Intraspecific Root Grafts and Clonal Growth Within *Ailanthus altissima* Stands Influence *Verticillium nonalfalfae* Transmission

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Abstract

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Verticillium nonalfalfae, causal agent of Verticillium wilt, is being considered as a biocontrol for the highly invasive *Ailanthus altissima* in Pennsylvania. This soilborne fungus is extremely virulent on *Ailanthus* and rapidly transmitted from diseased to healthy trees within *Ailanthus* stands. The rapid transmission of the fungus could be facilitated by root grafts, but neither root graft formation in *Ailanthus* nor *Verticillium* transmission by root grafts in trees has been reported. Here, *V. nonalfalfae* transmission between diseased and healthy *Ailanthus* trees via intraspecific root grafts and clonal growth is evaluated. Using air-spade excavation, dye

translocation, and root graft inoculations, functional root grafts were detected between *Ailanthus* trees and transmission of *V. nonalfalfae* across root grafts demonstrated. Inoculation of one *Ailanthus* parent stem resulted in 187 root sprouts showing Verticillium wilt symptoms 12 months after inoculation. This study revealed that clonal growth and root grafts, normally advantageous growth habits, leave *Ailanthus* stands vulnerable to widespread *V. nonalfalfae* infection. This study also broadens the understanding of the *Ailanthus*-*Verticillium* pathosystem, growth strategies of invasive *Ailanthus*, and epidemiology of Verticillium wilt within trees.

Tree-of-heaven, *Ailanthus altissima* (Mill.) Swingle, is an aggressive exotic invader of forest and urban environments across the United States. Since introduction into Philadelphia in 1784, this shade-intolerant tree has become especially populous in the Mid-Atlantic region (11,22). Historically, *Ailanthus* was mainly found along transportation corridors and in urban areas, where it remains problematic. In addition, *Ailanthus* has recently invaded and increased in number within forested areas (22), where fast growth, prolific seed production, clonal proliferation, and allelopathy has enabled *Ailanthus* to displace desirable native plant species (8,17,31). Presently, attempts to control *Ailanthus* rely primarily on the use of chemical herbicides, which are expensive, laborious, often ineffective, and require repetitive applications. In recent years, Verticillium wilt caused by *Verticillium nonalfalfae* Inderbitzin et al., a soilborne, vascular wilt pathogen, has been proposed as an effective, host-adapted, biological control for *Ailanthus* (24,25,40).

V. nonalfalfae, previously classified as *V. albo-atrum* Reinke and Berthold, has been reported to infect hops, spinach, solanaceous crops, and various trees in the United States, the United Kingdom, Canada, Slovenia, and Japan (16,19,24,25,35,41). In Pennsylvania, Virginia, and Ohio, *V. nonalfalfae* has been isolated from wilting and dying *Ailanthus* trees and appears native to eastern forests (25,37,43,44). Infected *Ailanthus* trees exhibit yellow vascular discoloration and acute foliage wilt, followed by defoliation, and eventual mortality. Etiology and efficacy studies have shown that *V. nonalfalfae* isolate VnAa140 is extremely virulent on *Ailanthus*, and comparative pathogenicity and host range testing revealed that the isolate appears to be host-adapted to *Ailanthus* (24,25,41). However, very low incidences of natural infection have been observed in striped maple (*Acer pensylvanicum* L.), staghorn sumac (*Rhus typhina* L.), and devil's walking stick (*Aralia spinosa* L.) within severely infected *Ailanthus* stands, indicating that these three species may serve as minor, native, alternative hosts (24).

In both naturally infected and artificially inoculated *Ailanthus* stands, *V. nonalfalfae* is rapidly transmitted from diseased to healthy trees by undetermined modes. During the spring seasons of 2006 to 2009, the inoculation of 100 *Ailanthus* trees across 12 stands resulted in the infection and mortality of >14,000 *Ailanthus* stems by fall of 2011. In one of these stands, *V. nonalfalfae* was transmitted from only 10 inoculated *Ailanthus* trees to >300 adjacent, formerly healthy *Ailanthus* trees within 1 year after inoculation (25). We hypothesized that the modes of rapid *V. nonalfalfae* transmission, both local and long-distance, may include insect vectors (ambrosia beetles); infected leaves, seeds, and wood; root contact (12,20,30,38); clonal root systems; and intraspecific root grafts (23,25,39). The latter two potential modes of transmission are the focus of this study.

Root grafts between trees are initiated when opposing mature roots first come in contact. The respective cortexes then rupture from the pressure caused by root diameter expansion that is constricted by soil and rock. Next, the cambia come into contact and vascular tissues fuse (4,27,32,33). Ecologically, root grafts between or among trees can influence tree growth, forest succession, stand development, and forest management practices (9,15,48). Root grafts are generally advantageous adaptations for trees (28), but transmission of many well-known forest pathogens can occur through root grafts (5,10). For example, *Ceratocystis fagacearum* (Bretz) J. Hunt, causal agent of oak wilt, and *Ophiostoma ulmi* (Buis.) Melin and Nannfeldt, causal agent of Dutch elm disease, can be transmitted from diseased to healthy trees by intraspecific root grafts (18,49). Other wilt diseases such as persimmon wilt, caused by *Acremonium diospyri* (Crand.) W. Gams, and laurel wilt, caused by *Raffaelea lauricola* T.C. Harr., Fraedrich, and Agaveya, might also be transmitted by root grafts and clonal root systems. However, evidence for root graft transmission of *A. diospyri* and *R. lauricola* is limited and requires further study (6,36).

