The Pennsylvania State University
The Graduate School
College of Agricultural Sciences

VERTICILLIUM WILT OF AILANTHUS ALTISSIMA

A Dissertation in
Plant Pathology

by

Mark J. Schall

© 2008 Mark J. Schall

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2008
The dissertation of Mark J. Schall was reviewed and approved* by the following:

Donald D. Davis  
Professor of Plant Pathology  
Dissertation Advisor  
Chair of Committee

Gary W. Moorman  
Professor of Plant Pathology

David M. Geiser  
Associate Professor of Plant Pathology

Maria del Mar Jimenez Gasco  
Assistant Professor of Plant Pathology

Dennis R. Decoteau  
Professor of Horticulture and Plant Ecosystem Health

James C. Sellmer  
Associate Professor of Ornamental Horticulture

Barbara J. Christ  
Professor and Department Head of Plant Pathology

* Signatures are on file in the Graduate School
ABSTRACT

Tree-of-heaven \textit{[Ailanthus altissima} (Miller) swingle], is an aggressive exotic invader of disturbed areas in urban and rural ecosystems throughout North America. Since \textit{Ailanthus} was introduced into North America in 1784, the proliferation of this botanical pest has continued unchecked and \textit{Ailanthus} is now found in nearly all 50 states of the USA. The need for a natural control of tree-of-heaven is of utmost importance, but the tree has few pests. However we recently observed several stands of \textit{Ailanthus} that were wilting and declining in south-central Pennsylvania. Isolations revealed that two species of \textit{Verticillium, V. albo-atrum and V. dahliae}, were associated with the disease. Inoculations of \textit{Ailanthus} potted seedlings in the greenhouse, and canopy trees in the field, showed that both species were pathogenic on \textit{Ailanthus}, but that \textit{V. albo-atrum} was much more virulent and aggressive. It is now known that \textit{Verticillium albo-atrum} is the primary pathogen causing extensive wilt of the invasive tree species \textit{Ailanthus} in south-central Pennsylvania. The potential for using \textit{V. albo-atrum} as a biocontrol agent is discussed.

Inoculation studies in both the greenhouse and field showed that \textit{Ailanthus} was extremely susceptible, but that the host range did not extend to northern red oak, chestnut oak, red maple, sugar maple, yellow-poplar, and white ash were not susceptible hosts. However, striped maple exhibited wilt symptoms following inoculation, and \textit{V. albo-atrum} was isolated from one wilted striped maple in the field. \textit{Ailanthus} trees inoculated with \textit{V. albo-atrum} died within the same growing season. In contrast, some \textit{Ailanthus} trees inoculated with \textit{V. dahliae} survived for 2 or more seasons. Soil persistence as melanized hyphal resting structures may not play a significant role in the survival of the
pathogen in south-central Pennsylvania, due to the highly acidic forest soils, which are unfavorable for persistence of *V. albo-atrum* in the soil. The pathogen likely survives within infected leaves on the forest floor, or possibly symptomless carriers.

Survival can occur within overwintering infected *Ailanthus* trees, on fallen *Ailanthus* leaves, or in soil, and possibly in symptomless hosts. Primary inoculum, in the form of germinating resting structures or conidia, is formed in spring, and inoculation takes place at time of leaf emergence. Dissemination may involve wind disseminated leaflets, seed transmission, and/or ambrosial beetle transmission. Dissemination by root grafts needs to be studied. *Ailanthus altissima* roots are predisposed to *V. albo-atrum* by wounding. During colonization, phenols are deposited in outer xylem parenchyma within one week of inoculation indicating that initial fungus colonization is circumferential. Colonization then proceeds both upward (rapidly) and downward (slowly) until host mortality occurs. In the field, rate of spread from tree to tree is rapid. Since 2000, approximately 10,000 *Ailanthus* trees have died in southern Pennsylvania from *Verticillium* wilt, likely mainly due to *V. albo-atrum*.

During the early 1980s, *Ailanthus* invaded an oak-dominated forest that had been logged and subsequently dominated stand openings, encompassing nearly 40% of stand basal area. In 2000 the stand was attacked by *V. albo-atrum* from an unknown source. By summer 2007, the pathogen had killed more than 7000 canopy *Ailanthus* trees in that stand. In severely affected parts of the stand, the entire overstory component of *Ailanthus* was removed and approximately half of the understory *Ailanthus* seedlings or sprouts were killed. Following decline of *Ailanthus*, the canopy composition is beginning to revert to a native state, as residual trees left from the salvage harvest expanded their
crowns and young red maple and black birch trees are recruited into the overstory. The large openings caused by dying *Ailanthus* were filled by seedlings of red maple, sweet birch, and striped maple.

In conclusion, we determined the causal agents of *Verticillium* wilt of the invasive *Ailanthus* tree in southern Pennsylvania to be caused by *V. albo-atrum* and *V. dahliae*. Of these, *V. albo-atrum* is far more virulent and aggressive on both seedlings and canopy trees of *Ailanthus*. Within the forest, dissemination of *Verticillium* likely involves windblown leaves, seeds, and ambrosial beetles. Host range is very limited. *Verticillium albo-atrum* did not require wounded roots to incite disease on *Ailanthus* seedlings, though root wounding did accelerate symptom development. In naturally infected dense stands of *Ailanthus*, *V. albo-atrum* is moving rapidly, and is drastically altering forest composition, indicating a high potential for use in biocontrol applications.
## TABLE OF CONTENTS

Acknowledgements .................................................................................................viii

Chapter I. LITERATURE REVIEW............................................................................1
  Introduction to *Ailanthus altissima*........................................................................1
  Positive attributes of *Ailanthus altissima*.............................................................2
  Negative attributes of *Ailanthus altissima*............................................................3
  Control of *Ailanthus altissima*................................................................................4

  Introduction to *Verticillium*..................................................................................6
  *Verticillium* Disease Cycle. ..................................................................................8
  Symptoms of *Verticillium* wilt..............................................................................11

  Literature cited.........................................................................................................12

Chapter II. *VERTICILLIUM* WILT OF *AILANTHUS ALTISSIMA*:

  EITIOLOGY .............................................................................................................14
  Abstract ....................................................................................................................14
  Introduction .............................................................................................................14
  Materials and Methods.........................................................................................17
  Results ....................................................................................................................24
  Discussion .............................................................................................................30
  Literature Cited.....................................................................................................33
  Figures and Tables.................................................................................................36

Chapter III. HOST RANGE OF *V. ALBO-ATRUM* ISOLATED FROM
  *AILANTHUS*......................................................................................................41
  Abstract ....................................................................................................................41
  Introduction .............................................................................................................41
  Materials and Methods.........................................................................................43
  Results ....................................................................................................................47
  Discussion .............................................................................................................50
  Literature Cited.....................................................................................................54
  Figures and Tables.................................................................................................56

Chapter IV. EPIDEMIOLOGY OF *VERTICILLIUM* WILT OF *AILANTHUS ALTISSIMA* IN SOUTH-CENTRAL PENNSYLVANIA..........................60
  Abstract ....................................................................................................................60
  Introduction .............................................................................................................60
  Materials and Methods.........................................................................................66
  Results ....................................................................................................................82
  Discussion .............................................................................................................92
  Literature Cited...................................................................................................106
  Figures and Tables...............................................................................................116
Chapter V. *VERTICILLIUM* WILT OF *AILANTHUS ALTISSIMA*: EFFECT ON FOREST COMPOSITION.................................................................................................................................137
  Abstract..........................................................................................................................137
  Introduction.....................................................................................................................138
  Materials and Methods.................................................................................................141
  Results..............................................................................................................................145
  Discussion.......................................................................................................................147
  Literature Cited...............................................................................................................150
  Figures and Tables.........................................................................................................152

Chapter VI. FUTURE WORK..................................................................................................160

Appendix A. Additional figures........................................................................................162
Appendix B. Chapter 3 Statistical data................................................................................185
Appendix C. Chapter 3 statistical data...............................................................................187
Appendix D. ITS sequence of *Verticillium* isolates used in inoculations..........................189
Appendix E. NCBI BLASTN results of isolates used in inoculations.................................190
Appendix F. GPS locations of plots..................................................................................192
Acknowledgements.

I first would like to send my deepest thanks to my loving wife, Lindsay, and boys, Ethan and Cooper. Their love and support have always driven me to achieve my greatest potential. I would especially like to thank my advisor Dr. Donald Davis, for his patience, his wisdom and the unrelenting time spent discussing theories and experiments. I would also like to thank all members of my committee: Dr. Gary Moorman, Dr. David Geiser, Dr. Maria del Mar Jimenez Gasco, Dr. Dennis Decoteau, and Dr. James Sellmer for their enormous input and support throughout my research. Without their guidance and resources this project would not have been possible.

Thanks to the USDA Forest Service and The Pennsylvania State University for financial support, as well as the Pennsylvania Bureau of Forestry for the use of the Tuscarora State Forest as a study area. Specifically, I would like to thank Stephen Wacker, Tuscarora State Forest assistant district forester, for his help finding specific target study areas and for his valuable discussions.

I would like to thank Dr. Robert J. Rabaglia for ambrosial beetle identification and Dr. John Halbrendt for nematode analyses. I would like to thank Dr. Sarah Melissa Witiak and Dr. Michele Mansfield, as well as everyone in Dr. Geiser’s lab, for their valuable comments and discussions. I would also like to thank Mathew Davis for tree-ring measurements, and Ruth Haldeman of the electron microscopy facility for her assistance and advice with histology.
Chapter I. Review of the Literature

Introduction to Ailanthus altissima. Tree-of-heaven [Ailanthus altissima (Miller) Swingle] is an invasive species that was introduced into North America from Asia in 1784, planted as an ornamental in an arboretum in Philadelphia. Ailanthus was later brought from Asia into the west coast by Chinese immigrants during the gold rush and railroad construction throughout the 1800s (Miller 1990, Dorland 2005).

Ailanthus is a successional pioneer species, and is intolerant of shade. The tree is fast-growing and easily established. Mature trees can reach a height of 27 m or more and a maximum diameter of 140 cm. Ailanthus is a short-lived tree, usually surviving for only 30 - 50 years (Miller 1990).

Ailanthus is easily identified by its large pinnately compound leaves, which may reach 1 m in length and are comprised of 11 to 25 leaflets. Each leaflet is lance-ovate, having 7 to 12 cm long with 2 to 4 coarse teeth near the base of the leaflet. Terminal buds are absent. Lateral buds are small, 0.5 cm or less long, half-spherical, reddish brown, downy, and have thick scales. The bark of Ailanthus is smooth and usually is light gray in color. Twigs are light brown and covered with velvet down (Dirr 1998, Miller 1990, Farrar 1995).

The tree species is dioecious and usually bears male and female flowers on different trees; however, some trees have both flowers. The yellowish flowers of Ailanthus appear in mid-April to July in large panicles at the ends of new shoots. Male trees produce three to four times more flowers than female trees (Miller 1990). After pollination, clusters of yellow-green seeds begin to grow on the female trees. Female trees may produce as many as 325,000 seeds/tree/year. Ailanthus has its highest seed
production at 12 - 20 years. Seeds are produced in flat twisted samaras with the seed in
the middle of the samara, which are very well developed for wind dispersal. Seeds can be
dispersed from the time the seeds ripen; however, the majority of the seeds usually persist
on the trees through winter. (Dirr 1998, Miller 1990)

Following epigeal seed germination, resultant seedlings grow vigorously and may
reach 1 to 2 m in height within the first growing season. Dense thickets of *Ailanthus* arise
through vegetative reproduction, root sprouts, cut stumps, and root fragments.

*Ailanthus* produces several quassinoids, which are the bitter chemicals
characteristics of the Simaroubaceae family, including ailanthone, amarolide, acetyl
amarolide, 2-dihydroailanthone, ailanthinone, chaparrin, chaparrinone, quassin,
neoquassin, shinjulactone, and shinjudilactone (Heisey 1996). These quassinoids
chemicals have allelopathic properties (Lin et al. 1995). The phytotoxic, allelopathic
compound ailanthone gives *Ailanthus* an advantage over other competing species by
inhibiting their growth. Ailanthone is found most concentrated in roots and bark, with
moderate levels in the leaves, and relatively low levels in the wood (Heisey et al. 2003).
Ailanthone has strong herbicidal activity on over 35 species of hardwoods and 34
coniferous species, but not white ash (*Fraxinus Americana* L.) (Mergen 1959).

**Positive attributes of *Ailanthus altissima***. *Ailanthus* was originally brought to the U.S.
as a horticultural planting for its visually pleasing foliage, drought tolerance, fast growth,
and tolerance to air pollution. These characteristics made the tree very popular in urban
settings, and the species was widely planted as an ornamental within many cities.
*Ailanthus* even inspired the writing of “A Tree Grows in Brooklyn” by Betty Smith.
However due to its negative characteristics, as explained later, *Ailanthus* is seldom recommended for ornamental cultivation.

One potential benefit of *Ailanthus* considered in the past was its use in production of pulpwood for paper products. *Ailanthus* wood is ring-porous and soft (Rawling and Staidl 1924), but had excellent characteristics for pulping (but not for lumber). Trees could be harvested for pulpwood in a 10 - 15 year rotation, producing about 1.1 to 1.5 m³/ha/yr of wood on average sites (Rawling and Staidl 1924). However, this species was never widely used for pulp and paper. Another interesting attribute is that honey made from *Ailanthus* fills a niche when honey is not available from other species. The honey initially has a foul taste, but through aging, becomes a remarkably good honey (Melville 1944).

*Ailanthus* may also have medicinal values. The quassinoids exuded by trees not only have allelopathic properties, but may have anticancer, antimalarial, amoebicidal, antiviral, insecticidal, and anti-inflammatory properties (Lin et al. 1995). Chinese have long used extracts from *Ailanthus* as folk medicine as a bitter aromatic drug and for treatment of colds and gastric diseases. *Ailanthus* is also currently being investigated for use as a natural medicine, as well as for use in “natural” post-emergent herbicides and pesticides.

**Negative attributes of Ailanthus altissima.** Despite the few positive attributes of *Ailanthus*, this invasive species has many more negative aspects. The pollen is a known allergen (Ballero et al. 2003) and the leaves and stems can cause contact dermatitis on sensitive people (Derrick and Darley 1994). The tree sheds numerous unsightly leaf stems. Practically every part of the tree, including the leaves and wood, has a very
disagreeable odor when crushed (Feret 1985). Even flowers of *Ailanthus* produce a foul odor, making the tree unpleasant for ornamental plantings (Miller 1990). Since *Ailanthus* is a vigorous competitor, it can quickly become established in many varied sites where it is unwanted. The species is difficult to exclude from roadsides, Christmas tree plantations, and agricultural fields. Because the tree growth is so rapid, the wood is light and weak, giving way to splitting and breakage in windstorms or when weighted down with snow or ice (Illick and Brouse 1926). This brittle wood makes the species especially dangerous along highways and in public areas. Sap may cause acute myocarditis (inflammation of the heart tissue) when entering the body through cuts or abrasions, such as on the hands when sawing or felling *Ailanthus* (Bisognano et al. 2005).

This non-native, invasive species grows practically anywhere and quickly establishes itself at the expense of other plant species. Almost half of the plant species listed as threatened or endangered under the U.S. Endangered Species Act, are so listed due to competition with non-native invasive species such as *Ailanthus* (Wilcove et al. 1998). Invasive non-native species have been estimated costing the American economy about $137 billion dollars per year, and inexpensive controls (including biocontrol) are urgently needed for invasive organisms (Pimentel et al. 2000).

