056. doi:10.1094/Phyto-67-1051

Snyder, A. L., Kasson, M. T., Salom, S. M., Davis, D. D., Griffin, G. J., & Kok, L. T. (2013). First report of Verticillium wilt of *Ailanthus altissima* in Virginia caused by *Verticillium nonalfalfae*. *Plant Disease*, *97*, 837. doi:10.1094/PDIS-05-12-0502-PDN

Stubbs, T. L., & Kennedy, A. C. (2012). Microbial weed control and microbial herbicides. In R. Alvarez-Fernandez (Ed.), *Herbicides-environmental impact studies and management approaches* (pp. 135–166). Rijeka: InTech-Open Access.

Talboys, P. W. (1960). A culture-medium aiding the identification of *Verticillium albo-atrum* and *V. dahliae*. *Plant Pathology*, *9*, 57–58. doi:10.1111/j.1365-3059.1960.tb01147.x

Wilhelm, S. (1955). Longevity of the Verticillium wilt fungus in the laboratory and field. *Phytopathology*, *45*, 180–181.

Wilson, B. F., & Fischer, B. C. (1977). Striped maple: Shoot growth and bud formation related to light intensity. *Canadian Journal of Forest Research*, *7*, 1–7. doi:10.1139/x77-001

Wilson, C. L. (1969). Use of plant pathogens in weed control. *Annual Review of Phytopathology*, *7*, 411–434. doi:10.1146/annurev.py.07.090169.002211

Yadeta, K. A., & Thomma, B. P. H. J. (2013). The xylem as battleground for plant hosts and vascular wilt pathogens. *Frontiers in Plant Science*, *4*, 97. doi:10.3389/fpls.2013.00097