Although root grafts have not been reported to transmit *Verticillium* spp. from diseased to healthy trees, Epstein (10) reviewed this possibility. Intraspecific root grafts have not been reported in *A. altissima*, but there is circumstantial evidence that such grafts occur. For example, Lewis and McCarthy (29) reported that herbicide applications of select *Ailanthus* trees resulted in the death of adjacent, untreated *Ailanthus* trees, presumably by herbicide translocation through root grafts between treated and untreated trees. Also, Schall (39) observed that noninoculated *Ailanthus* trees adjacent to *Ailanthus* trees artificially inoculated with *V. nonalfalfae* developed wilt symptoms within

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2 weeks after the inoculated trees wilted. These observations indicate that intraspecific root grafts and/or clonal root systems may be important in the rapid, localized transmission of *V. nonalfalfae* from diseased to healthy *Ailanthus* trees. Herein we provide the first evidence of functioning intraspecific root grafts in *Ailanthus* and movement of *Verticillium* through the grafts.

The main objectives of this study were to (i) determine if *Ailanthus* naturally forms functional intraspecific root grafts, (ii) estimate the frequency of intraspecific root grafts within *Ailanthus* stands, (iii) determine if *V. nonalfalfae* can be transmitted from tree-to-tree by intraspecific root grafts, and (iv) determine if *V. nonalfalfae* can be transmitted through clonal root systems of *Ailanthus*.

Materials and Methods

Study sites. Three *Ailanthus* stands, differing in age and size of *Ailanthus* trees, located in south-central Pennsylvania among mixed-hardwood dominated forests, were selected for the root graft studies.

The first *Ailanthus* stand, LV, was even-aged and located in Locke Valley near Shade Gap, PA, within Rothrock State Forest (Huntingdon Co.). The mean age of *Ailanthus* trees was 11.8 years and mean diameter at breast height (DBH, 1.4 m) was 11.7 cm. The mean basal area for *Ailanthus* trees within the stand was 23.0 m²/ha.

The second stand, SGL 322, was a mixed-aged stand located on State Game Lands 322 near Petersburg, PA (Huntingdon Co.). The mean age of *Ailanthus* trees was 37.6 years, mean DBH was 12.2 cm, and mean basal area for *Ailanthus* trees was 22.5 m²/ha. This stand also contained many small *Ailanthus* saplings and young root sprouts, in addition to a few old large canopy *Ailanthus* trees.

The third stand, SGL 281, was located on State Game Lands 281 near Newport, PA (Perry Co.). This even-aged stand was larger, denser, and older than the other two study stands. Mean *Ailanthus* tree age was 38.9 years, mean DBH was 17.3 cm, and mean basal area for *Ailanthus* trees was 32.6 m²/ha.

Detection of root grafts using dye translocation. Methods used to detect functional intraspecific root grafts in *Ailanthus* stands were modified from protocols reported by Bormann and Graham (4) and Graham (14). In May 2013, five circular 5-m radius plots per stand were established in stands LV, SGL 322, and SGL 281.

An *Ailanthus* tree in the middle of each plot was selected, based on size and proximity to neighboring *Ailanthus* stems, and designated as plot center. Diameters of all *Ailanthus* stems >2.54 cm DBH in each plot were recorded, as well as distance of each stem from the plot center. In each plot, the plot center stem was felled, leaving an approximately 30-cm tall “donor” stump. A 30-cm tall dye reservoir was constructed on each donor stump using vinyl roof flashing, staples, and caulk. Reservoirs were filled with 0.15% aqueous acid fuchsin dye solution. Each reservoir was capped and covered with plastic sheeting to prevent evaporation (Supplementary Fig. 1). Dye was maintained at a depth of 13 cm for 6 weeks, at which time a 20-cm wide strip of bark around the circumference on all *Ailanthus* stems within each plot was removed. Stems that received dye from the donor stump were designated “receptor” stems and those that did not were designated “non-receptor” stems. The ages of all receptor stems and felled donor stems were determined at DBH using 5.15-mm diameter increment borers (Haglof Company Group, Madison, MS). Extracted cores were mounted, sanded, and aged by counting annual growth rings as described by Kasson et al. (22).

Air-spade excavation of *Ailanthus* root systems. In early June 2013, an air-spade (Guardair Corp., Chicopee, MA) and a towable 185 CFM air compressor (Sullair, Michigan City, IN) were used to excavate the root systems of 16 *Ailanthus* trees in stand LV to determine presence and estimate frequency of intraspecific root grafts. Stand LV was judged most suitable for excavation due to its accessibility and lack of large stones. *Ailanthus* trees to be excavated were selected based on size and proximity to other *Ailanthus* trees. Herbaceous vegetation, if present, within a 2-m radius around each selected stem was removed and soil excavated to a depth of approximately 40 cm with the air-spade. Any two *Ailanthus* roots, each originating from a separate stem, that appeared to be grafted and could not be easily separated by hand were tallied as intraspecific root grafts.

Culture maintenance, inoculum preparation, and isolation.

Protocols for *V. nonalfalfae* culture maintenance and inoculum preparation followed those reported by Kasson et al. (25) and Schall and Davis (40). *V. nonalfalfae* was grown in petri dishes containing plum extract agar (PEA) (46) amended with streptomycin sulfate and neomycin sulfate (PEA+SN) (25,40) in a controlled environment chamber maintained at 23°C with a 12-h photoperiod.