**Control of *Ailanthus***. In concept, there are several intensive control measures that can be used to control *Ailanthus*, including physical, prescribed burning, and chemical means. Two types of physical control are manual and mechanical. Manual control entails procedures like hand-pulling, hand-felling of trees, hand-digging, and girdling of stems. Where mechanical controls are applicable, mowing and chipping above-ground portions have been implemented for removal of *Ailanthus* trees (TNC 1995). Physical control
measures usually need to be followed by chemical controls like herbicide stump
treatments, or the roots will sprout aggressively, and result in clonal thickets. Burning is
another inexpensive control option. However, *Ailanthus* trees often resprout after
burning, requiring additional chemical controls for complete eradication. However,
 systemic herbicidal control seems to be most efficient control at the current time (TNC
1995).

Due to our growing agricultural needs, chemical pesticides have been widely
incorporated into our agricultural and forestry management systems. Herbicides such as
glyphosate, triclopyr, dicamba, imazapyr, 2,4-D + picloram, and metsulfuron methyl,
have been effective controlling *Ailanthus* (TNC 1995). Chemicals have gained popularity
in weed control, primarily because they often have immediate and effective control.
However, use of chemicals in the environment has come under increasingly rigid
restrictions. Also invasive plant species, through repeated spraying, can develop
resistance to chemicals. Biocontrol may be the most promising option for future control
of *Ailanthus*.

Biocontrol of undesired plant species with plant pathogens such as fungi has been
considered for decades (Te Beest et al. 1992). Current biocontrol efforts have been
classified into two strategies, the classical tactic and the bioherbicide tactic. The classical
tactic represents a biocontrol that survives from one year to the next, eliminating the need
for annual reintroduction of the biocontrol agent. This results in a long-term relationship
between the host plant and the pathogen (Te Beest et al. 1992). For example, the strategy
for control of weeds entails the application of a biocontrol in the same manner that
chemical herbicides are applied. The control measures are applied each year where
control of weeds (primarily on annual crops) is needed immediately to lower weed populations below economic thresholds (Te Beest et al 1992).

However, biocontrol for *Ailanthus* has not been addressed past the theoretical stages, due to the lack a suitable biocontrol pathogen. Field observations indicate that fungi potentially have greater potential for biocontrol of *Ailanthus*, as compared to other organisms such as bacteria and insects (Donald Davis, personal communication). Farr et al. (1989) listed 44 fungi known to occur on *Ailanthus*. The fungi include some saprophytes, as well as pathogenic fungi that cause leafspots, twig and branch dieback, decay, cankers, root rot, and wilts. However, none of these have been reported to be especially lethal to *Ailanthus*. In contrast, our field observations have revealed wilts may, indeed, be very lethal to this undesirable, invasive species. Of the fungal wilts, *Fusarium* wilt and *Verticillium* wilt appear to have the most potential to serve as a practical biocontrol for *Ailanthus*. Both *Fusarium* and *Verticillium* can be very aggressive and virulent, readily disseminated, and survive long periods in the soil. Our research and field observations indicate that *Verticillium* is the better of these two potential biocontrol agents. We hypothesize that *V. albo-atrum* is most promising species of *Verticillium* to control *Ailanthus*.

**Introduction to *Verticillium***. *Verticillium* is a genus of the kingdom Fungi, the phylum Ascomycota, subphylum Pezizomycotina, class Sordariomycetes, and order Phyllachorales (Fradin and Thomma 2006). Many species of *Verticillium* are root pathogens and cause wilting of plants (Agrios 2005). Both *V. albo-atrum* and *V. dahliae* cause vascular wilt in broadleaved shade and ornamental trees, as well as shrubs (Sinclair and Hudler 1998). However, in a critical review of *Verticillium* wilts, Harris and
Heimstra (1998) reported that *V. dahliae* was the species most often causing wilting in woody plants.

Both *V. albo-atrum* and *V. dahliae* produce similar mycelium, as well as verticillate, whored, blastic, and phialidic conidiophores. Hyphae and conidia are mostly haploid and monokaryotic; but hyphal tips may be multinucleate in both *V. albo-atrum* and *V. dahliae* (Pegg and Brady 2002). Conidia are borne in clusters in mucilaginous substance on the phialides (Pegg and Brady 2002). Each individual conidium is oval-shaped and most contain two readily visible lipid droplets. In all species of *Verticillium*, the first conidium is produced holoblastically and conidia formed thereafter are produced enteroblastically.

There are several characteristics that can be used to differentiate these two common *Verticillium* species based on morphology: hyaline conidiophores, conidiophore and conidia size, and the presence of unique resting structures. *Verticillium albo-atrum* also produces larger conidiophores (compared to *V. dahliae*) with darkly pigmented bases. *Verticillium dahliae* produces clusters of heavily subspherical to elongate, melanized, thick-walled resting structures which separate and form microsclerotia. In contrast, *V. albo-atrum* produces hyphal sections of thick-walled melanized cells, best described as thickened mycelium. Use of fresh cultures when making morphological comparisons is important, because most *Verticillium* species will lose some of their unique characteristics in older cultures (Pegg and Brady 2002, Harris and Hiemstra 1998).

Vegetative compatibility groups (VCGs) have been used to distinguish among different populations within *V. dahliae*. Studies conducted using nitrate non-utilising (*nit*)
mutants have shown the existence of four major VCGs and several sub-groups within *V. dahliae*, but only two vegetative compatibility groups in *V. albo-atrum*. In *V. dahliae*, most cotton-wilting isolates, along with most American isolates isolated from woody hosts, belong to VCG1. Our isolate, since it was isolated from the woody *Ailanthus*, may be in the VGC1 group (Harris and Heimstra 1998).

Molecular markers, such as RFLPs, RAPDs, and ITS sequences, have been used to determine relationships among *V. dahliae* strains. Molecular variation is detectable in populations of *V. dahliae* and this variation may result in somewhat discrete groups (Harris and Heimstra 1998).

**Verticillium Disease Cycle.** *Verticillium dahliae* and *V. albo-atrum* are soilborne pathogens, typically associated with agricultural or nursery settings. *Verticillium dahliae* is found only in those forest lands that have been farmed in the past. Both species usually gain ingress into plant roots through wounds, or openings where secondary roots emerge, but may penetrate directly. *Verticillium dahliae* survives in the soil by means of resting microsclerotia, which can live 10 or more years in the soil. However, in *V. albo-atrum*, segments of hyphae specialize and become melanized creating resting structures of thick-walled melanized cells that can survive from 9 months to 4 years in the soil (Pegg and Brady 2002). Other means of soil persistence include the colonization of dicotyledonous weeds, legume and cereal crops, and “volunteer” crop plants that act as agents for bridging between agricultural crops (Harris and Heimstra 2002). *Verticillium* also may be spread from plant to plant through root grafts, root contact, and budding (Agrios 2005). A general disease cycle for *V. dahliae* is illustrated in Figure A19.
Resting structures are formed within infected plant tissue. As infested plant tissues fall to the soil, they become incorporated into the soil and germinate in response to root exudates. Not only do susceptible host root exudates induce germination, but some immune monocots can also induce germination. In addition germination also may occur in response to the addition or seepage of nutrients into the soil. Numbers of *Verticillium* propagules (resting structure inoculum level) in the soil can be estimated through collection of soil samples and counting resulting colonies from soil assays (Termorshuizen et al. 1998).

Once the resting structures germinate, resulting hyphae penetrate the host root cortex. Wounds to the roots aid in the ingress of *Verticillium* into the host. Plant parasitic nematodes and cropping equipment are examples of common wounding agents in agricultural situations. Following penetration, *Verticillium* grows into the root stele (Harris and Heimstra 1998), where the fungus colonizes the vascular tissue and spreads upward into the stem through the plant xylem via either hyphal growth or conidia (Brady and Pegg 2002). *Verticillium dahliae* causes monocyclic disease, cycling once during the growing season. However, *V. albo-atrum* may produce conidia on infected plant tissue and conidia may become airborne causing multiple infections per growing season, and therefore may become a polycyclic disease (Fradin and Thomma 2006).

Once inside the stem, conidia move rapidly through the xylem. Conidia may become trapped in vessel end walls or pit cavities, germinate, penetrate adjacent vessel elements, and sporulate to continue colonization (Fradin and Thomma 2006). Banfield (1941) showed that conidia released within the vascular tissue could move 1.5 m up an elm tree in 8 to 15 seconds. At each newly infected site, high numbers of conidia are
produced on simple conidiophores, or through budding, resulting in a rapid spread of *Verticillium* spores through the host plant. Conidial production is initially favored over hyphal growth in the host. *Verticillium* may recognize the chemical and physical environment specific to xylem, such as the low oxygen levels and negative water potential, which favor conidial production (Harris and Heimstra 1998). After initial colonization, the fungus induces the host to produce tyloses or gums within the xylem that block water uptake and cause wilting (Brady and Pegg 2002).

*Verticillium* species also produce cell wall degrading enzymes that may play a role in pathogenesis. Pectinolytic enzymes may play the primary role in pathogenesis, since they aid in the breakdown of pectin containing vessel end walls, allowing the fungus to move through the plants vascular system (Fradin and Thomma 2006). In support of this hypothesis, *V. albo-atrum* mutants, known to produce lower levels of pectinases, have reduced virulence on tomato plants than wild type strains, despite similar degrees of colonization, as compared to isolates with higher levels of pectinases (Durrands and Cooper 1988). *Verticillium albo-atrum* and *V. dahliae* have also been shown to produce phytotoxins and other cell death inducing molecules. However, the role of these phytotoxins is unclear (Fradin and Thomma 2006).

*Verticillium* diseases are known to infect more than 200 different dicotyledonous plants, including such diverse crops as nuts, vegetables, herbs, root crops, and oilseed crops, as well as soft, pome, and stone fruits (Fradin and Thomma 2006). Other suscepts include legumes, more than 60 genera of woody and herbaceous ornamentals, and some shade tree species. In addition to these important species, *Verticillium* also infects weeds
and other plants that show no disease symptoms. These non-hosts can be important
disease reservoirs.

*Verticillium dahliae* and *V. albo-atrum* are soil-borne plant pathogens dispersed in
the soil primarily through resting structures and root contact, but can also be spread
through the air, water, seeds, vegetative propagation, pruning, and insect vectors. Resting
structures are normally found with the roots in the upper layers of the soil, but also may
be found near the soil surface, a location that facilitates dispersal by water and/or wind.
Another important means of dispersal is wind-blown infected host leaves and leaflets,
which can be carried considerable distances to infest new areas (Harris and Heimstra
1998).

**Symptoms of Verticillium wilt.** Three main symptoms of *Verticillium* wilt include leaf
symptoms (wilt, chlorosis, necrosis, defoliation), vascular symptoms (discoloration of the
xylem and plugging of the vessels), and decline (stunting, dieback, death). Depending on
the host species, any combination of the three may occur. Within most infected host
species, wilting and defoliation are usually the most common symptoms. Sapwood of
infected plants exhibits a vascular discoloration that occurs in bands or streaks throughout
the infected tissue. This vascular discoloration can appear as a faint tan, grayish-green,
brown, orangish-brown, or black depending on the host species (Harris and Heimstra
1998). Other common symptoms include tree dieback, decline, and death, appearing after
defoliation. Adventitious shoots occur commonly at the base of wilting plants. Some
infected plants can overcome the fungus and recover fully the following year, but even
some recovering plants may be reinfected the following year and die. Recovered trees
exhibit vascular discoloration within the previous year’s growth ring.
Literature Cited


Illick, J.S., and Brouse, E.F. 1926. The *Ailanthus* tree in Pennsylvania. Pennsylvania


Rawling F.G. and Staidal, J.A. 1924. The pulping value of *Ailanthus*. USDA Forest Service Forest Products Laboratory and University of Wisconsin. L-10:270.


Chapter II. *Verticillium* wilt of *Ailanthus altissima*: etiology

Mark J. Schall, and Donald D. Davis,  Department of Plant Pathology, The Pennsylvania State University, University Park 16802

ABSTRACT

Tree-of-heaven [*Ailanthus altissima* (Miller) Swingle] is an aggressive exotic invader of disturbed areas in urban and rural ecosystems throughout North America. Since *Ailanthus* was introduced into North America in 1784, this botanical pest has proliferated and *Ailanthus* is now found in nearly all 50 states of the USA. The need for a natural control of tree-of-heaven is of utmost importance, but the tree has few known pests. We recently observed several stands of *Ailanthus* that were wilting and declining in south-central Pennsylvania. Isolations revealed two species of *Verticillium*, *V. albo-atrum* and *V. dahliae*, associated with the disease. Inoculations of *Ailanthus* potted seedlings in the greenhouse and canopy trees in the field showed that both species were pathogenic on *Ailanthus*, but that *V. albo-atrum* was much more virulent, spreading rapidly within plants, and aggressive, rapidly spreading spatially. The potential for using *V. albo-atrum* as a biocontrol agent is discussed.

Tree-of-heaven [*Ailanthus altissima* (Miller) Swingle] is an exotic, highly invasive tree species introduced into North America from Asia in 1784. This tree species has negative impacts in both urban and forested areas. The pollen of *Ailanthus* is a known allergen (Blumstein 1943) and leaves and stems cause contact dermatitis (Derrick and Darley 1994). The tree sheds numerous leaf stems, which are considered unsightly to
homeowners. Practically every part of the tree, including leaves and wood, has a very disagreeable odor when crushed (Feret 1985). Even the flowers of *Ailanthus* produce a foul odor, making the tree unsuitable for most ornamental plantings (Miller 1990). Since *Ailanthus* is a vigorous competitor, it becomes quickly established, thriving in many varied sites and on all soil types. *Ailanthus* seedlings and trees are difficult to exclude from areas such as roadsides, Christmas tree plantations, and agricultural fields. Because growth is rapid, the wood is light and weak, giving way to splitting and breakage in windstorms or when weighted down with snow or ice (Wyman 1965). Weak, brittle wood makes the species especially dangerous along highways and in public areas since *Ailanthus* is prone to limb fall and stem breakage.

Although *Ailanthus* is a short-lived tree, usually surviving for only 30 - 50 years (Miller 1990), it is very fast growing and can reach a height of 27 m (88 ft) or more and a maximum diameter of 100 cm (40 in). Female trees are prolific seed producers, and are capable of producing more than 300,000 seeds/tree as on trees as young as 12 years. Seeds are produced in flat twisted samaras, with the seed in the middle, are very light and well developed for long-range wind dispersal. Seeds can be dispersed from the time the seeds ripen; however, the majority of the seeds usually persist on the trees through winter (Dirr 1998, Miller 1990). Seeds do not require stratification for germination, and resultant seedlings grow vigorously, often reaching a height of 1-2 m (3-6 ft) within the first growing season. Dense thickets of *Ailanthus* arise through root sprouts and cut stumps.

*Ailanthus altissima* produces several quassinoids including ailanthone, amarolide, acetyl amarolide, 2-dihydroailanthone, ailanthinone, chaparrin, chaparrinone, quassin, neoquassin, shinjulactone, and shinjudilactone (Heisey 1996). These quassinoids posses
phytotoxic allelopathic properties (Lin et al. 1995) that give *Ailanthus* a distinct advantage over competing plant species. Ailanthone has strong herbicidal activity on more than 35 hardwoods and 34 coniferous species (Mergen 1959).

Many measures have been attempted to control *Ailanthus*, including pulling and digging; tree felling or girdling; mowing and chipping above-ground portions; and burning. Such control measures usually need to be accompanied by chemical herbicide stump treatments or roots will sprout aggressively (TNC, 1995). However, use of chemicals has come under increasingly rigid restrictions. Also, through repeated chemical spraying, weeds may develop resistance to herbicides. Biocontrol may be the most promising option for future control of *Ailanthus*.