After 4 weeks, 5 to 10 ml sterile distilled water was added to the surface of actively growing cultures. Mycelia and conidia were loosened from the agar with a sterile spatula and pipetted in 3-ml aliquots into 10-ml scintillation vials containing a 1:1:1 mixture (by volume) of sterile potting soil-perlite-peat moss. The mixture had been previously autoclaved for 1 h on at least three or more consecutive days and prewetted with 2 ml sterile distilled water. Vials were loosely capped and stored on the laboratory bench at room temperature for 2 weeks to allow *V. nonalfalfae* colonization of the mixture. After 2 weeks, the vials were tightly capped and stored at 4°C. To remove isolates from storage, soil particles were tapped from vials onto fresh PEA+SN petri dishes. Within 3 to 5 days, resulting colonies were transferred to new PEA+SN dishes. *V. nonalfalfae* isolates stored following this protocol remain viable after >4 years of storage (E. S. O’Neal, unpublished data).

To produce inoculum, 3-week-old *V. nonalfalfae* isolate VnAa140 cultures were flooded with 10 ml sterile distilled water and scraped with a sterile spatula to loosen mycelia and conidia. The resultant suspension was collected, vortexed, and passed through a milk filter (KenAg, Ashland, OH) to remove mycelial fragments. Conidial concentration was determined using a hemocytometer and adjusted to 10⁷ conidia ml⁻¹ by adding sterile distilled water to the suspension. Viability of resulting inoculum was determined by counting colony forming units (CFU) from 10-fold serial dilutions on PEA+SN dishes. Inoculum exhibiting >80% conidial viability was stored at 4°C until used in the field. Pathogenicity of *V. nonalfalfae* isolate VnAa140 was maintained by annually inoculating 1-month-old potted *Ailanthus* seedlings in a greenhouse with VnAa140 conidial suspension. Following inoculation, symptomatic stems were destructively sampled, disinfested with 70% ethanol, and inserted into fresh PEA+SN dishes, where *V. nonalfalfae* was subsequently reisolated into pure culture for use as inoculum.

To confirm infections following field inoculations and symptom development, *V. nonalfalfae* was isolated by removing bark and excising discolored wood chips from symptomatic roots or stems with a disinfested knife. Chip samples were bagged, placed in coolers, and surface-disinfested in the laboratory with 70% ethanol. Samples were reduced in size with sterile cutting instruments to remove outer surfaces, exposing the inner wood, and inserted into PEA+SN dishes. After 10 days, emerging colonies exhibiting morphological characteristics of *V. nonalfalfae* (e.g., verticillate conidiophores and melanized resting mycelia) were transferred to fresh PEA+SN dishes to obtain pure cultures that were stored and later used for molecular identification.

DNA extraction and molecular identification of isolates. Genomic DNA was extracted from air-dried mycelial plugs harvested from potato dextrose broth (PDB) (BD, Franklin Lakes, NJ) using a Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI), following procedures used by Kasson et al. (25). Isolate identification was based on PCR protocols reported by Inderbitzin et al. (19). A Nyx Technik ATC 201 Programmable Thermal Cycler (Nyx Technik, Inc., San Diego, CA), GoTaq PCR kits (Promega Corp., Madison, WI), and Platinum Taq (Invitrogen, Carlsbad, CA) were used to amplify three loci: the protein-coding genes elongation factor 1- α (EF), glyceraldehyde-3-phosphate dehydrogenase (GPD), and tryptophan synthase (TS). Amplified PCR products were electrophoresed in 2% agarose gels and stained with EZ-Vision Three DNA Dye & Buffer 6 $\times$ (AMRESCO, Solon, OH) to visualize bands. PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA). At the Pennsylvania State University Genomics Core Facility, Sanger sequencing was conducted on an ABI 3730 XL automated DNA sequencer (Applied Biosystems, Carlsbad, CA). Sequencher 4.8 (Gene Codes, Ann Arbor, MI) was used to revise the raw sequence data and assemble consensus sequences before used as BLAST queries to search against the NCBI GenBank database for isolate identification.

Intraspecific root graft inoculations. In late May to mid-June 2013, 12 previously discovered intraspecific root grafts were fully excavated within *Ailanthus* stands SGL 281 and LV. Vegetation and soil were removed from around the roots to expose the graft unions (Fig. 1A), and each grafted root was traced to its parent stem. Grafts were rinsed with distilled water to remove any additional soil. One root, designated the “donor” root, was severed on both sides of the graft union. This created a free root segment connected to an intact root, designated the “receptor” root, only by the graft union (Fig. 1B). Severing the donor roots was conducted to ensure that *V. nonalfalfae* would enter the receptor roots and stems only via the graft unions.

Both cut surfaces of the donor root were sealed with grafting wax (Trowbridge’s, Walter E. Clark & Son, Orange, CT) to reduce desiccation. Ten donor roots were each inoculated with a conidial suspension (10^7 conidia ml^{-1}) of *V. nonalfalfae* isolate VnAa140 at two locations, between each cut surface and the graft union. Inoculation points received 0.5 ml inoculum in a wound made with a 0.75-cm diameter cork borer (Fig. 1C).

A variation of this protocol was used on intraspecific root grafts that formed within the base of *Ailanthus* stems, where a root grew into and fused with the base of a neighboring stem. In those cases, the

donor root was severed, creating a root stub protruding from the stem base. This stub was inoculated only in one location, between the stem base and the cut surface, with 0.5 ml conidial suspension (Fig. 1D). The remaining two donor roots served as negative controls and were treated with sterile distilled water. A plastic sheet was placed under each intraspecific root graft during inoculation and for the duration of the experiment to prevent inoculum from contacting the soil.