Biocontrol of undesired plant species with plant pathogens such as fungi has been considered for decades (Te Beest et al. 1992). However, biocontrol for *Ailanthus* has not progressed beyond the theoretical stages, due to a lack of a suitable biocontrol pathogen. Farr et al. (1989) listed 44 fungi that occur on *Ailanthus*. However, many of the fungi listed are saprophytes and some of the pathogenic fungi cause only minor diseases that do not result in death of *Ailanthus*. However, in 2002 we discovered a small stand of *Ailanthus* that was wilting and dying within a state forest in south-central Pennsylvania. By 2003, most *Ailanthus* trees in this stand were dead. Also in 2003, personnel of the Pennsylvania Bureau of Forestry showed us a larger stand in which *Ailanthus* was also exhibiting severe wilt symptoms and mortality. The objectives of this paper were to determine the etiology of “Ailanthus wilt” and determine the potential of the causal agent as a biocontrol of invasive and undesirable *Ailanthus* trees.
MATERIALS AND METHODS

Study Area. The study area is located at 40°10’00” N latitude and 77°35’00” W longitude and lies along Second Narrows Road and Conococheague Creek within the Tuscarora State Forest in south-central Pennsylvania (Fig. A1). The area is an oak-dominated, mixed-hardwood, 56-ha stand that contained actively wilting and dying Ailanthus seedlings, root sprouts, saplings, and canopy trees. Associated cohort species included American basswood (Tillia americana L.), black locust (Robinia pseudoacacia L.), black oak (Quercus velutina Lam.), chestnut oak (Quercus montana Willd.), northern red oak (Quercus rubra L.), red maple (Acer rubrum L.), shellbark hickory (Carya laciniosa Michx. f.), striped maple (Acer pensylvanicum L.), sugar maple (Acer saccharum Marsh.), sweet birch (Betula lenta L.), white ash (Fraxinus americana L.), and yellow-poplar (Liriodendron tulipifera L.), as well as an extensive acreage of Ailanthus in the more open portions of the stand. The understory consisted primarily of seedlings and sprouts of Ailanthus, sweet birch, and red maple.

The study area receives approximately 100 cm of rainfall annually, with an average temperature of 18°C, and an average growing season (April 1 to August 31) temperature of 26°C (Zarichansky, 1986). Soils are Dystrochrepts bouldery, Meckesville very stony silt loam, Hazelton extremely stony sandy loam, Buchanan very stony loam, and Weikert shaly silt loam (Long 1975, Zarichansky 1986).

Stand mapping using a Global Positioning System (GPS), revealed that Ailanthus was concentrated in an area that had been defoliated by gypsy moth (Lymantria dispar L.) in the early 1980s. Oak mortality and subsequent salvage harvesting in 1984 had resulted in an open stand ideal for subsequent Ailanthus invasion. Aerial photographs and
forestry stand maps (Figs. A2, A3), later obtained from the Pennsylvania Department of Conservation and Natural Resources, Bureau of Forestry, confirmed these observations. Date of *Ailanthus* establishment in the stand was also estimated by felling and counting annual growth rings from 50 of the largest *Ailanthus* trees. Ring counts showed that *Ailanthus* likely invaded the stand in 1984 or 1985. The location of the original seed source, assuming initial stand invasion was from seed, was likely on adjacent ridgetops from which seed could be easily dispersed.

**Isolation and culturing from natural infections.** Leaves, stems, and xylem chips from symptomatic *Ailanthus* seedlings and trees were collected during summers of 2005 and 2006. Samples were returned within 6 hr to the laboratory and refrigerated. On the following day, isolations were made onto Potato Dextrose Agar (PDA), a general non-selective medium for fungi (Radisek et al. 2003). Cultures were grown in the dark within a controlled environment chamber at 21°C for 3 wks. Culture morphology was examined using light and scanning electron microscopy. *Verticillium* species were consistently isolated from initial samples (Figs. A4, A5). Therefore, all additional samples were flame-sterilized and placed (one per petri dish) onto plum extract agar (PEA; 900 ml distilled water, 20 g agar, 100 ml concentrated plum extract, 1 g yeast, 5 g lactose, pH 5.6 - 6.0), a culture medium more consistent for *Verticillium* growth and identification (Bejarano-Alcazar et al. 1996). To reduce bacterial contamination, PEA was amended with streptomycin and neomycin. Previously collected *Verticillium* isolates were transferred from PDA to PEA and grown at 22 °C in the dark on PEA (Bejarano-Alcazar et al. 1999).
Molecular identification of *Verticillium* fungi. Mycelium from PEA cultures was transferred to sterile culture tubes containing potato dextrose (PD) broth (EM Science, Gibbstown, NJ) and incubated at room temperature for 14 – 21 days without shaking (Geiser et al. 2005). Mycelium was recovered from the broth by filtration, lyophilized (Perez-Artes et al. 2000), and DNA extracted using a DNeasy Plant Minikit (Qiagen Inc., Hilden, Germany). DNA concentration was determined by electrophoresis in a 1% agarose gel.

A standard polymerase chain reaction (PCR) protocol was used to amplify the ITS region of the nuclear ribosomal RNA gene repeat. Reverse primer ITS4 and forward primer ITS5 (White et al. 1990) were used in the PCR reaction to amplify a 566 bp product of the ITS region (Fahleson et al. 2004). PCR was performed in a PTC-100 Programmable Thermal Cycler (MJ Research Inc., Waltham, MA) using: 5 μL 10X PCR buffer, 5 μL 10X dNTP, 1μL 10 μM primer ITS4, 1μL 10 μM primer ITS5, 0.2 μL 1.25 U Taq DNA polymerase and 4 μL of 10 to 50 ng template DNA for each sample. PCR parameters were: 2 min at 95 °C; followed by 35 cycles of 1 min at 94 °C, 1 min at 53 °C, and 1 min at 72 °C; followed by 5 min at 72 °C. Products were separated by electrophoresis in a 1.0% agarose gel, stained with ethidium bromide for visualization, and purified using a EXOSAP-IT PCR Purification Kit (USB Corporation, Cleveland, OH). Purified amplification products were sequenced at the Pennsylvania State University Nucleic Acid Facility on an ABI 3730XL automated DNA sequencer, using both the ITS5 and ITS4 primers.

Consensus sequence data were assembled and edited using Sequencher 3.1 (Gene Codes, Ann Arbor, MI) from both forward and reverse DNA sequences. Consensus
sequences were placed into NCBI BLASTN (National Center for Biotechnology Information, Bethesda, MD) as an identification tool.

**Confirmation of pathogenicity using Koch’s postulates**

*Ailanthus altissima* seeds, which had formed in fall of 2004, were collected from four mature trees during March of 2005 from the Pennsylvania State University campus. Seeds were placed in flats containing a blend of 20% Hagerstown silt loam topsoil, 80% Metro Mix 200 (The Scotts Company, Marysville, OH) and maintained in the greenhouse. Seeds germinated immediately, requiring no further stratification. Approximately 650 resultant seedlings were transplanted to 6 x 25 cm tubes and maintained in the greenhouse using an automatic watering regime. Seedlings were fertilized once with 12:12:12, N:P₂O₅:K₂O Osmocote (ScottsMiracle-Gro, Marysville, OH).

Based on 2005 preliminary inoculation experiments, using an isolate of *V. albo-atrum* isolate PSU 140 (GenBank accession # FJ424082) and one of *V. dahliae* isolate PSU 154 (GenBank accession # FJ424083), we fulfilling Koch’s postulates, confirming that both *V. albo-atrum* and *V. dahliae* were pathogenic on *Ailanthus* seedlings. We also concluded that stem-inoculations method (Bugbee and Presley 1967), as compared to a root-clipping/dipping method (Bhat and Subbarao 1999), were best for assessing disease severity based on symptoms.

**2006 seedling inoculations.** Based on the promising results of the 2005 preliminary inoculations, more comprehensive stem-inoculation studies were performed in 2006 using both *Verticillium* species. To ensure the isolates had not lost pathogenicity in culture, *Ailanthus* seedlings were inoculated with both species of *Verticillium* in
January 2006 and *Verticillium* re-isolated from symptomatic plants. Only re-isolated “fresh” isolates were used in the following studies. Standardized techniques were used to establish uniform levels of inoculum; spore suspensions were harvested from plates and concentrations adjusted to $10^7$ conidia/ml (Pastor-Corrales et al. 1987).

A completely randomized experimental design was used, in which treatments were stem-inoculated with *V. albo-astrum* (PSU 140, GenBank accession # FJ424082) or *V. dahliae* (PSU 154, GenBank accession # FJ424083), and controls were stem-inoculated with sterile water. One-hundred *Ailanthus* seedlings, in four blocks of 25 were stem-inoculated for each species of *Verticillium* in the Pennsylvania State University, Forestry Resources greenhouse. Seedlings were inoculated with 0.1 ml of a conidial suspension containing $10^7$ spores/ml in a 1-ml syringe at two points at the base of the stem (Bugbee and Presley 1967). Twenty-five control plants were injected at two points at the base of the stem with sterile distilled water and maintained in the same greenhouse as inoculated plants. Following inoculation, treated and control plants were examined weekly for 3 months. Wilting severity was rated using a relative 0 - 4 scale, which followed the natural progression of disease symptoms, where 0 = healthy leaves, 1 = chlorotic leaves, 2 = necrotic leaf tips, 3 = wilting leaves, and 4 = dead or defoliated seedlings (Bejarano-Alcazar et al. 1996). Seedling heights were measured weekly. At the end of each experiment, tissue samples were taken from all plants and cultured for *Verticillium* onto PEA. Each treatment was replicated three times.

All plants inoculated with *V. albo-astrum* died by the end of the 2006 growing season, and therefore no additional measurements were made the following year. However, many plants (84%) inoculated with *V. dahliae* survived the 2006 growing
season. They were allowed to go dormant for the winter, and were brought out of dormancy on March 2007 to evaluate leaf emergence. Leaf emergence was measured 3 weeks after plants were brought out of dormancy. Each seedling was rated from 0 to 3, based on emergence characteristics of the apical bud, where 0 = bud dormant, 1 = bud swollen, 2 = bud swollen but leaves unexpanded and 3 = bud broken and leaves fully expanded. Final disease severity rating and final height growth also recorded at the end of the 2007 growing season.

**2006 Canopy tree inoculations.** Ten stands were located for treatments. Stand selection was based primarily on presence of adequate numbers of *Ailanthus* trees, and lack of disease symptoms. Four stands were used for *V. albo-atrum* (PSU 140, GenBank accession # FJ424082) inoculations, five for *V. dahliae* (PSU 154, GenBank accession # FJ424083) inoculations, and one as a control stand. In each stand five co-dominant *Ailanthus* trees (average height = 20 m, diameter = 16.5 cm) were randomly selected. For treatments, a randomized complete block experimental design was used. Treatments were *V. albo-atrum* stem-inoculation, *V. dahliae* stem-inoculation, and sterile water stem-inoculation. On each tree, the root crown (at the stem/root intersection) was surface-sterilized with 70% ethanol and allowed to dry. Each tree was wounded with a sterilized ax at three locations on the upper surface of the root crown. Approximately 1.5 ml of $10^7$/ml conidia was aspirated or injected into each of the three wounds. Five trees in the control stand received 1.5 ml of sterile distilled water aspirated into each sterile wound. *Verticillium albo-atrum* stem-inoculated treatments were replicated three times, *V. dahliae* inoculated treatments were replicated four times, and controls were not replicated. The number of replicates was based on the number of suitable *Ailanthus* trees.
Wilting severity was rated from 21 April 2006 to 10 September 2006 using the previously described 0 - 4 scale, where 0 = healthy leaves, 1 = chlorotic leaves, 2 = necrotic leaf tips, 3 = wilting leaves, and 4 = dead or defoliated trees.

Trees that died from inoculations were felled during January 2007 and a cross-section disk removed from each stem at 1.4m from original ground line for tree ring analysis (McClenahen 1995). The effect of *V. albo-atrum* on diameter growth, during the time period between inoculation and mortality, was determined and compared to non-inoculated controls (Davis et al. 2006). Trees inoculated with *V. dahliae* were not felled since they did not die following inoculation, but will be felled and sampled in future studies.

**2007 Canopy tree inoculations.** Inoculations were conducted again on canopy *Ailanthus* trees in 2007 to evaluate symptom development more closely in the northern Tuscarora State Forest. However, in this study trees were inoculated with *V. albo-atrum* only. Inoculations (10⁷ conidia/ml) were applied on the root crown using a Hypohatchet injection tool (Forestry Suppliers, Jackson MS) (Fig. A10). Preliminary experiments revealed that the Hypohatchet was a more efficient tool than an ax for tree inoculations. The Hypohatchet, traditionally used for herbicide injection, injects 1 ml of suspension/tree with each “hit”.

A completely randomized experimental design was used, where treatments were *V. albo-atrum* stem-inoculation and sterile water stem-inoculation. Five trees at each of five locations were inoculated with *V. albo-atrum* (n=25). Controls were not replicated (n=5). Inoculations were made at three areas on the root crown, applying a total of 3 ml inoculum/tree. Control trees were treated with sterile distilled water in wounds made by a
sterile ax. Inoculated and control trees were observed biweekly and rated using the previously described 0 – 4 scale of disease severity.

**Statistical analyses.** Analysis of variance and Tukey’s mean comparisons were used to detect significant differences (p=0.001) due to treatment (Minitab 2007). Disease severity index (DSI), which ranged from 0 to 100 %, was calculated as, DSI = (I x S)/M, where I = is the percentage of diseased plants, S = mean severity of foliar symptoms in diseased plants, and M = maximum severity value (M = 4 in our experiments) (Bejarano-Alcazar et al. 1996). To determine if plant size was related to resistance or susceptibility to *V. albo-astrum*, linear regression was used to determine relationships between seedling height or canopy tree diameter on severity of wilt symptoms. Since *V. dahliae* induced few symptoms, only data from *V. albo-astrum* inoculations were used in regression analyses.

**RESULTS**

**Pathogen identification based on culture morphology.** Initial findings indicated that two different species of *Verticillium* were associated with *Ailanthus* wilt, and that the species were likely *V. albo-astrum* and *V. dahliae*. Preliminary identification of *Verticillium* species was made using culture morphology, conidiophore type, and presence of resting structures produced on PEA (Bejarano-Alcazar et al. 1996). The fungus most consistently isolated from symptomatic tissue of *Ailanthus* seedlings and trees produced white, fluffy colonies and verticillate conidiophores, morphologies characteristic of *Verticillium* (Fig. A6). Most cultures formed heavily melanized hyphae in culture, characteristic of *V. albo-astrum* (Fig. A6). Some cultures produced abundant
microsclerotia, characteristic of *V. dahliae*. Morphology of subcultures agreed with our putative identifications of *V. albo-atrum* and *V. dahliae*.

**Pathogen identification based on molecular techniques.** Our morphological identifications were checked by BLAST analysis of ITS sequences representing the two morphotypes identified as *V. albo-atrum* and *V. dahliae*. A BLAST search (National Center for Biotechnology Information, Bethesda, MD) of the ITS sequence from a putative *V. albo-atrum* (isolate PSU 140) matched most closely with the ITS sequence of *V. albo-atrum* (GenBank accession no. AB458830; strain no. VA001; e-value = 0.0) (appendix E) with only a single nucleotide difference in 512 bp. The ITS sequence of putative *V. dahliae* isolate PSU 154 531-bp showed an exact match with an isolate previously identified as *V. dahliae* (GenBank accession no. AF104926; strain cotton001; e-value = 0.0) (appendix E). This confirmed our putative identifications of both *V. albo-atrum* and *V. dahliae* that had been based on culture morphology on PEA. Amplified PCR confirmed that the most common and virulent (based on severity of field symptoms) *Verticillium* species that was killing *Ailanthus* was *V. albo-atrum*. Of the 48 *Verticillium* isolates collected from symptomatic *Ailanthus*, we sequenced the ITS gene region of 19 isolates. Of those 19 isolates, 15 isolates matched *V. albo-atrum* and 4 matched *V. dahliae*. The *V. albo-atrum* isolates were all associated with a single large study area of wilting *Ailanthus* trees. The *V. dahliae* isolates were associated with several very small groups of wilting *Ailanthus* trees.