Once the donor root absorbed the inoculum, the severed root faces and inoculation wounds were sealed with grafting wax to reduce desiccation (Fig. 1C and D). Each graft, including the entire donor root, was wrapped in a white cotton cloth to minimize *V. nonalfalfae* contact with soil and reduce root desiccation. When the parent stem of the receptor root, designated the “receptor” stem, exhibited acute wilt symptoms ($>15\%$ wilt of the total foliage) the bark was removed from the receptor stem and root, in addition to the donor root to inspect for yellow vascular discoloration. Wood chip samples were taken from discolored wood in the receptor stems and roots, as well as associated donor roots. These samples were then cultured on PEA+SN dishes to reisolate *V. nonalfalfae*. In addition, the root grafts involved in *V. nonalfalfae* transmission were destructively sampled and examined for shared vascular tissues (13). All receptor stems were monitored biweekly for Verticillium wilt symptoms until June 2014, at which time remaining inoculated root grafts were destructively sampled and examined to determine if they were functional, indicated by shared vascular tissues.

***Ailanthus* clone inoculation.** In early June 2013, a fourth study site was selected to study *V. nonalfalfae* transmission within a relatively small, nearly pure *Ailanthus* clone. This new site was located on the Raystown Lake Project, U.S. Army Corps of Engineers (USACE) land near Hesston, PA (Huntingdon Co.). The stand consisted of two male (non-seed producing) *Ailanthus* parent stems and >200 suspected *Ailanthus* clonal root sprouts <2.54 cm DBH. Root sprouts differ from root grafts in that a root sprout is a vegetative shoot that arises directly from parent stem’s roots, thus already sharing vascular tissues.

The larger of the two male parent stems (24.3 cm DBH, 14 years old) at the stand center was inoculated by injecting 3 ml *V. nonalfalfae* isolate VnAa140 conidial suspension (10^7 conidia ml^{-1}) into three basal wounds (1 ml/wound) with a hypo-hatchet (OEM Fabricators Inc., Woodville, WI). All surrounding *Ailanthus* stems >0.5 m in height, as well as both parent stems, a total of 213 stems, were flagged for monitoring. As stems exhibited Verticillium wilt symptoms, they were flagged with a different color and tallied. Stems that exhibited $>15\%$ wilt of the total foliage were rated as symptomatic; stems that had $<15\%$ wilted foliage were rated as asymptomatic. A tally of symptomatic stems was generated monthly (with an additional tally generated in mid-September 2013) through June 2014 to monitor the spread of *V. nonalfalfae* throughout the *Ailanthus* clone. In May 2014, wood samples were taken from 10 symptomatic stems surrounding the inoculated parent stem to reisolate *V. nonalfalfae*.

Results

Detection of root grafts in three stands using dye translocation.

Results from the dye translocation study revealed that *Ailanthus* frequently forms functional intraspecific root grafts, even within young stands like LV. Dye translocation was observed within all three study stands, as evidenced by purple dye in the xylem of *Ailanthus* receptor stems (Fig. 2). Thirteen of 15 donor stumps (87%) donated dye to at least one receptor stem, and nine of the 13 donor stumps (69%) donated dye to the nearest *Ailanthus* stem. Thirty-five receptor stems were observed: nine in stand LV, nine in stand SGL 322, and 17 in stand SGL 281 (Tables 1, 2, and 3). Occasionally, multiple dark streaks of dye were observed in single stems (Fig. 2, Supplementary Fig. 2). Dissection of donor stumps revealed that downward dye movement occurred only in a few outer growth rings, even though the entire cut stump surface was in contact with dye (Supplementary Fig. 3).

In the youngest *Ailanthus* stand, LV, four of the five *Ailanthus* donor stumps donated dye to at least one receptor stem (Table 1). No visible dye was observed in any potential receptor stems in the remaining plot. The mean number of receptor stems was 1.8 receptor

Fig. 1. Methods used to inoculate *Ailanthus* intraspecific root grafts with *Verticillium nonalfalfae* isolate VnAa140. The diagram shows **A**, the excavation of the intraspecific root graft from soil; **B**, severing of the donor root and designation of the receptor root and stem; **C**, inoculation points, each receiving 0.5 ml *V. nonalfalfae* isolate VnAa140 conidial suspension (10^7 conidia ml^{-1}), sealing of the inoculation points and wounds created by severing the donor root with grafting wax; and **D**, a modified inoculation method used to inoculate intraspecific root grafts that occur within the base of an *Ailanthus* stem.

stems per donor stump. The mean stem DBH of the five donor stumps was 15.1 cm (range: 12.6 to 22.1 cm). The mean receptor stem DBH was 11.8 cm (range: 6.8 to 16.3 cm). The mean distance between the donor stumps and receptor stems was 1.24 m (range: 0.40 to 2.15 m). The four donor stumps that donated dye did so to the nearest *Ailanthus* stem (Table 1).

In the mixed-aged stand, SGL 322, all five *Ailanthus* donor stumps donated dye to at least one receptor stem, and had a mean of 1.8 receptor stems/donor stump (Table 2). The mean stem DBH of the donor stumps was 18.2 cm (range: 14.6 to 24.3 cm). The mean receptor stem DBH was 18.5 cm (range: 8.2 to 25.6 cm). The mean distance between donor stumps and receptor stems was 1.5 m (range: 0.65 to 2.20 m). Unlike stand LV, only two of the five donor stumps donated dye to the nearest *Ailanthus* stem (Table 2).