**Koch’s postulates**

**2006 seedling inoculations.**
The final height increase (HT) data were not normally distributed and were normalized using a square root transformation prior to statistical analyses, for both Verticillium species. Final disease severity rating data (DIS) were normally distributed. There were no significant differences among replicates (n = 25 each) for any treatment, so replicate data were combined for analysis (n = 100). Analysis of variance (ANOVA) revealed significant differences among treatments for DIS and final HT (Fig. 1 and 2). Tukey’s multiple comparison test revealed significant differences in DIS between controls and treatments using both V. albo-atrum and V. dahliae inoculations, as well as between DIS of V. albo-atrum vs. V. dahliae inoculated seedlings (Fig. 2).

Significant differences in final HT were observed between non-inoculated controls and V. albo-atrum inoculated seedlings, but not for V. dahliae inoculated seedlings (p = 0.070), although the trend was nearly significant (Fig. 1). Significant differences were also observed between final HT of V. albo-atrum inoculated vs. V. dahliae inoculated seedlings. Linear regression revealed no significant relationship between weekly measure of seedling heights and week of wilting symptoms of V. albo-atrum inoculated seedlings (p = 0.439, R² = 0.60%).

Wilt symptoms appeared suddenly on plants inoculated with V. albo-atrum. Seedlings often became chlorotic after approximately four weeks, approximately one week before wilting, and would defoliate during the following week. Defoliation usually involved loss of the entire compound leaf, with leaflets still attached to the rachis (Fig. A7). Compared to non-inoculated controls, seedlings inoculated with V. albo-atrum exhibited 74.8% less height growth (HT) only 2 weeks after inoculation (Fig. 1), and visible foliar symptoms measured by the disease severity index increased significantly
(8.6%) between weeks 0 to 4 (Fig. 2). Inoculated plants began to wilt and defoliate by week 6, when disease severity index reached 79.1% and HT was 83% less than non-inoculated seedlings. By week 9, all seedlings inoculated with *V. albo-atrum* replicates were dead.

*Verticillium dahliae* induced symptoms different from those induced by *V. albo-atrum*. Leaflets on seedlings injected with *V. dahliae* first became chlorotic, starting with the oldest leaflets at the base of the petiole. Chlorotic leaflets progressively wilted and defoliated along the rachis towards the leaf apex (Fig. A8). After all leaflets had dropped, the entire rachis would drop from the seedling. Seedlings inoculated with *V. dahliae* exhibited a 4.1% increase in disease severity by week 5 (Fig. 2) and less cumulative HT (8.7%) by week 7 (Fig. 1). Only 16% of inoculated seedlings had died by week 15. Disease severity index at week 15, which includes mortality, had increased 32.3%, based on final disease severity rating (DIS) was significant. At week 15 *V. dahliae* HT had decreased by 20.9% less than non-inoculated controls, but was not significant (p=0.070).

**Overwintering of seedlings inoculated with *V. dahliae***. Many of the *Ailanthus* seedlings, 84%, that had been inoculated with *V. dahliae* during spring 2006 survived until the end of the growing season, and then survived overwintering until spring of 2007. However, the 84 surviving seedlings exhibited 55.7% dieback of terminal growth, while non-inoculated controls (n=25) had significantly less dieback (1.3%) (Fig. 3) at the end of the 2006 season and prior to 2007 leaf emergence. Three weeks following normal bud break in 2007, 56.0 % of the non-inoculated control plants had fully expanded leaves, but only 7.2% of the living, inoculated seedlings had fully expanded leaves (Fig. 4). At this
time 47.4% of the inoculated seedlings remained in the dormant bud stage, whereas only 4.0% of the control seedlings remained in this undeveloped growth stage.

Parameters on surviving plants were measured again at the during the 2007 growing season. Data that were not normally distributed were normalized using square root transformations. Analysis of variance (ANOVA) revealed no significant differences between controls and *V. dahliae* treatments for final height (HT) increase and disease severity rating (DIS) during the 2007 growing season: final DIS (p = 0.129) and final HT (p = 0.136). ANOVA did reveal a significantly that *V. dahliae* treatments retained greater terminal dieback as compared to control seedlings.

**Canopy tree inoculations**

**2006 Canopy tree inoculations.** Disease rating data from the four replicates (n = 5 each) using *V. albo-atrum*, as well as the five replicates (n = 5 each) using *V. dahliae*, were not significantly different from each other, so data from respective replicates were combined (n=20 and n=25, respectively). ANOVA revealed significant (p = 0.000) differences between final DIS ratings among treatments. Tukey’s multiple comparison showed significant differences between the final DIS ratings of controls and trees inoculated with *V. albo-atrum* (p = 0.000) and those inoculated with *V. dahliae* (p = 0.000). Significant differences (p = 0.000) also occurred between DIS ratings for *V. albo-atrum* and *V. dahliae*.

Results from inoculation of canopy *Ailanthus* trees with *V. albo-atrum* in the field were very similar to those from seedling inoculations with *V. albo-atrum* in the greenhouse. Canopy trees inoculated with *V. albo-atrum* exhibited sharply increasing disease severity index (index=79) by week 7. By week 7, symptoms appeared throughout
the entire crown of inoculated trees, including sudden wilting of entire compound leaves, with leaflets and rachis intact, followed by defoliation. By week 10, more trees had died, and by week 18, all trees inoculated with *V. albo-atrum* were dead (Fig. 5).

Canopy trees inoculated with *V. dahliae* developed symptoms more slowly, than those inoculated with *V. albo-atrum*, similar to our observations on greenhouse inoculated seedlings. Canopy trees inoculated with *V. dahliae* exhibited only a slight increase in disease severity index (index=1.4) by week 8 (Fig. 5). By week 10, disease severity index increased to 17, and as of week 16, the disease severity index was at 42 (Fig. 5). However, trees inoculated with *V. dahliae* wilted irregularly throughout the canopy, with symptoms often appearing on branches on opposite ends of the crown. On other inoculated trees, the upper canopy remained asymptomatic, while the lower branches exhibited defoliation. On some inoculated trees, the entire canopy wilted only slightly. Later, we observed that trees inoculated with *V. dahliae* survived overwintering, and began to show recovery during the following growing season (2007).

All canopy *Ailanthus* trees that had been inoculated with *V. albo-atrum* in 2006 were dead by the end of the growing season. Dead trees were felled, cross-sectional disks collected, and tree rings measured to determine if inoculations had any effect on 2006 stem diameter growth prior to mortality. Average diameter increment (DIA) data were not normally distributed and square root transformations were used to normalize the data. Data from replicates were not significantly different, based on ANOVA and Tukey’s multiple comparison, (p = 0.411), so replicate data were combined for analysis (n = 20). Two sample t-tests revealed that trees inoculated with *Ailanthus* trees with *V. albo-atrum* had significantly less 2006 growth prior to mortality as compared to control trees. In the
2006 growing season following inoculation of *V. albo-atrum*, control trees averaged 3.293 mm of diameter growth, while *V. albo-atrum* inoculated trees averaged only 1.062 mm (a reduction of 67.75%) until they died by the end of the growing season (Figs. 5, 6).

Linear regression revealed that tree diameter was not related to week of wilting (p = 0.309, R² = 4.5%). The rate of wilting was similar for all diameters of trees, although the range in diameter may have been too narrow (10 to 19 cm) to detect significant difference.

**2007 Canopy tree inoculation.** Canopy trees inoculated with *V. albo-atrum* in 2007 exhibited considerable disease symptoms, as compared to the non-inoculated controls which were asymptomatic (Fig. 5). A rapid increase in disease severity on inoculated trees was observed between weeks 2 and 4 following inoculation. Disease severity increased slightly on week 6, and then leveled off. During this period, from week 6 to 16, inoculated trees defoliated, but epicormic sprouts refoliated, forming a new crown that was approximately 5% leaf volume of the original crown. As, the new epicormic sprouts on inoculated trees wilted, other sprouts would appear, however, epicormic sprouts never comprised more than 5% of the crown. Tree mortality began to appear by week 6. These epicormic sprouts were likely fed by reserves in the upper branches.

At the end of the experiment on 23 August 2007, ANOVA and Tukey’s multiple comparison revealed that all five replicates (n = 5 each) of *V. albo-atrum* field inoculated trees were not significantly different so replicate data was combined (n = 25). Analysis of variance (ANOVA) revealed significant differences in final disease severity between controls and trees inoculated with *V. albo-atrum*. 
DISCUSSION

The greater virulence (disease within a plant) and aggressiveness (spatial disease spread) of *V. albo-atrum* as compared to *V. dahliae* on *Ailanthus* is consistent with reports on one tree species, yellow-poplar, as well as hop (*Humulus lupulus* L.), cotton (*Gossypium* spp.), lucerne (*Medicago sativa* L.), potato (*Solanum tuberosum* L.), strawberry (*Fragaria* spp.), sainfoin (*Onobrychis* spp.), clover (*Trifolium* spp.), tomato (*Lycopersicon lycopersicum* L.), and cucumber (*Cucumis sativus* L.) (Morehart et al. 1980, Issac 1967).

*Verticillium albo-atrum* and *V. dahliae*, were isolated from naturally infected, wilting *Ailanthus* in mixed-hardwood stands in south-central Pennsylvania. Isolation and subsequent inoculation of *Ailanthus* potted greenhouse seedlings and canopy trees in the field with both *Verticillium* species induced typical wilt symptoms. Reisolation of the two *Verticillium* species fulfilled Koch’s postulates (Agrios 2005), proving that both *V. albo-atrum* and *V. dahliae* were pathogenic and capable of inducing *Ailanthus* wilt. However, *V. albo-atrum* was much more virulent and aggressive, causing severe and widespread wilt and mortality on *Ailanthus* seedlings and trees where *V. dahliae* did not.

*Ailanthus* seedlings that were stem-inoculated with *V. albo-atrum* exhibited severe wilting symptoms by week 5 and were dead by week 9 (Fig. 2). In contrast, seedlings that were inoculated with *V. dahliae* showed only slight symptoms by week 5 and 8, and were still alive at the end of the experiment (week 16). Seedlings inoculated with *V. dahliae* survived over winter until the next spring. Canopy tree inoculations also revealed that *V. albo-atrum* was much more virulent and aggressive than *V. dahliae* in mature trees. Canopy trees inoculated with *V. albo-atrum* exhibited severe wilting
symptoms by week 4 (Fig. 5) and many trees were > 50% defoliated by week 4. In contrast, canopy trees inoculated with \textit{V. dahliae} exhibited less severe symptoms that were induced at a slower rate (Fig. 5). As with the seedlings, canopy trees inoculated with \textit{V. dahliae} in 2006 were still alive at the end of the summer, and many recovered during the 2007 and 2008 growing seasons. Sinclair et al. (1981) reported that recurrence of symptoms caused by \textit{V. dahliae} in maple trees relied on radial movement of the pathogen through the maple stems, overcoming resistance mechanisms in the vertical vessel elements of the xylem. They also suggested that major dieback and death of large maple trees due to \textit{V. dahliae} were likely due to infections that occurred during several previous growing seasons. Similar to what was found with our \textit{V. dahliae} isolate and \textit{Ailanthus}, in yellow-poplar, Morehart et al. (1980) reported that \textit{V. dahliae} caused less disease in inoculated yellow poplars than \textit{V. albo-atrum}.

Use of \textit{V. albo-atrum} isolate PSU 140 should be considered as a biocontrol for \textit{Ailanthus} in areas where this invasive tree species is unwanted. The use of plant pathogens as biocontrols, or biological silvicides in forests, has been explored in several pathosystems where undesirable trees have been controlled with an endemic plant pathogen. Persimmon wilt, caused by \textit{Cephalosporium diospyri} Crandall, has been applied to control persimmon in pastures in Arkansas for decades (Wilson 1965). Oak wilt, caused by \textit{Ceratocystis fagacearum} (Bretz) Hunt, has been used to control low-value oak trees on sites better suited for pine production in Minnesota (French and Schroeder 1969). However, additional studies need to be conducted in this \textit{Verticillium}-\textit{Ailanthus} pathosystem, including susceptibility of non-\textit{Ailanthus} forest trees, and agricultural crops, presence of other isolates of each \textit{Verticillium} species, as well as
determining means of dissemination, and conducting risk analyses.
LITERATURE CITED


xylarioides (anamorph: Fusarium xylarioides), a causative agent of coffee wilt disease in Africa, is a previously unrecognized member of the G. fujikuroi species complex. Mycologia 97:191-205.


LIST OF FIGURES

Figure 1. Average cumulative height (cm) of potted *Ailanthus* seedlings at various times after stem injection with 0.5 ml (10^7 spores/ml) *V. albo-atrum* or *V. dahliae* conidia. Control plants were injected with distilled water.

Figure 2. Disease severity index of potted *Ailanthus* seedlings at various times after stem injection with 0.5 ml (10^7 spores/ml) *V. albo-atrum* or *V. dahliae* conidia. Control plants were injected with distilled water, and disease severity index did not significantly increase from 0. Index of 0 to 100, where 0 = all plants were healthy and 100 = all plants were dead. DSI = (I x S)/M, where I = is the percentage of diseased plants, S = mean severity of foliar symptoms in diseased plants, and M = maximum severity value (M = 4 in our experiments).

Figure 3. Average height growth (cm) for the second growing season and average dieback (cm) from season 1 to season 2, of *Ailanthus* seedlings inoculated with *V. dahliae*.

Figure 4. Percentage of *Ailanthus* seedlings inoculated with *V. dahliae* that had delayed leaf emergence at the beginning of the second growing season.

Figure 5. Average disease severity index of 2006 and 2007 canopy trees inoculated in the field with 3 ml of 10^7 *V. albo-atrum* or *V. dahliae* conidia/ml. Control trees were inoculated with sterile distilled water and remained asymptomatic. Index of 0 to 100, where 0 = all plants were healthy and 100 = all plants were dead. DSI = (I x S)/M, where I = is the percentage of diseased plants, S = mean severity of foliar symptoms in diseased plants, and M = maximum severity value (M = 4 in our experiments).

Figure 6. Average annual diameter growth (mm) of 2006 controls and *V. albo-atrum* trees, inoculated with 4.5 ml (10^7 spores / ml) of conidia.
Fig. 1. Average cumulative height (cm) of potted Ailanthus seedlings at various times after stem injection with 0.5 ml (10^7 spores/ml) *V. albo-atrum* or *V. dahliae* conidia. Control plants were injected with distilled water.

Fig. 2. Disease severity index of potted Ailanthus seedlings at various times after stem injection with 0.5 ml (10^7 spores/ml) *V. albo-atrum* or *V. dahliae* conidia. Control plants were injected with distilled water, and disease severity index did not significantly increase from 0. Index of 0 to 100, where 0 = all plants were healthy and 100 = all plants were dead. DSI = (I x S)/M, where I = is the percentage of diseased plants, S = mean severity of foliar symptoms in diseased plants, and M = maximum severity value (M = 4 in our experiments)
Fig. 3. Average height growth (cm) for the second growing season and average dieback (cm) from season 1 to season 2, of *Ailanthus* seedlings inoculated with *V. dahliae*.