Lastly, in the older even-aged stand, SGL 281, four of five *Ailanthus* donor stumps had at least one receptor stem. The mean number of receptor stems was 3.4 stems/donor stump (Table 3). The mean stem DBH of the donor stumps was 22.3 cm (range: 20.7 to 25.3 cm). Mean receptor stem DBH was 19.6 cm (range: 5.4 to 29.1 cm). Greater distances between donor stumps and receptor stems were observed in this stand, as compared with the other two stands. The mean distance between the donor stumps and the receptor stems was 2.60 m (range: 0.95 to 5.00 m), which was double the mean distance between stems in stand LV (Tables 1 and 3). Eight of 17 (47%) receptor stems were ≥ 2.5 m from their donor stumps. In addition, three of four donor stumps that donated dye did so to the nearest *Ailanthus* stem. There were no receptor stems in plot 2 (Table 3), even though a large-diameter neighboring stem (28.0 cm DBH) was present within a few meters (1.95 m) from the large donor stump (21.8 cm DBH).

Air-spade excavation of *Ailanthus* root systems. Of the 16 *Ailanthus* root systems excavated, 12 (75%) formed at least one intraspecific root graft with a neighboring *Ailanthus* stem (Table 4). Excavation revealed a total of 28 intraspecific *Ailanthus* root grafts, which represented a mean of 1.8 root grafts per stem (Table 4). The number of root grafts excavated per stem ranged from one to seven. The mean distance between root-grafted stems was 1.08 m (range: 0.55 to 2.04 m). Seventy-five percent of the stems that formed intraspecific root grafts formed them with the nearest *Ailanthus* stem. Root systems of stems 9 and 14 formed root grafts with the nearest *Ailanthus* stem and one additional nearby stem (Table 4). Intraspecific root grafts were not observed in the root systems of stems 1, 2, 4, and 12 (Table 4). Six (38%) of the 16 excavated *Ailanthus* root systems formed a total of 11 self-grafts. Interspecific root grafts between *A. altissima* and other tree species were not observed.

Intraspecific root graft inoculations. In May 2013, 10 excavated *Ailanthus* intraspecific root grafts were inoculated with *V. nonalfalfae* isolate VnAa140. Of the inoculated root grafts, three receptor

stems (30%) displayed symptoms characteristic of *Verticillium* wilt (Fig. 3A), becoming symptomatic at 2, 4, and 12 weeks postinoculation (WPI). The three symptomatic stems developed yellow vascular discoloration that extended distally into the receptor roots, graft unions, and donor roots (Fig. 3B and C). *V. nonalfalfae* was successfully isolated from symptomatic receptor trees and their associated donor roots, confirming transmission of the pathogen. The remaining seven receptor stems were asymptomatic through June 2014, at which time sampling and dissection revealed that four of the seven grafts were not functional. The two control stems remained asymptomatic.

Inoculation of the *Ailanthus* clone. Inoculation of one *Ailanthus* parent stem with *V. nonalfalfae* isolate VnAa140 revealed that *Verticillium* can spread rapidly from a parent stem into clonal root sprouts. At 1 month postinoculation (MPI), 12 of the 213 (5.6%) monitored stems (>0.5 m tall), including the inoculated parent stem, were symptomatic (Fig. 4). By 2 MPI, the number of symptomatic stems increased to 48 (22.5%), and wilting sprouts were observed 10 m from the inoculated parent stem. At 3 MPI, the second suspected parent stem, which had not been inoculated and was located 3.5 m from the inoculated parent stem, became symptomatic. By mid-September 2013, 3.5 MPI, 129 stems (60.6%) were symptomatic. At this time, the inoculated parent stem displayed 99% crown defoliation (Fig. 4) and many additional unmonitored, smaller root sprouts (<0.5 m tall) were wilting and defoliating as a result of the one original inoculation. In June 2014, 12 MPI, the inoculated parent stem was dead and the total number of symptomatic stems increased to 187 (87.8%) (Fig. 4). *V. nonalfalfae* was successfully reisolated from all wood chip samples taken from 10 symptomatic stems.

Discussion

The objectives of this study were to determine if *Ailanthus* forms functional intraspecific root grafts, how frequently they form, and whether *V. nonalfalfae* isolate VnAa140 can be transmitted through *Ailanthus* intraspecific root grafts and clonal root systems.

The dye translocation study revealed that *Ailanthus* forms functional intraspecific root grafts, like many other tree species. A total of 35 receptor stems received dye from just 13 donor stumps, suggesting that *Ailanthus* not only forms intraspecific root grafts, but that grafts are common and abundant, especially in dense stands. Within the 11-year-old *Ailanthus* stand LV there were nine receptor stems, indicating that *Ailanthus* forms functional intraspecific root grafts at a young age, likely resulting from its rapidly expanding lateral root systems early in stand development (34). In the older and denser *Ailanthus* stand SGL 281, there were 17 receptor stems, indicating that the number of functional intraspecific root grafts and interconnected trees increases with stand age and density, as within other trees species. These direct relationships likely occur because larger root

Table 1. Dye translocation observed within the even-aged 11-year-old *Ailanthus* stand, LV

Plot ID	Donor stump DBH (cm) ^b	Receptor stems ^a		Nearest non-receptor stem	
		DBH (cm)	Distance from donor stump (m)	DBH (cm)	Distance from donor stump (m)
1	12.6	12.0	0.90	14.0	1.05
2	15.3	-	-	7.1	1.10
3	13.1	16.0	0.80	14.5	1.05
		8.6	1.40		
		15.5	2.00		
4	12.6	12.5	0.40	7.2	0.50
		6.8	1.05		
		7.1, 10.3, 11.7	1.25		
5	22.1	16.3	1.25	15.3	2.75
		13.2	2.15		
Average	15.1	11.8	1.24	11.6	1.29

^a For multiple stemmed receptor stems, the DBH values for every stem are listed. “-” indicates there were no receptor stems.