Fig. 4. Percentage of *Ailanthus* seedlings inoculated with *V. dahliae* that had delayed leaf emergence at the beginning of the second growing season.
Fig. 5. Average disease severity index of 2006 and 2007 canopy trees inoculated in the field with 3 ml of $10^7$ V. albo-atrum or V. dahliae conidia/ml. Control trees were inoculated with sterile distilled water and remained asymptomatic. Index of 0 to 100, where 0 = all plants were healthy and 100 = all plants were dead. DSI = (I x S)/M, where I = is the percentage of diseased plants, S = mean severity of foliar symptoms in diseased plants, and M = maximum severity value (M = 4 in our experiments).

Fig. 6. Average annual diameter growth (mm) of 2006 controls and V. albo-atrum trees, inoculated with 4.5 ml ($10^7$ spores / ml) of conidia.
Chapter III. Host Range of \textit{V. albo-atrum} isolated from \textit{Ailanthus altissima}.

Mark J. Schall, and Donald D. Davis, Department of Plant Pathology, The Pennsylvania State University, University Park 16802

\textbf{ABSTRACT}

\textit{Verticillium albo-atrum} is the primary pathogen causing extensive mortality of the invasive tree species \textit{Ailanthus altissima} in south-central Pennsylvania. Inoculation studies, using \textit{V. albo-atrum} isolate PSU 140, in both the greenhouse and field showed that \textit{Ailanthus} seedling and canopy trees were extremely susceptible. However, stem-inoculations of potted greenhouse seedlings and canopy trees in the field revealed that northern red oak, chestnut oak, red maple, sugar maple, yellow-poplar, and white ash were not susceptible hosts under the study conditions. However, striped maple exhibited wilt symptoms following inoculation, and \textit{V. albo-atrum} was isolated from one wilted striped maple in the field.

\textit{Verticillium} diseases have been reported in more than 200 different dicotyledonous plants, including such diverse crops as nuts, vegetables, legumes, herbs, root crops, and oilseed crops, as well as soft, pome, and stone fruits (Fradin and Thomma 2006, Sinclair and Lyon 2005). Perennial woody suscepts include more than 60 ornamental genera and shade tree species (Sinclair and Lyon 2005). In addition to these economically important species, \textit{Verticillium} species also occur in weeds and other “non-host” tolerant plants that may be important disease reservoirs (Pegg and Brady 2002).
However, biological mechanisms of plant susceptibility to *Verticillium* are not well defined (Robb 2007). Colonization of dicotyledonous weeds that act as agents for bridging between generations of susceptible hosts (Harris and Heimstra 1998, Vallad 2005, Wisler and Norris 2005).

*Verticillium dahliae* (Klebahn) and *V. albo-atrum* (Reinke & Berthold) are soilborne pathogens, typically associated with agricultural or nursery settings. *Verticillium dahliae* is almost exclusively associated within agricultural crops, and seldom, if ever, occurs as a forest pathogen on sites where agriculture hasn’t been practiced (W. Sinclair personal communication). In contrast, *V. albo-atrum* is capable of infecting trees on forest soils that have never been farmed (Harrington and Cobb 1984, Morehart et al. 1980, Morehart and Melchoir 1982, Sinclair and Lyon 2005).

The use of plant pathogens as biocontrols, or biological silvicides in forests, has been explored in several pathosystems where undesirable trees have been controlled with an endemic plant pathogen. Persimmon wilt, caused by *Cephalosporium diospyri* Crandall, has been applied to control persimmon in pastures in Arkansas for decades (Wilson 1965). Oak wilt, caused by *Ceratocystis fagacearum* (Bretz) Hunt, has been used to control low-value oak trees on sites better suited for pine production in Minnesota (French and Schroeder 1969). *Chondrostereum purpureum* (Fr.) Pouzar strain PFC 2139 is a registered EPA biopesticide for inhibition of sprouting and regrowth of hardwood tree stumps. However, *C. purpureum* is a pathogen of many plant species, such as economically important apples and plums (Sinclair and Lyon 2005, Wall 1990). Despite the risk involved to economically important hosts, *C. purpureum* is a ubiquitous endemic
pathogen throughout North America and applicators of the biopesticide must be aware of potential risks to non-target hosts (De Jong et al. 1990)

The main purpose of this chapter was to determine the host range, beyond *Ailanthus*, of the virulent *V. albo-atrum* (isolate PSU 140). Results from this study could also contribute information regarding the possibility of using this strain of *V. albo-atrum* as a biocontrol of *Ailanthus*, similar to the use of persimmon wilt described above. Evaluation susceptibility of non-*Ailanthus* species is an important part of risk analysis (DeJong et al. 1980). Any future biocontrol efforts must be aware of any threat this pathogen poses to economically and ecologically important forest trees.

Specific objectives of this chapter were to: 1) determine susceptibility of three (non-*Ailanthus*) species of seedlings to *V. albo-atrum* (isolate PSU 140); 2) determine susceptibility of seven (non-*Ailanthus*) species of canopy trees to *V. albo-atrum* (isolate PSU 140); and 3) evaluate field symptoms on non-*Ailanthus* canopy trees within severely infested areas.

**MATERIALS AND METHODS**

**Study Area.** The study area is within an oak-dominated mixed-hardwood stand in south-central Pennsylvania that contained actively wilting and dying *Ailanthus* canopy trees, saplings, root sprouts, and seedlings. The stand is located within the Tuscarora State Forest, near the Perry-Franklin County border and is approximately 56 ha (138 ac) in size. Oaks in the stand had been defoliated by gypsy moth (*Lymantria dispar* L.), and the stand was salvage-harvested in the early 1980s. The resulting open stand was invaded by *Ailanthus*, which later became severely infected by *V. albo-atrum*. The study site has been described in detail (Chapter II). All inoculations discussed in the following sections
were performed with *V. albo-atrum* isolate PSU 140. Levels of significance in all studies were tested at $p = 0.05$.

**Inoculum production.** For inoculations, conidial suspensions were prepared by growing 20 plates of *V. albo-atrum* (PSU 140, GenBank accession # FJ424082) on plum extract agar (PEA; 900 ml distilled water, 20 g agar, 100 ml concentrated plum extract, 1 g yeast, 5 g lactose, pH 5.6 - 6.0) (Bejarano-Alcazar et al. 1996) for 3 weeks at 22°C in the dark (Correll et al. 1988). Six-week-old cultures were washed with sterile distilled water and the wash strained through several layers of cheesecloth (Bejarano-Alcazar et al. 1996). Spore concentration was measured using of a hemocytometer and suspension adjusted to $10^7$ spores/ml.

**Seedling inoculations in the greenhouse.** In the spring of 2006, we evaluated the pathogenicity of a *V. albo-atrum* on three native forest species: white ash (*Fraxinus americana* L.), northern red oak (*Quercus rubra* L.), and sugar maple (*Acer saccharum* Marsh.). Three-year-old seedlings were obtained from Penn Nursery, Centre Co., PA, potted in 15.24 cm (6 in) plastic pots (Fig. A9) filled with MetroMix 200 (Sungro Horticulture, Bellevue, WA), and maintained in a greenhouse. Inoculated *A. altissima* seedling used in previous studies (Chapter II), were used as positive controls. Seedlings were fertilized once with Osmocote 12-12-12 N:P₂O₅:K₂O (The Scotts Company, Marysville, OH), and watered automatically for 2 min/day. On June 1, 20 seedlings of each species were injected at two points at the base of the stem with 0.5 ml of spore suspension ($10^7$ spores/ml) using a sterile syringe (Bugbee and Presley 1967). Five control seedlings of each species were stem-injected with sterile distilled water and maintained in the greenhouse.
The experiment used a completely randomized experimental design, wherein treatments were *V. albo-atrum* stem-inoculation and sterile water stem-inoculation. *Verticillium* was inoculated into 10 seedlings of each tree species and the study was replicated once, for a total of 20 *V. albo-atrum* treated plants for each tree species. Following inoculation, plants were examined weekly for 3 mo. Wilt severity was rated using a relative scale of 0 to 4, where 0 = healthy leaves, 1 = chlorotic leaves, 2 = necrotic leaf tips, 3 = wilting leaves, and 4 = dead or defoliated seedlings (Bejarano-Alcazar et al. 1996). Analysis of variance (ANOVA) and Tukey’s mean comparisons were used to determine if severity ratings of non-*Ailanthus* seedling species differed significantly from that of *Ailanthus* (Minitab 2007).

**Forest trees inoculated in the field.** In early May 2007, seven forest tree species, plus *Ailanthus*, were inoculated in the field with *V. albo-atrum* isolate (PSU 140) at a concentration of $10^7$ spores/ml. Species inoculated were chestnut oak (*Quercus montana* Willd.), northern red oak, red maple (*Acer rubrum* L.), sugar maple, striped maple (*Acer pennsylvanicum* L.), white ash, and yellow-poplar (*Liriodendron tulipifera* L.). We included *Ailanthus* in with the canopy tree inoculations as a positive control. For each species, six individual trees, showing no signs of disease, were located at a minimum of two locations (two replicates), with three locations for red and striped maple (three replicates) (Fig. A11), and marked with a numbered aluminum tag. Five trees/species were inoculated and one tree served as a non-inoculated control. Inoculated trees were selected at random. Treatments were stem inoculation with *V. albo-atrum* or stem inoculation with sterile water in a completely randomized design. Trees were struck three times at the base with a Hypo-hatchet® (Forestry Suppliers, Jackson MS) (Fig. A10) that
injected 1 ml inoculum into a 6-cm wound. Non-inoculated control trees were wounded with a surface-sterilized axe at three locations on the base, and 1 ml of sterile distilled water applied to each wound using a sterile syringe. Disease severity was rated monthly using the relative 0 to 4 scale, previously used to rate inoculated seedlings.

To ensure that the Hypo-hatchet® was dispensing liquid, Cake Mate® blue food coloring (Signature Brands, Ocala, FL) (Fig. A12) was added to the spore suspension and wounds observed for a blue food color. To test inoculum viability and to determine conidia germination rate, and to ensure that the food coloring had no effect on spore germination and viability, we plated ten-fold dilutions onto plates of prune lactose yeast agar and counted resultant colonies of *Verticillium* from suspensions prepared before and after the addition of blue food coloring.

Stem-inoculations may bypass root defenses (Huisman 1988) and may not allow an accurate determination whether a plant is susceptible or resistant. Therefore, a series of plots were established to evaluate the health of non-*Ailanthus* tree species within a severely infested area. In July - August 2007, 12 circular permanent plots were established throughout the study area (Fig. A13). Each plot was 0.081ha (0.2 acre) in size with a 16.06 m (52.7 ft) radius. Plots were located approximately 200 m apart, with steel stakes used to establish plot centers, ensure sampling of the entire infested area. Location of plot centers and boundaries was determined by GPS and coordinates recorded.

Tree species evaluated for wilt symptoms included *Ailanthus*, American basswood (*Tilia americana* L.), American elm (*Ulmus americana* L.), black ash (*Fraxinus nigra* Marsh.), black gum (*Nyssa sylvatica* Marsh.), black locust (*Robinia pseudoacacia* L.), butternut (*Juglans cinerea* L.), chestnut oak, red maple, red oak,
sassafras (*Sassafras albidum* (Nutt.) Nees), shellbark hickory (*Carya laciniosa* (Michx. f.) L.oud.), striped maple, sweet birch (*Betula lenta* L.), white ash, witch hazel (*Hamamelis virginiana* L.), and yellow-poplar (Fig. 2).

Trees located within each permanent plot boundary were rated on a 0 to 2 scale, where 0 = healthy, 1 = wilting, and 2 = dead. Samples were taken from trees exhibiting wilt and isolations made onto PEA (Bejarano-Alcazar et al. 1996). Cultures were grown at 22 °C in the dark (Correll et al. 1988). Morphological characteristics were used to confirm presence or absence of *V. albo-astrum*.

On each plot, data from tree species that comprised < 2% or less of the total trees/ha were grouped and labeled miscellaneous hardwoods (MISC) (Fig. 2). This group included white ash, yellow-poplar, black gum, chestnut oak, red oak, sassafras, American elm, butternut, and black ash. Analysis of variance (ANOVA) and Tukey’s mean comparisons were used to determine if severity ratings of non-*Ailanthus* tree species differed significantly from that of *Ailanthus* (Minitab 2007).

**RESULTS**

**Effect of food coloring.** Addition of blue food coloring to conidial suspensions confirmed that the Hypohatchet was properly dispensing liquid. Food coloring had no significant effect on spore germination rate compared to suspension plated prior to the addition of blue food coloring, which was approximately 76.7% in 42-day-old cultures on PEA plates.

**Seedling inoculations in the greenhouse.** Data from replicates did not significantly differ for each treatment, so data from replicates were pooled. ANOVA revealed no significant difference in final disease severity (week 9) among controls and the three
species of seedlings [red oak (p = 0.431), white ash (p = 0.155), and sugar maple (p = 0.627)]. One inoculated white ash and one inoculated sugar maple died during the potted seedling experiment. However, *V. albo-astrum* was not isolated from leaf and stems of the dead seedlings. In addition, two white ash control seedling died. Isolations from those seedlings were also negative for *V. albo-astrum*. No other seedling mortality was observed.

**Forest tree inoculation in the field.** Data from the final disease severity ratings were not normally distributed and were normalized using a square root transformation. Data from replicates did not significantly differ and were pooled. ANOVA revealed significant differences among species in the final disease ratings for species. Only striped maple had a final disease severity rating that did not significantly differ from that of *Ailanthus* (p = 0.206). Data from all other inoculated species (red maple, sugar maple, red oak, chestnut oak, yellow-poplar, and white ash) were not significantly different from each other, but were statistically different from the inoculated *Ailanthus* trees (Fig. 1).

Striped maple developed significant disease severity ratings in response to *V. albo-astrum* stem-inoculations (Fig. 1). The disease severity index for striped maple reached a value of 50 by week 8 and 99.2 by week 16 (Fig. 1). Isolations from symptomatic striped maple trees confirmed the pathogen as *V. albo-astrum*. Inoculated striped maples were dead at the end of the growing season. Inoculated white ash exhibited a slight rise in symptom severity during July (Fig. 1). However, this increase in disease severity rating appeared to be due to a twig dieback diagnosed as ash anthracnose caused by *Gnomoniella fraxini* Redlin & Stack (Sinclair and Lyon 2005). No other
inoculated tree species showed increased disease severity during the 2007 growing season.

During the summer of 2008, plots were revisited and inoculated trees rated as in 2007. There was no increase in final disease severity in 2008 as compared to the final ratings taken on 21 September 2007. The inoculated striped maple trees, which were rated as dead in 2007, had developed no stump or root sprouts, indicating that the entire tree including the root system was dead. In addition, *Verticillium* wilt had not spread from the inoculated striped maple trees to adjacent healthy striped maple trees.

**Permanent Plots.** Disease severity data were not normally distributed and were normalized using square-root transformations. ANOVA revealed significant differences in disease severity rating among tree species. However, Tukey’s mean comparisons revealed that black locust was the only species whose disease severity did not significantly differ from that of *Ailanthus* (*p* = 0.950). Ratings of all other species (striped maple, black locust, red maple, black birch, witch-hazel, shellbark hickory, American basswood, and the miscellaneous group) were not statistically different.