^b The DBH of the stem prior to felling.

Table 2. Dye translocation observed within the mixed-aged 37-year-old *Ailanthus* stand, SGL 322

Plot ID	Donor stump DBH (cm) ^a	Receptor stems		Nearest non-receptor stem	
		DBH (cm)	Distance from donor stump (m)	DBH (cm)	Distance from donor stump (m)
1	20.0	12.8	1.55	5.0	1.00
2	15.2	18.7	0.65	10.6	0.60
		20.9	2.05		
3	24.3	25.6	0.75	18.6	1.70
		20.7	1.60		
		23.6	2.20		
4	16.7	13.8	1.65	2.7	1.25
5	14.6	8.2 ^b	1.40	3.4	1.45
		22.3	1.55		
Average	18.2	18.5	1.5	8.1	1.2

^a The DBH of the stem prior to felling.

^b Stem was possibly of clonal origin and may not have received dye via intraspecific root graft.

Fig. 2. Dye translocation between *Ailanthus* stems in *Ailanthus* stand SGL 281, showing four receptor stems that received dye from the donor stump (shown in the foreground fitted with a covered dye reservoir), indicating the presence of intraspecific root grafts. The receptor stem on the extreme right was 4.0 m from the donor stump. Multiple streaks of dye were occasionally observed in receptor stems indicating multiple intraspecific root grafts may have been involved in dye translocation.

Table 3. Dye translocation observed within the even-aged 38-year-old *Ailanthus* stand, SGL 281

Plot ID	Donor stump DBH (cm) ^b	Receptor stems ^a		Nearest non-receptor stem	
		DBH (cm)	Distance from donor stump (m)	DBH (cm)	Distance from donor stump (m)
1	21.3	15.2	1.00	19.4	0.60
		29.1	1.30		
2	21.8	-	-	28.0	1.95
3	25.3	5.4 ^c	0.95	30.2	1.95
		23.2	1.35		
		16.9	2.65		
		24.0	3.00		
		20.9	3.00		
		21.3	4.70		
		5.5 ^c	4.75		
4	20.7	13.3 ^c	5.00	5.5	2.15
		23.5	1.75		
		21.7	2.40		
		25.7	2.50		
5	22.3	21.2	4.00	22.7	1.15
		19.2	1.10		
		23.0	2.35		
Average	22.3	23.5	2.35	21.2	1.56
		19.6	2.60		

^a “-” indicates there were no receptor stems.

^b The DBH of the stem prior to felling.

^c Stem was possibly of clonal origin and may not have received dye via intraspecific root graft.

Table 4. Number of intraspecific root grafts per excavated *Ailanthus* root system in stand LV

Excavated root systems			Root-grafted stems ^a		Nearest non-grafted stem	
Stem ID	DBH (cm)	Number of intraspecific root grafts	Distance(s) from excavated stem (m)		Distance from excavated stem (m)	
			DBH (cm) ^b	Distance from excavated stem (m)	DBH (cm) ^b	Distance from excavated stem (m)
1	12.2	0	-	-	10.2	0.75
2	14.3	0	-	-	18.8	1.05
3	12.0	1	10.3	1.35	12.4	0.85
4	10.4	0	-	-	15.1	1.15
5	14.0	2	15.5	1.50	12.4	1.85
6	20.2	1	12.6	0.79	17.0	1.10
7	14.7	1	13.0	1.33	12.5	1.35
8	13.1	1	20.0	2.04	12.4	1.00
9	15.0	3	13.2, 13.7	0.75, 0.85	10.2	1.10
10	16.2	2	17.4	1.02	8.3 ^c	0.90
11	12.6	2	8.1	0.55	5.2	1.05
12	17.2	0	-	-	11.5	1.40
13	15.4	7	17.6	1.06	7.2 ^c	1.65
14	12.3	4	15.5, 13.8	0.93, 1.1	10.4	2.10
15	12.3	3	8.7	0.65	12.1	1.35
16	14.3	1	23.9	1.14	14.4	1.35
Average	14.1	1.8	13.4	1.08	11.7	1.25

^a Excavated root systems that formed intraspecific root grafts with multiple neighboring stems have multiple DBH and distance values listed respectively for each root-grafted stem. “-” indicates that no root intraspecific root grafts were discovered.

^b For multiple stemmed trees, initial multiple DBH values were each converted to basal area (m²) values ($BA = 0.00007854 \times DBH^2$), summed, and converted back to a single DBH (cm) value ($DBH = \sqrt{(BA/0.00007854)}$).

^c Stem was dead.

systems of older trees have a greater probability of coming into contact and forming root grafts with other trees. Also, older trees have had more time to form functional root grafts (1,7,13,45). To our knowledge, this is the first report of functional intraspecific root grafts in *A. altissima*.

Dye translocation studies do not allow for exact quantification of functional root grafts between or among trees, and generally underestimate the abundance of root grafts. Bormann and Graham (4) presented three ways in which the number of root grafts between trees might be underestimated in dye translocation studies. First, root grafts are only detected by the dye, and the actual number of grafts

cannot be accurately quantified, since one or more root grafts can contribute to dye translocation from the donor stump to receptor stems. For example, we observed multiple streaks of dye within the xylem of some receptor stems (Fig. 2), suggesting multiple root grafts were involved in dye translocation. Secondly, streaks of dye can occur deeper in the xylem, where they cannot be observed without felling the trees. Since all *Ailanthus* trees within the dye translocation plots were not felled, we could not detect possible dye translocation in deeper xylem tissues of all stems. Thirdly, when

several potential receptor stems are grafted to each other, but perhaps only one is directly grafted to the donor stump, the closest receptor stems may preferentially absorb the dye, leaving little for the more distal receptor stems. Dye translocation is at least partially caused by the transpirational pull of receptor stems, which is likely inversely related to distance between the donor stump and receptor stem. Thus, the small amounts of dye translocated to distal receptor stems might not be observed. However, the interconnected trees may still be able to receive agents other than dye, such as wilt pathogens, via translocation through grafted root systems.

In contrast, vegetative root sprouts could receive dye if their parent stem is selected as a donor. Dye may travel from the donor stump, along its root system, and upward into the vegetative sprouts. This coloration might be misinterpreted as translocation via root grafts, resulting in an overestimation of root graft frequency. This form of dye translocation likely occurred within stands SGL 322 and SGL 281 (Tables 2 and 3), where some receptor stems were suspected to be of clonal origin. However, even if some of these stems were indeed clonal, the vast majority (88.5%) of the observed receptor stems appeared to receive dye via intraspecific root grafts.

Although dye translocation experiments provide valuable observations and data, they must be considered preliminary in furthering our understanding of *Ailanthus*' underground growth strategies. Therefore, to physically observe root grafts and further document their frequency within *Ailanthus* stands, we excavated root systems of 16 *Ailanthus* stems in the youngest *Ailanthus* stand, LV. Air-spade excavation allowed for targeted excavations that do not damage or disrupt root grafts, unlike previous studies using hydraulic excavating (7,21,47).

Similar to the results of the dye translocation experiment, the excavations revealed that intraspecific root grafts among *Ailanthus* trees were very common, even in young stands. The 16 excavated root systems in stand LV yielded 28 *Ailanthus* intraspecific root grafts (1.8 grafts per stem), 75% of which formed at least one intraspecific root graft, usually with the nearest *Ailanthus* stem. Self-grafts within *Ailanthus* root systems were also observed, but potential for interspecific graft formation was likely hindered by *Ailanthus*' allelopathy, as well as stand LV's lack of tree species diversity.

Proximity appeared to be an important factor governing root graft frequency among *Ailanthus* stems, as reported for other tree species (9,42,47). If excavation had been feasible and conducted in stand SGL 281, which was denser than stand LV and contained larger and older *Ailanthus* trees, we hypothesize that perhaps double the number of root grafts would have been observed, as indicated by the difference in the number dye receptor stems between stands LV (nine stems) and SGL 281 (17 stems) (Tables 1 and 3). In support of this idea, our observations and preliminary hand excavations in

Fig. 4. Disease progression of *Verticillium* wilt in an artificially inoculated parent stem and surrounding clonal stems. The parent stem was inoculated with *Verticillium nonalfalfae* isolate VnAa140 in June 2013. All 212 nearby stems (>0.5 m tall), as well as the inoculated parent stem, were monitored for *Verticillium* wilt symptoms until 12 months postinoculation. Stems were tallied as symptomatic when the total foliage exhibited >15% wilt. As a part of each tally, a percentage of symptomatic monitored stems was calculated.

Fig. 3. Transmission of *Verticillium nonalfalfae* isolate VnAa140 by an *Ailanthus* intraspecific root graft, showing **A**, wilting foliage of a receptor stem, as compared with the foliage of adjacent healthy *Ailanthus* crowns; **B**, yellow vascular discoloration in the donor root ("a"), associated with the inoculation wound (area of detail), the receptor root ("b"); and **C**, the receptor stem ("c"). *V. nonalfalfae* was successfully reisolated from the donor root and receptor stem.

stand SGL 281 revealed seven intraspecific *Ailanthus* root grafts, as well as two living *Ailanthus* stumps. A living stump is created when a stem dies or is felled, but its root system continues to live and function via root grafts to a nearby healthy tree, and thus is a good indicator of root graft presence (2,15,50).

With intraspecific root grafts, living stumps, and clonal growth, *Ailanthus* likely forms vast interconnected root systems capable of colonizing large areas, contributing to its invasive nature and ability to compete. In this manner, *Ailanthus* is similar to trembling aspen (*Populus tremuloides* Michx.), which is capable of forming large clones that include living stumps and living root systems from stems that have been dead for decades (7,21).

Kowarik (26) reported that clonal growth allows *Ailanthus* to produce vegetative sprouts that quickly fill canopy gaps within forested environments. Likewise, the interconnectedness of *Ailanthus* trees via root grafts and living stumps may act similarly. Interconnected root grafts and living stumps likely help *Ailanthus* maintain a competitive advantage over present or recently established non-*Ailanthus* tree species by utilizing space and absorbing water and nutrients that competitors would otherwise utilize. Though undoubtedly beneficial for *Ailanthus*, root grafts can also be detrimental to survival.

Our inoculations confirmed that *V. nonalfalfae* isolate VnAa140 can move from diseased to healthy *Ailanthus* trees via functional root grafts. However, root graft sampling and dissections conducted in June 2014 revealed that four of the 10 inoculated root grafts were not functional, and thus were not capable of transmitting *V. nonalfalfae*, likely due to the young age of the parent stems. Therefore, only three of the six root graft inoculations, involving functional grafts, resulted in successful transmission to receptor stems, indicating other factors may have limited the success of this novel root graft inoculation technique.