*Ailanthus altissima* trees were in severe decline throughout the study area. Many *Ailanthus* trees were symptomatic, having either wilting (19.6% of *Ailanthus* trees) or mortality (46.7% of *Ailanthus* trees). Although the disease severity rating of black locust was not significantly different from *Ailanthus*, black locust trees were not exhibiting wilt. Black locust trees exhibited only mortality (58.4%) or top dieback (Fig. 3), associated with the locust borer (*Megacyllene robiniae* Forster) (Fig. A15), resulting in stem girdling and mortality. Dead and dying black locust trees were also heavily colonized by fungal saprophytes, precluding isolation. A significant proportion (21.8%) of striped
maple in the study area were also dead. However most striped maple trees (67.3%) had stem cankers (Fig. A16). Isolations from cankers consistently yielded one fungus, which was isolated in pure cultures. This fungus was identified using PCR followed by DNA sequencing, and comparison of the ITS region with others in NCBI’s BLASTN database (NCBI, Bethesda, MD) as cankers was *Botryosphaeria dothidea* [(Moug.:Fr) Ces & De Not], a common opportunistic fungus that attacks many stressed plant species (Fig. A18) (Sinclair and Lyon, 2005).

**DISCUSSION**

**Host Range.** Of the seven non-*Ailanthus* species evaluated, only striped maple appeared to be susceptible to *V. albo-atrum*. However, the stem-inoculation technique may have bypassed extravascular root defenses (Talboys 1972). Therefore, the perceived susceptibility of stem-inoculated striped maple trees could have been an artifact of the inoculation procedure. This finding was reinforced by the low isolation incidence of *V. albo-atrum* from declining striped maple in the permanent plot study, indicated that other factors may be playing a significant role in wilt and decline of striped maple in the field. It is likely that the opportunistic fungus *B. dothidea* caused much of the striped maple wilt and mortality. Although occasional striped maple trees were observed to be wilting on the permanent plots, *V. albo-atrum* was isolated from only one wilting striped maple (in a non-inoculated plot). Striped maple wilting likely occurred when the *Ailanthus* overstory died from *V. albo-atrum*, exposing the understory striped maple to bright sunlight and possible drought stress (Crist and Schoeneweiss 1975). Foresters in the study area have observed understory striped maple wilting in a similar manner following partial canopy harvests.
Non-inoculated black locust trees had statistically similar disease severity rating as compared to *Ailanthus*. However, black locust trees are prone to high mortality from locust borer. The high incidence of locust borer damage in the stand, coupled with normal short life span of the tree species (Boring and Swank 1984), was likely responsible for the high level of black locust mortality (Figs. 3, A15).

Lack of symptom development within inoculated non-*Ailanthus* trees, as well as trees observed in the permanent plot study, does not necessarily mean that they are not hosts for *V. albo-atrum*. Plants that appear tolerant to *Verticillium* infection can be colonized systemically by *Verticillium*, but show little or no external symptoms (Robb 2007). However, the importance of such endophytes or “symptomless carriers” is largely unknown in forest stands. However, such symptomless carriers may contribute to the persistence of *V. albo-atrum* in forests. Additional research is needed to evaluate the importance of symptomless non-*Ailanthus* hosts that are apparently tolerant to *Verticillium* infection, and the role of such symptomless carriers in the persistence of *V. albo-atrum* in southern Pennsylvania forests.

Weed host reservoirs also contain *Verticillium* species. Some weeds contain internal *Verticillium* resting structures, in leaves while remaining symptomless (Pegg and Brady 2002, Vallad 2005). Weed species observed to grow among diseased *Ailanthus* trees in the study area include: American pokeweed (*Phytolacca americana* L.), common blackberry (*Rubus allegheniensis* Porter ex L.H. Bailey), garlic mustard (*Alliaria petiolata* Bieb.), hay-scented fern (*Dennstaedtia punctilobula* Michx.), nettle (*Urtica* spp.), Pennsylvania smartweed (*Polygonum pennsylvanicum* L.), and Virginia creeper (*Parthenocissus quinquefolia* L.). Blackberry, nettle, and Pennsylvania smartweed are
reported as potential hosts or symptomless carriers of *Verticillium* species (Pegg and Brady 2002, Vallad 2005). Japanese creeper (*Parthenocissus tricuspidata* (Siebold & Zucc.) Planch.) is a known host of *V. dahliae* (Pegg and Brady, 2002), indicating that Virginia creeper, which is in the same genus and is very common in the study area, should be investigated as a symptomless carrier for *Verticillium*. In addition to symptomless forest trees, such weed hosts could be involved in persistence and survival of *V. albo-atrum* in this forest ecosystem.

It is apparent that *Ailanthus* is very susceptible to *V. albo-atrum*. However, on the permanent plots, many (80.6%) of the dead *Ailanthus* trees also had *Armillaria* spp. rhizomorphs at the base of the tree. *Armillaria mellea* has been reported to be a pathogen of *Ailanthus* (Fig. A17) (Miller 1990), but is often opportunistic, attacking after a primary pathogen, such as *Verticillium*, has weakened trees. *Armillaria* may also act as a saprophyte, decaying dead *Ailanthus* trees (Sinclair and Lyon 2005). Research is needed to determine if whether *Armillaria* plays a role in the *Verticillium-Ailanthus* pathosystem.

In conclusion, *V. albo-atrum* isolate PSU 140, previously isolated from severely wilting *Ailanthus* trees, does not cause visible disease symptoms on other ecologically or economically hardwood trees, including northern red oak, chestnut oak, red maple, sugar maple, white ash, and yellow-poplar under the study conditions here. These species are not likely to be attacked by naturally occurring populations of *V. albo-atrum*, nor if this isolate of *V. albo-atrum* is released as a potential biocontrol agent. Striped maple, however, deserves further study as a possible host for *V. albo-atrum*. Interestingly, striped maple is an undesirable understory plant that is often treated with herbicides in
economically managed forests. Management strategies that use *V. albo-atrum* as a biocontrol of *Ailanthus* might also consider this pathogen as a biocontrol of striped maple. However, this may or may not be feasible.
F. Literature Cited


LIST OF FIGURES AND TABLES

**Figure. 1.** Average disease severity index chart of *Ailanthus*, striped maple, red maple, sugar maple, white ash, yellow-poplar, red oak, and chestnut oak field inoculations of *V. albo-atrum*. Only *Ailanthus* and striped maple exhibited significant susceptibility. Red maple, sugar maple, white ash, yellow-poplar, red oak, and chestnut oak did not develop significant disease symptoms. DSI = (I x S)/M, where I = is the percentage of diseased plants, S = mean severity of foliar symptoms in diseased plants, and M = maximum severity value (M = 4 in our experiments).

**Figure. 2.** Percent trees/ha of species within the permanent plot survey.

**Figure. 3.** Percent of species wilting, dead, or healthy trees on permanent plots.

**Figure. 4.** Percent of striped maples in the study area in four health ratings, comparing striped maple trees with cankers vs. trees without cankers.

**Table 1.** Percent striped maple in 4 different health classes, comparing striped maple with and without stem cankers in permanent plots.
Fig. 1. Average disease severity index chart of Ailanthus, striped maple, red maple, sugar maple, white ash, yellow-poplar, red oak, and chestnut oak field inoculations of V. albo-atrum. Only Ailanthus and striped maple exhibited significant susceptibility. Red maple, sugar maple, white ash, yellow-poplar, red oak, and chestnut oak did not develop significant disease symptoms. DSI = (I x S)/M, where I = is the percentage of diseased plants, S = mean severity of foliar symptoms in diseased plants, and M = maximum severity value (M = 4 in our experiments).
Fig. 2. Percent trees/ha of species within the permanent plot survey.

Fig. 3. Percent of species wilting, dead, or healthy trees on permanent plots.
Fig. 4. Percent of striped maples in the study area in four health ratings, comparing striped maple trees with cankers vs. trees without cankers.

Table 1. Percent striped maple in 4 different health classes, comparing striped maple with and without stem cankers in permanent plots.

<table>
<thead>
<tr>
<th></th>
<th>Striped maple without cankers</th>
<th>Striped maple with cankers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>29.1%</td>
<td>29.6%</td>
</tr>
<tr>
<td>Dieback</td>
<td>1.5%</td>
<td>17.6%</td>
</tr>
<tr>
<td>Wilting</td>
<td>0.0%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Dead</td>
<td>2.0%</td>
<td>18.6%</td>
</tr>
</tbody>
</table>
Chapter IV. Epidemiology of *Verticillium* wilt of *Ailanthus altissima* in south-central Pennsylvania

Mark J. Schall, and Donald D. Davis, Department of Plant Pathology, The Pennsylvania State University, University Park 16802

ABSTRACT

*Verticillium albo-atrum* and *V. dahliae* cause severe wilt and mortality of the invasive tree species *Ailanthus altissima* in southern Pennsylvania. *Verticillium albo-atrum* is more virulent and aggressive to *Ailanthus* than *V. dahliae*, as shown by both greenhouse and field inoculations. The pathogen can survive within overwintering infected *Ailanthus* trees, on fallen *Ailanthus* leaves, in soil, or in symptomless hosts. Primary inoculum, in the form of germinating resting structures or conidia, is formed in spring, and inoculation takes place at time of leaf emergence. Dissemination may involve wind disseminated leaflets, seed transmission, and/or ambrosial beetle transmission. *Ailanthus altissima* roots are predisposed to *Verticillium* by wounding. During colonization, phenols are deposited in outer xylem parenchyma within 1 week of inoculation indicating that initial fungus colonization is circumferential. Colonization then proceeds both upward and downward until host mortality occurs. In the field, rate of spread from tree to tree is rapid. Since 2000, approximately 10,000 *Ailanthus* trees have died in southern Pennsylvania from *Verticillium* wilt.

We previously reported that *Verticillium dahliae* (Klebahn) and *V. albo-atrum* (Reinke & Berthold) caused wilt of *Ailanthus altissima* (Mill.) Swingle, although *V. albo-
"atrum" was the more virulent and aggressive of the two *Verticillium* species (Chapter II).
The purpose of this chapter is to study the epidemiology of Ailanthus wilt. The following introduction will discuss various aspects of the *Verticillium* disease cycle (see Fig. A19), including survival, dissemination, and colonization.

**Survival.** *Verticillium dahliae* survives in the soil by means of microsclerotia resting structures, which can persist at least 14 years in field soils (Wilhelm 1955). In *V. albo-atrum*, segments of hyphae specialize and become resting structures as thick-walled melanized cells (Fig. A6), which can survive in the soil from 9 months to 4 years, or possibly more in fallow soils (Pegg and Brady 2002). Both types of resting structures persist through mycostasis, awaiting plant root exudates that stimulate germination (Pegg and Brady 2002). Conidia and hyaline hyphae are much shorter lived, persisting for only 3 to 4 weeks within infested soils outside of plants (Schreiber and Green 1963, Pegg and Brady 2002). Other means of *Verticillium* survival include colonization of dicotyledonous weeds, legume and cereal crops, and within “volunteer” crop plants that act as bridging agents between species (Vallad 2005, Wisler and Norris 2005).

Soil environmental factors have various effects on wilt severity and survival of *Verticillium*. Increased nitrogen and soil salinity generally increases *Verticillium* wilt severity in many pathosystems; conversely, the addition of potassium to potassium-deficient soils reduces wilt severity (Elmer and Ferrandino 1994, Pegg and Brady 2002, DeVay et al. 1997). Trace elements such as cobalt, manganese, copper, molybdenum, aluminum polyphosphate, and copper sulfate amendments have a negative effect on wilt severity (Pegg and Brady 2002). Soil pH may severely impact survival and persistence of *Verticillium* resting structures in soils. Soils with a pH <5.5 can reduce microsclerotal
production and survival of *V. dahliae* (Baard and Pauer 1982, Guba 1934, Pegg and Brady 2002).

*Verticillium albo-atrum* is highly sensitive to soluble aluminum in the soil. Aluminum concentrations of 8 μg/g Al\(^{3+}\) caused decreased pathogenicity, occurrence, and pigment development as related to *Verticillium* resting structures (Orellana et al. 1974). However, effects of aluminum are likely confounded by soil pH. Availability of aluminum in most soils is strongly related to pH. Soluble aluminum concentration increases exponentially as soil pH decreases, and most soluble aluminum is immobilized at soil pH >5 (Reuss et al. 1990, Mulder et al. 1989). However, Baard and Pauer (1982) surmised that aluminum played a minor role in disease suppression via increasing resting structure mycostasis, and concluded that the negative effect of acidic soils on *V. dahliae* was due to other, unknown soil pH-related factors besides aluminum.

Numbers of *Verticillium* propagules (resting structure inoculum level) in the soil can be estimated through collection of soil samples and counting resultant colonies (Goud and Termorshuizen 2003). Soil inoculum densities of *V. albo-atrum* needed to cause wilt of *Ailanthus* are not known. However an inoculum density of 17 to 23 *V. albo-atrum* propagules/g soil was necessary for significant yield reductions in potato fields (Nnodu and Harrison 1979).

Overwintering through inhospitable cold periods in Pennsylvania is imperative for year-to-year survival of *Verticillium*. In addition to survival in the soil, *Verticillium* can also overwinter within infected perennial host plants that survive until the following spring, when leaves emerge and plant growth is resumed (Fradina and Thomma 2006). *Verticillium* may also survive within infected tolerant plants or symptomless carriers,
resembling an endophyte. Such plants exhibit few or no visible *Verticillium*-induced symptoms (Robb 2007, Pennypacker et al. 1985).

**Germination.** Resting structures are formed within infected plant tissue such as leaves and stems. As infected plant tissues fall to the soil, the resting structures become incorporated into the soil, and may later germinate in response to root exudates. Roots from both dicots and monocots, host and non-host, can induce germination (Pegg and Brady 2002). Germination also may occur in response to increased nutrients in the soil (Fradin and Thomma 2006). However, the zone of influence that plant roots have on resting structures is usually only a few millimeters (Pegg and Brady 2002).

**Dissemination.** *Verticillium albo-atrum* and *V. dahliae* are soil-borne plant pathogens dispersed within the soil primarily through movement of resting structures or hyphal growth, but can be disseminated externally to the soil by wind, water, seeds, vegetative propagation, pruning equipment, and insect vectors (Pegg and Brady 2002, Vallad 2005, Kalb and Millar 1986). Resting structures are normally found within the soil layer where the roots are found, but also may occur on or near the soil surface, which facilitates propagule dispersal by water and/or wind. Wind-blown, infected leaves and seeds can be carried considerable distances to infest new areas (Rijkers et al. 1992, Harris and Heimstra 1998, Vallad 2005, Wisler and Norris 2005, du Toit et al. 2005). *Verticillium* also may be spread from plant to plant through root grafts and root contact (Agrios 2005, Isaac 1953).

*Verticillium dahliae* usually causes a monocyclic disease, producing primary inoculum once during the growing season, usually late in the season at plant senescence. However, *V. albo-atrum* may produce airborne conidia on infected plant tissue, and may
occasionally cause multiple infections per growing season, inciting a polycyclic disease (Fradin and Thomma 2006).

**Penetration.** Germinated resting structures produce hyphae that penetrate host root cortex, often through wounds or openings where secondary roots emerge. In agricultural areas, plant parasitic nematodes and cropping equipment are common wounding agents, but *Verticillium* apparently does not utilize nematode wounds as points of entry into plant roots (Bowers et al. 1996).

**Colonization.** Following penetration, *Verticillium* grows into the root stele (Harris and Heimstra 1998), where the fungus colonizes the vascular tissue and spreads up into the stem via either hyphal growth or transport of conidia (Brady and Pegg 2002). Once inside the stem, conidia move rapidly through the xylem in the transpiration stream. *Verticillium* may recognize the chemical and physical environment specific to xylem vessels, such as the low oxygen levels and negative water potential, which favor conidial production (Harris and Heimstra 1998). In a similar system, Banfield (1941) reported that conidia released within the vascular tissue of an elm tree could move 1.5 m upward in 8 to 15 sec (Harris and Heimstra 1998). Conidia may become trapped in vessel end walls or pits, germinate, penetrate adjacent vessel elements, and subsequently sporulate and continue colonization (Pegg and Brady 2002, Fradin and Thomma 2006). At each newly infected site, high numbers of additional conidia are produced on simple conidiophores or through budding, resulting in a rapid spread of *Verticillium* propagules through host xylem vessels. Initially, conidial production within xylem vessels is favored over hyphal growth until plant death, at which time hyphal growth continues to colonize the dead host tissues and conidial production typically ceases (Heinz et al. 1998, Sewell and Wilson 1964).
During colonization, the fungus also induces the host to produce tyloses and gums within the xylem, blocking water uptake and resulting in foliar wilt (Brady and Pegg 2002).