Severing and sealing of the inoculated *Ailanthus* donor roots may have slowed or stopped the transpirational pull across the graft unions, decreasing translocation of *V. nonalfalfae* inoculum from the donor roots to the receptor roots and stems. Furthermore, severing and sealing the donor roots may have caused the fungus to grow more slowly toward the graft unions, and might help explain time differences observed between inoculation and symptom development among the three symptomatic receptor *Ailanthus* stems. However, severing the donor roots was essential to ensure that *V. nonalfalfae* would enter the receptor roots and stems only via the graft unions.

Nevertheless, three of the root graft inoculations were successful and confirm that *V. nonalfalfae* can be transmitted from a diseased root to a healthy root by way of intraspecific root grafts. To our knowledge, this is the first report confirming *Verticillium* transmission from diseased to healthy trees via root grafts for any tree species.

To what level and how quickly *Verticillium* spp. transmission occurs within *Ailanthus* stands under natural conditions is difficult to predict, since the speed and efficacy of transmission depends on both frequency and functionality of root grafts between or among *Ailanthus* trees. Even two *Ailanthus* trees connected by functional root grafts may not share resources equally, and the deprived tree may decline and eventually die (3,4,9). In addition, if the dominant tree becomes infected first with *V. nonalfalfae*, that tree may decline and *V. nonalfalfae* conidia may be redirected into the previously deprived tree, which may then become the new dominant tree. In contrast, if the inferior *Ailanthus* tree becomes infected first, *V. nonalfalfae* may be transmitted into the dominant tree rapidly due to its stronger transpirational pull. However, regardless of which tree becomes infected first, both connected *Ailanthus* trees would likely succumb to *V. nonalfalfae* infection soon after the initial infection.

The mean number of receptor stems per donor stump ratio of 2.3 appears to be a useful measure of *Ailanthus* tree-to-tree connectivity. Use of this ratio allows us to further demonstrate that root graft transmission is likely a major mode of rapid, local *V. nonalfalfae* transmission in high density stands. For example, if 10 trees became infected with *V. nonalfalfae*, they could potentially transmit the pathogen to 2.3 additional trees each. Then all 23 newly infected trees could transmit *V. nonalfalfae* to another additional 2.3 trees each, and so forth in a geometric progression. Since *V. nonalfalfae* can be transmitted to noninoculated healthy trees within 2 to 4 weeks following

initial infections (39), root grafts are likely responsible for the rapid epidemics observed within the first growing season in dense stands. Also, root grafts may remain a major mode of *V. nonalfalfae* transmission in following years as the pathogen continues to spread throughout a stand until all grafted *Ailanthus* have been killed. At that time, long range dispersal of the pathogen, possibly by ambrosia beetles (23,24), may be critical to maintain a more regional epidemic, or to infect isolated *Ailanthus* trees in the forest or in urban areas.

V. nonalfalfae isolate VnAa140 transmission was also evaluated within an *Ailanthus* clone comprising two parent stems and numerous vegetative root sprouts/suckers. Following inoculation of one parent stem, 128 surrounding sprouts were symptomatic by 3.5 MPI. Symptomatic sprouts occurred at distances ranging from 0.5 to 10 m from the inoculated parent stem. These results demonstrate that *V. nonalfalfae* is rapidly transmitted from infected parent stems, through their root systems, to surrounding clonal root sprouts. Therefore, the normally advantageous dense clonal growth of invasive *Ailanthus* serves as a conduit for rapid *V. nonalfalfae* transmission, which can kill entire *Ailanthus* clones within one or two growing seasons.

Ailanthus root grafts likely play positive roles in site invasion, stand development, dominance over native species, survival, and persistence. However, if an *Ailanthus* tree comes in contact with a previously unencountered pathogen, such as *V. nonalfalfae*, for which it has not evolved tolerance, root grafting and/or clonal growth habits in dense stands can threaten survival of an entire interconnected population. This weakness can be exploited, allowing for efficient and effective biocontrol of an entire *Ailanthus* stand using *V. nonalfalfae*.

However, understanding the rapid local transmission of *V. nonalfalfae* through root grafts and clonal root systems is paramount if isolate VnAa140 is to be used as a biological control to curtail growth and spread of the invasive *Ailanthus*. Transmission of the pathogen in dense stands through connected *Ailanthus* roots allows for optimized use of applied inoculum of the biocontrol agent. That is, not every *Ailanthus* tree in a dense stand has to be inoculated with *V. nonalfalfae*, since the pathogen spreads quite rapidly through root grafts and clonal root systems. To efficiently establish *V. nonalfalfae* infections throughout an *Ailanthus* stand, we propose that large *Ailanthus* trees with their extensive root systems, in close proximity to other large *Ailanthus* trees, should be targeted for initial inoculation. Also, entire *Ailanthus* clones may be controlled by simply inoculating suspected parent stems.

Collectively, this study has expanded knowledge regarding the biology of the invasive *Ailanthus* tree, as well as transmission of *V. nonalfalfae* through *Ailanthus* root systems. This knowledge can help optimize the use of *V. nonalfalfae* as a biocontrol agent against *Ailanthus*. The better we understand transmission of the proposed biocontrol agent, the better we can assess any risks or hazards related to its use.

The results of this study help explain and further demonstrate the effectiveness and efficiency of *V. nonalfalfae* isolate VnAa140 in controlling *Ailanthus*. In addition to recent host range studies and demonstration that isolate VnAa140 may have become host-adapted to *Ailanthus* (24,25,41), our results further indicate that low risk is associated with its use in Pennsylvania's forests. *V. nonalfalfae* should become an effective biological control option for the highly invasive *A. altissima*, since chemical herbicide applications remain expensive, laborious, and often ineffective.

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