*Verticillium* produces cell wall degrading enzymes that may play a role in pathogenesis. Pectinolytic enzymes likely play a primary role in pathogenesis, since these compounds aid in breakdown of pectin in vessel end walls, allowing the fungus to move efficiently through the plant vascular system (Fradin and Thomma 2006). *Verticillium albo-astrum* mutants that produce lower levels of pectinases exhibit reduced virulence on tomato plants as compared to wild-type plants, despite similar degrees of colonization (Durrands and Cooper 1988). *Verticillium albo-astrum* and *V. dahliae* also produce phytotoxins and other cell death-inducing molecules. Phytotoxins stimulate ethylene production in susceptible plants, which induces disease symptoms (Mansoori and Smith 2005). However, the complete roles of these phytotoxins are unclear in *Verticillium* wilt (Fradin and Thomma 2006).

The overall objective of this chapter is to gain insight into the disease cycle and epidemiology of *Ailanthus* wilt. Specific objectives are to determine 1) a simple molecular protocol for sensitive detection of both *V. albo-astrum* or *V. dahliae*, 2) means of *V. albo-astrum* overwintering in our study area, 3) the rate of *V. albo-astrum* spread in small and large stands of *Ailanthus*, 4) potential for dissemination of *Verticillium* in leaves, seeds, and ambrosial beetles, 5) the influence of root wounding on predisposition to *Verticillium* infection, and 6) the rate at which *V. albo-astrum* colonization occurs within inoculated *Ailanthus* seedlings.
MATERIALS AND METHODS

Study area. The study area was in an oak-dominated, mixed-hardwood stand in south-central Pennsylvania (Fig. A1) that contained wilting and dying *Ailanthus* canopy trees, saplings, root sprouts, and seedlings. The study area was described in detail in chapter II. We previously reported that the *Ailanthus* wilt in the study area was caused by *V. albo-atrum* and *V. dahliae* (Chapter II).

The non-native, highly invasive *Ailanthus* had invaded an area within a forest that had been defoliated by gypsy moth (*Lymantria dispar* L.) and subsequently salvage harvested in the early 1980s. The resulting open stand was ideal for *Ailanthus* invasion. Tree-ring counts of the oldest *Ailanthus* trees indicate that *Ailanthus* invaded the forest stand in 1984, shortly after the salvage operation. The source of the original *Ailanthus* seed is unknown, but was likely large female *Ailanthus* trees on adjacent ridgetops. The infested area appeared to have a defined infection center with disease spreading to the southwest and northeast. The epidemic was progressing rapidly, indicating that pathogen dissemination is efficient, and may involve wind or insects, and does not entirely rely on slower methods of transmission such as soil transmission (Vallad 2005, Kalb and Millar 1986, Bengough et al. 2006). Heavy infestations of ambrosial beetles were observed in dead and declining *Ailanthus* trees in the study area. It is unknown what role, if any, they play in dissemination.

Permanent plots. During late July and early August 2007, 0.081ha (0.2 acre), 16.06 m (52.7 ft) radius permanent plots were established throughout the study area (Fig. A13). Plots were established approximately 200 m apart and steel stakes marked plot centers.
Each plot was located by GPS and coordinates recorded. Species and diameter of all woody vegetation > 2.54 cm (1 in) diameter within each plot was recorded.

Plot trees were evaluated and rated on a 0 to 2 scale, where 0 = healthy, 1 = wilting, and 2 = dead. Other general characteristics were recorded for each tree, such as canker severity, beetle infestation severity, crown class, and presence or absence of *Armillaria* rhizomorphs on dead trees.

Wood chips, twigs, petioles, and leaf samples were taken from wilting plot trees. Samples were flame-sterilized and placed (one per petri dish) onto plum extract agar (PEA; 900 ml distilled water, 20 g agar, 100 ml concentrated plum extract, 1 g yeast, 5 g lactose, pH 5.6 - 6.0), a selective growth medium for *Verticillium* (Bejarano-Alcazar et al. 1996). Cultures were grown in controlled environment chambers on PEA in the dark at 22°C (Correll et al. 1988).

**Detection of *Verticillium albo-atrum* and *V. dahliae* using nested PCR.** Nested polymerase chain reaction (PCR) is a sensitive tool used for detection of fungal pathogens in plant materials, seeds, and soils (Mercado-Blanco et al. 2001, Mercado-Blanco et al. 2003, Karajeh and Masoud 2006, Perez-Artes et al. 2005). A nested PCR protocol was developed for molecular detection of *V. albo-atrum* within *Ailanthus* seeds and ambrosial beetles, to gain insight into seed and beetle dispersal of the pathogen.

**DNA extraction.** Mycelium from cultures on PEA was transferred to sterile culture tubes containing potato dextrose (PD) broth (EM Science, Gibbstown, NJ) and incubated for 14 – 21 days without shaking. Mycelium was separated from broth by filtration and lyophilized (Perez-Artes et al. 2000). *Ailanthus* seeds and ambrosial beetles were collected, placed into 1.5 ml microcentrifuge tubes and immediately frozen. Seed
and beetle samples were lyophilized and stored at -4 °C until DNA extraction. DNA from all samples was extracted using a DNeasy Plant Minikit (Qiagen Inc., Hilden, Germany). DNA concentration was determined by electrophoresis in a 1% agarose gel.

**Primer building.** For first-round amplification of nested PCR, the previously developed primer pair NESF18S, 5'-CCTCATAACCCTTTGTGAACC-3' and NESR28S, 5'-CCGAGGTCACCCGTGCCC-3' by Volossiouk et al. (1995) was used to amplify a 452 bp fragment of DNA immediately internal of the universal primer pair ITS5 and ITS4 (Volossiouk et al. 1995, Karajeh and Masoud 2006, Karajeh 2006; White et al. 1990). Differences in the internal transcribed spacer (ITS) region between *V. albo-atrum* and *V. dahliae* allow differentiation between the two *Verticillium* species for the development of species-specific probes and primers (Nazar et al. 1991, Leivens et al. 2003). ITS region sequences (primers ITS5 to ITS4) had been obtained during molecular identification of fungal isolates (Chapter II) and aligned using MEGA v. 4 software (Tamura et al. 2007). Species-specific primers were developed for *V. albo-atrum*, FVAL6/RVA (FVAL6 5'- CGGTACATCAGTCTCTTTATTC-3' and RVA 5'-TCCGATGCGAGCTGTAAT -3') and *V. dahliae*, FVDA5/RVD (FVDA5 5'-CGGTCCATCAGTCTCTCTGTATTT-3' and RVD 5'- TCCGATGCGAGCTGTATAAC -3'), creating a 329 bp DNA fragment used in the second round of the nested PCR (Karajeh and Masoud 2006). Both primer pairs were either created or adapted from primer pair FVD/RVD (Table 1) (Masoud 2002). OligoAnalyzer 3.1 (Integrated DNA Technologies, 2008) was used to analyze primer kinetics (Table 1).

**Optimizing the PCR.** A standard PCR protocol was used to amplify the ITS gene region. Primer pairs FVAL6/RVA and FVDA5/RVD were used in a PCR reaction to
amplify a 329 bp product of the ITS gene region (Fahleson et al. 2004). PCR was performed in a PTC-100 Programmable Thermal Cycler (MJ Research Inc., Waltham, MA) using: 27.8 μL PCR water, 5 μL 5X Colorless goTaq® PCR buffer (Promega Corp, Madison, WI), 5 μL 5X Green goTaq® PCR buffer (Promega Corp, Madison, WI), 5 μL 10X dNTP, 4 μl 25mM MgCl2, 1 μL 10 μM primer FVAL6, 1 μL 10 μM primer RVA, 0.2 μL 500 u GoTaq® DNA polymerase (Promega Corp, Madison, WI) and 1 μL template DNA for each sample (Geml et al. 2005).

To optimize annealing temperatures, the internal primer pairs FVAL6/ RVA (V. albo-atrum) and FVDA5/RVD (V. dahliae) were tested in a PCR reaction using a gradient cycler (MJ Research PTC – 200) with pure extracted fungal DNA from our V. albo-atrum type isolate (PSU 140, GenBank accession # FJ424082) and V. dahliae type isolate (PSU 154, GenBank accession # FJ424083). PCR parameters were: 2 min at 94 °C; followed by 35 cycles of 30 sec at 95 °C, 30 sec at a gradient of 59 to 66°C, and 30 sec at 72 °C; and followed by 3 min at 72 °C. Products were electrophoresed in a 1.0% agarose gel and stained with ethidium bromide to visualize DNA. For each primer set, the other Verticillium species (either V. albo-atrum or V. dahliae) was used as a negative control. MgCl2 concentrations were optimized to reduce the chance of amplifying non-specific PCR products. PCR was optimized for annealing temperature (Ta), primer concentration (1 or 10μM), and MgCl2 concentration (1.5, 2, or 2.5 mM), with our V. albo-atrum primer set FVAL6/RVA (Rochelle et al. 1997). For V. dahliae primer set FVDA5/RVA, primer concentrations and MgCl2 concentrations were not optimized, since acceptable species differentiation was achieved by optimizing Ta (Rochelle et al. 1997).
Conducting nested PCR. The nested PCR protocol developed by Karajeh (2006) was adapted to detect low levels of *V. albo-atrum* and *V. dahliae* in *Ailanthus* seeds, and *V. albo-atrum* in ambrosial beetles. In the first-round PCR, NESF18S and NESR28S primers were used as the first-round PCR in a nested PCR where species-specific primers were used in the second PCR amplification (Volossiouk et al. 1995, Karajeh 2006). First-round PCR (50 μl) used 27.8 μL PCR water, 5 μL 5X colorless goTaq® PCR buffer (Promega Corp, Madison, WI), 5 μL 5X green goTaq® PCR buffer (Promega Corp, Madison, WI), 5 μL 10X dNTP, 4 μl 25 mM MgCl₂, 1 μL 10 μM primer NESF18S, 1 μL 10 μM primer NESR28S, and 0.2 μL 500 U GoTaq® DNA polymerase (Promega Corp, Madison, WI) (Geml et al. 2005). First-round PCR was performed in a PTC-100 Programmable Thermal Cycler (MJ Research Inc., Waltham, MA), set at 4 min at 94°C; followed by 35 cycles of 1 min at 94 °C, 1 min at 56°C and 1 min at 72 °C; and followed by a 6-min extension at 72 °C (Karajeh 2006). Products were electrophoresed in a 1.0% agarose gel and stained with ethidium bromide to visualize DNA.

*Verticillium albo-atrum* primer pairs (FVAL6/RVA) were used in the second-round PCR, with the product of the first PCR as template, our optimized MgCl₂, and the optimum annealing temperature; 26.8 μL PCR water, 5 μL 5X Colorless goTaq® PCR buffer (Promega Corp, Madison, WI), 5 μL 5X Green goTaq® PCR buffer (Promega Corp, Madison, WI), 5 μL 10X dNTP, 5 μl 25 mM MgCl₂, 1 μL 1 μM primer FVAL6, 1 μL 1 μM primer RVA, and 0.2 μL 500 u GoTaq® DNA polymerase (Promega Corp, Madison, WI). Second-round PCR was performed in a PTC-100 Programmable Thermal Cycler (MJ Research Inc., Waltham, MA), set at 4 min at 94°C; followed by 30 cycles of 1 min at 94 °C, 1 min at 61°C and 1 min at 72 °C; followed by 10 cycles of 1 min at 94
°C, 1 min at 58°C and 1 min at 72 °C; and a 6-min extension step at 72 °C. Products were
electrophoresed in a 1.0% agarose gel and stained with ethidium bromide to visualize
DNA.

Verticillium dahliae primer pair (FVDA5/RVD) for the second-round PCR, using
the product of the first PCR as template, utilized our optimized annealing temperature
protocol: 27.9 μL PCR water, 5 μL 5X Colorless goTaq® PCR buffer (Promega Corp,
Madison, WI), 5 μL 5X Green goTaq® PCR buffer (Promega Corp, Madison, WI), 5 μL
10X dNTP, 4μl 25mM MgCl2, 10μL 1 μM primer FVDA5, 10μL 1 μM primer RVD, 0.1
μL 500 u GoTaq® DNA polymerase (Promega Corp, Madison, WI). Second-round PCR
was performed in a PTC-100 Programmable Thermal Cycler (MJ Research Inc.,
Waltham, MA) set at 4 min at 94 °C; followed by 30 cycles of 1 min at 94 °C, 1 min at
64°C and 1 min at 72 °C; followed by 10 cycles of 1 min at 94 °C, 1 min at 61 °C and 1
min at 72 °C; and a 6-min extension step at 72 °C. Products were electrophoresed in a
1.0% agarose gel and stained with ethidium bromide to visualize DNA.

PCR sensitivity to low amounts of DNA. Preliminary studies indicated that V.
albo-atrum and V. dahliae might be present at extremely low levels in seed and
ambrosial beetle samples. The protocol of Karajeh (2006) was used to determine the
sensitivity of primer pairs FVAL6/RVA and FVDA5/RVD. Conidia, rather than extracted
purified DNA, were used directly in the first PCR reaction. As a test of sensitivity, 2x10⁴,
2x10³, 2x10², 20 and 2 conidia were added per reaction. Nested PCR was conducted as
described previously.

Using PCR to screen Verticillium isolates. Purified DNA from mycelial extracts
of Verticillium isolates collected from wilting Ailanthus and other hosts were also
screened to determine if isolates were *V. albo-atrum* or *V. dahliae*. Isolates included *V. dahliae* isolates from *Ailanthus*, sugar maple, Norway maple (*Acer platanoides* L.), and Japanese maple (*Acer palmatum* Thunb.), as well as *V. albo-atrum* isolates from *Ailanthus* and striped maple. Nested PCR was conducted as described previously to ensure that the primer sets were effective to detect each *Verticillium* species.

**Evaluation of various aspects of the disease cycle.** A general disease cycle of *Verticillium* wilt disease cycle was presented in Fig. A19. Our studies in this chapter addressed the following parts of the disease cycle: survival (overwintering), dissemination (leaves, seeds, beetles), inoculation and infection (including wounding), and colonization. In addition, portions of the disease cycle that were not studied for lack of time are discussed.

**Survival**

**Within *Ailanthus***. Several large *Ailanthus* trees within the naturally infested study area were monitored to evaluate the possibility of *V. albo-atrum* surviving (overwintering) within the trees. Several lightly diseased *Ailanthus* trees were located during spring and summer 2006. Wood chips, twigs, and leaves were sampled from symptomatic trees that were still alive in the spring of 2006 to detect overwintering *Verticillium* within *Ailanthus* trees.

**In non-*Ailanthus* hosts.** Results from permanent plots (Fig. A13), as well as field and greenhouse inoculations (Chapter III), involving non-*Ailanthus* species were evaluated to determine the potential for *V. albo-atrum* to survive in non-*Ailanthus* seedlings and trees.
In soils. In 2006 a survey was conducted within three sub-areas of the study area (Chapter III). Based on the relative amount of *Ailanthus* disease, the sub areas are designated as: control (non-diseased), moderate disease incidence, and high disease incidence sub-areas (Fig. A14). Twelve established sample plots (Fig. A14) were to evaluate survival of *Verticillium* in soils. At each plot center, three soil samples were taken and composited for an assay of microsclerotia or melanized hyphae. Soil samples were air-dried for 2 weeks, and wet-sieved through 125-um sieve, and then through a 37-um sieve (Huisman and Ashworth, 1974). Material on the 37-um sieve was washed with sterile-distilled water into a 50-ml centrifuge tube (Sarstedt Inc., Newton, NC) and spun at 3200 rpm for 5 min. Supernatant was removed and solid materials separated further through density floatation (Huisman and Ashworth 1972) using a solution of 70% sucrose (wt/wt) constructed by dissolving sucrose in sterile-distilled water. Sucrose suspensions were cooled under constant stirring until temperature dropped to 40°C. Approximately 40 ml sucrose solution was added to each 50-ml centrifuge tube containing the spun down sample, and supernatant resuspended in the sucrose solution. Tubes were centrifuged at 2900 rpm for 15 min. Supernatant, containing the microsclerotia and melanized hyphae, was collected and 15 ml sucrose solution placed into three 50-ml centrifuge tubes. Approximately 30 ml of sterile distilled water was added to each tube and supernatant resuspended. Tubes were spun a final time at 3200 rpm for 10 min. Supernatant was discarded and solid materials resuspended into 10 ml sterile distilled water. The resulting suspensions were plated onto a pectate substrate agar that is selective for *Verticillium* (Huisman and Ashworth 1974) in 3-ml portions and incubated for 11 days at 21C. Black colonies, representative of *Verticillium* on this selective agar, were observed and counted.
To confirm that the black colonies were *Verticillium* spp., subcultures were made onto PEA (Bejarano-Alcazar et al. 1996). Morphology of colonies on PEA was examined, to confirm identification of *Verticillium*.

Excess soil samples from each sub-area were composited and analyzed for pH, exchangeable aluminum, nitrogen, phosphate, potash, magnesium, and calcium levels by Penn State Agricultural Analytical Services Laboratory. Data from the three samples were pooled and average levels reported.

**Dissemination**

**Rate of spread.** Tree-ring analysis (dendrochronology), was used to determine the initial year of the wilt epidemic, temporal and spatial patterns of mortality development, and to relate *Ailanthus* tree diameter growth with disease development (McClenahen 1995, Stokes and Smiley 1996). To estimate year of initial *Ailanthus* establishment, tree-ring counts were also used to determine age of the oldest *Ailanthus* trees in the study area. To approximate general location of disease focus, and to determine annual disease progression, we projected a 61 x 61 m grid onto a stand map. In the field, we sampled *Ailanthus* (Fig. A20) trees at each projected intersection point. The *Ailanthus* tree (living or dead) nearest to the projected intersection point was felled and cross-section disks removed from the stem at 1.4 m. Samples were taken only at intersecting points with standing *Ailanthus* trees. Disks were air-dried and sanded with a progressively finer sequence of sandpapers (80, 120, 180, 240 grit) to enhance visualization of annual ring boundaries (Fig. A21).

Age of living and dead trees was determined by counting the annual rings on each disk from the outer bark to the pith. Age of the largest, living diameter trees was used to
approximate initial year of *Ailanthus* establishment. We hypothesized this year would be approximately 1985, immediately following the salvage operation, that would have further opened the stand and scarified the soil, enhancing *Ailanthus* seed germination and seedling establishment. Year of death was used to approximate advancement of *V. albo-atrum*-induced mortality throughout our study area, by producing mortality-based maps using ArcMAP v. 9.2 (ESRI, Redlands, CA).

Growth rate of all trees was determined from the sample disks. The annual growth ring widths were measured with a Velmex (East Bloomfield, NY) measuring device. Tree ring data was organized using MeasureJ2X (Voor Tech Consulting, Holderness, NH). Although the tree-ring chronologies involved very few years, they were evaluated using the quality control program COFECHA to verify that there were no missing or extraneous rings (Grissino-mayer 2001, Holmes 1983). Visual comparisons were made between growth rates of *Ailanthus* trees living at time of felling vs. those dead at time of felling, in order to estimate effect of *Verticillium* on tree growth prior to death, and also to determine if trees that had died were growing at a naturally slower rate prior to death and were possibly predisposed.

The rate of disease spread was also assessed among two inoculated dense stands of *A. altissima*, to gain perspective regarding the temporal and spatial dynamics of *Verticillium* wilt. Five *Ailanthus* trees in the dense stands were inoculated in each stand with *V. albo-atrum* (PSU 140, GenBank accession # FJ424082) on 4 May 2007. Azimuths and distances from plot center to each *Ailanthus* tree in the stand, inoculated or non-inoculated, were recorded using a compass and 50-m tape. On 9 June 2008, all
Ailanthus trees in each plot were rated on a 0 to 2 scale, where 0 = healthy, 1 = wilting, 2 = dead. A plot map was developed using ArcMap v. 9.2 (ESRI, Redlands, CA).

**Dissemination in leaves.** In the greenhouse, 10 potted Ailanthus seedlings were stem-inoculated with *V. albo-atrum* (PSU 140) and 10 seedlings inoculated with *V. dahliae* (PSU 154) on 1 June 2006. On 9 August 2006, symptomatic leaves with attached leaflets were removed from the seedlings. A 1x1 cm section was excised from the center of symptomatic leaflets, and 1 cm sections removed from leaf raches. Samples were flame-sterilized and plated onto PEA (Bejarano-Alcazar et al. 1996) to evaluate the presence of *Verticillium* within diseased leaves.

**Dissemination in seeds.** In July 2006 and 2007 seeds were collected from naturally infected canopy trees exhibiting *Verticillium* wilt symptoms. Leaves adjacent to the seed clusters on symptomatic trees were collected and cultured onto PEA. Initial identification of *V. albo-atrum* or *V. dahliae* was determined using culture morphology. Upon putative identification of *V. albo-atrum* or *V. dahliae* infection within the seed trees, 160 seeds from trees infected with *V. albo-atrum*, and 160 seeds from trees infected with *V. dahliae*, were placed individually into 1.5 ml microcentrifuge tubes, lyophylzed, and stored at -12C until DNA extraction. From each set of 160 seeds, 50 were selected for DNA extraction and *Verticillium* molecular detection. All positive PCR products from nested PCR were sequenced and analyzed to confirm that gel bands were due to *V. albo-atrum* or *V. dahliae* DNA. Positive nested PCR reactions were purified using a ExoSAP-IT® (USB Corp., Cleveland, Ohio). Purified amplification products were sequenced using the Applied Biosystems (ABI) BigDye v. 3.0 terminator kit and an ABI 377 automated DNA sequencer (Perkin-Elmer, Foster City, CA) (Geml et al. 2005).
Consensus sequence data was assembled and edited using Sequencher 3.1 (Gene Codes, Ann Arbor, MI) from both forward and reverse DNA sequences (Geml et al. 2005). Consensus sequences were placed into NCBI’s BLASTN (National Center for Biotechnology Information, Bethesda, MD), to aid molecular identification (Geiser et al. 2005).

**Dissemination by ambrosial beetles.**

**Ambrosia beetle species identification.** The most common insects associated with wilting *Ailanthus* trees in the study area were unknown species of ambrosial beetles. Fifty beetles were collected from six wilting or dead *Ailanthus* trees and placed into glass vials containing 70% ethanol. Observed beetles were associated with tunnels or entrapped in sticky *Ailanthus* sap. Beetles (Figs. A22, A23) were sent to Dr. Robert J. Rabaglia, USDA Forest Service, Washington D.C., for identification.

**Frequency of beetle species.** On 18 June 2008 a field survey was conducted within a stand with severe *V. albo-astrum* wilt to determine beetle frequency on *Ailanthus* trees. Different species of ambrosial beetles produce different size holes in the tree stem. Numbers of ambrosial beetle holes of specific size diameter classes (0.75, 1.0, 1.5, 2.0-mm) were counted within a 15 x 15 cm square placed at 1.37 m height on the north side of stems of 15 dead or dying *Ailanthus* trees. Hole diameters were measured with a dial caliper (Battenfeld Technologies, Columbia, MO), number of ambrosial brood-chamber holes were counted, and data recorded. Twenty beetles associated with most common beetle hole size class (2 mm) were collected for identification.

**Non-Verticillium fungi associated with ambrosial beetles.** *Euwallacea validus* (Eichhoff) was the most common ambrosial beetle on *V. albo-astrum* infected trees (see
results, Dr. Robert Rabaglia, personal communication). Since beetle fungal symbionts are capable of causing wilts in trees (Fraedrich et al. 2008), we attempted to identify the fungi associated with *E. validus* using three methods. First, *Ailanthus* stem sections infested with 2-mm ambrosial beetle holes were collected and placed in a moist chamber. Fungi growing out of the ambrosial beetle holes, externally on the stem (Fig. A24), were single-spore cultured onto potato dextrose agar (PDA), then molecularly identified as described in chapter II using BLASTN (National Center for Biotechnology Information, Bethesda, MD). Secondly, fungi lining 12 beetle holes (Fig. A25), possibly the symbiont, were sampled. Fungi from beetle holes were cultured onto PDA and identified using sequencing of ITS gene and BLASTN comparisons. Thirdly, *E. validus* beetles were surface-sterilized for 30 sec in 70% ethanol, placed in a watch glass and ethanol allowed to evaporate. Eighteen beetles, still alive, were placed onto petri plates of PDA and allowed to walk on the agar surface for 5 min. Beetles were removed and resultant cultures on the PDA plates identified, following a 7-day incubation, using BLASTN.

*Verticillium albo-atrum* on or in ambrosial beetles. *Verticillium albo-atrum* species associated with *E. validus* beetles were evaluated using isolation onto PEA (Begarano-Alcazar et al. 1996) and nested PCR detection. Thirty male *E. validus* beetles were collected from the surface of the bolt of *Ailanthus* infected with *V. albo-atrum*, washed for 1 min in 70% ethanol, placed into a sterile 1.5 ml microcentrifuge tube containing 70% ethanol (1 ml) and vortexed for 1 min. Beetles were placed in a sterile watch glass to allow evaporation of ethanol and placed onto PEA culture plates. Beetles, still alive, walked on the plates for 15 min (Fig. A26) and were removed. Culture plates were placed within controlled environment chambers in the dark at 22C and observed
daily for *Verticillium* colonies. In addition, 50 *E. validus* beetles were collected from stem sections infected with *V. albo-atrum* and placed individually into 1.5 ml microcentrifuge tubes, lyophilized, and stored at -12C. Beetles were ground in a microcentrifuge tube with a microcentrifuge pestle, DNA extracted and a nested PCR procedure performed. All positive PCR products from the nested PCR were sequenced and analyzed. Positive reactions were purified using ExoSAP-IT® (USB Corp., Cleveland, Ohio) and purified amplification products sequenced using Applied Biosystems (ABI) BigDye v. 3.0 terminator kit and an ABI 377 automated DNA sequencer (Perkin-Elmer, Foster City, CA) (Geml et al. 2005). Consensus sequence data was assembled and edited using Sequencher 3.1 (Gene Codes, Ann Arbor, MI) from both forward and reverse DNA sequences. Consensus sequences were placed into GenBank BLAST to aid molecular identification (Geiser et al. 2005) of *V. albo-atrum* obtained from in or on the beetles.

**Inoculation and infection**

**Root wounding.** Since root wounding is known to increase the incidence of some *Verticillium* diseases (Pegg and Brady, 2002), we conducted wounding vs. non-wounding inoculation experiments using both *V. albo-atrum* (PSU 140, GenBank accession # FJ424082) and *V. dahliae* (PSU 154, GenBank accession # FJ424083). In 2006, 128 1-year-old *Ailanthus* seedlings were grown in a greenhouse in 6x25 cm tubes. Seedlings transferred to 11x15 cm pots, grown in MetroMix 200 (The Scotts Company, Marysville, OH) soil mix, and allowed to grow for 18 days to heal of any roots wounds incurred during transfer.
Seedlings were wounded and/or inoculated using a randomized complete block design involving six treatments. Treatments were 1) roots wounded followed by *V. albo-atrum* inoculation, 2) roots not wounded followed by *V. albo-atrum* soil drench inoculation, 3) roots wounded followed by *V. dahliae* inoculation, 4) roots not wounded followed by *V. dahliae* inoculation, 5) roots wounded and non-inoculated, and 6) roots not wounded and non-inoculated.

Twenty-four hours prior to treatment, pots were watered to saturation. For wounding treatments, roots were wounded by inserting a 15-cm soil knife once into the potting mix of each pot. For inoculation treatments, a 20-mL spore suspension, adjusted to $10^7$ conidia/ml (Pastor-Corrales et al. 1987) of *V. albo-atrum* or *V. dahliae*, was poured onto the potting mix in each pot. Pots containing wounded and non-wounded control plants were treated with sterile distilled water. Thirty minutes after inoculation, all plants were misted for 5 min to minimize spore desiccation in the potting mix of inoculated plants (Graner et al. 2003). The study was replicated 3 times.

Plant heights and symptom severity were measured weekly. Height (cm) was measured from surface of the potting mix to the apical bud. Symptoms were rated weekly on a 0 - 4 scale, where 0 = healthy plant, 1 = leaf chlorosis, 2 = leaf tip necrosis, 3 = leaf wilting, and 4 = leaf drop and plant death. Tissue samples were taken from all plants and cultured onto PEA to detect *Verticillium*. A disease severity index (DSI), ranging from 0 to 100 percent, was calculated as $DSI = (I \times S)/M$, where $I$ = percentage of diseased plants, $S$ = mean severity of foliar symptoms on diseased plants, and $M =$ maximum severity value ($M = 4$) (Bejarano-Alcazar et al. 1996). Parameters measured at the end of the growing season (week 15), were final disease severity rating (DIS) and
height growth since inoculation (HT). Treatments were coded as *V. albo-atrum* plus root wounding (VA+W), *V. albo-atrum* non-wounded roots (VA), *V. dahliae* plus root wounding (VD+W), *V. dahliae* and non-wounded roots (VD), control with wounding (C+W) and control with non-wounded roots (C). Data were analyzed using ANOVA and Tukey’s mean comparisons (p = 0.05) (Minitab 2007).

Soil nematodes. Soil samples were collected on 23 June 2005 from two locations: beneath wilting *Ailanthus* within the *V. albo-atrum* infested area and beneath healthy *Ailanthus* trees located approximately 150 m outside the infested area (Fig. A30). On 19 July 2006, two additional soil samples were collected within a healthy forest located approximately 2 km from the study area, where samples were collected under healthy *Ailanthus* trees and under healthy northern red oak trees. Soil samples were taken to a maximum depth of 10 cm with a soil sampling tube, composited, and placed in an insulated chest. Samples were transported to the Penn State Fruit Research and Extension Center, Biglerville, for identification and quantification by Dr. John Haldbrent.

Colonization

Colonization was evaluated using histological examination of inoculated, stem sections of *Ailanthus* seedlings. On 22 May 2008, *Ailanthus* seedlings were stem-inoculated with 0.1ml of 10^7 conidia ml\(^{-1}\) of *V. albo-atrum* (PSU 140, GenBank accession # FJ424082) using a hypodermic needle. At 1, 2, and 4 weeks following inoculation, plants were sampled at the site of inoculation and at 2.5-cm intervals on the stem above and below the inoculation point. At each point, 5 mm thick cross-sections were removed and fixed in 3% glutaraldehyde in 0.025 M sodium phosphate buffer (SPB), pH 7.4, and maintained at 4C for 32 hr. Samples were washed in SPB twice and dehydrated using the
following ethanol gradient: 25% ethanol for 40 min, 50% for 40 min, 70% for 1 hr, 80% for 30 min, 95% for 30 min, 95% for 8 hrs, 100% for 1 hr, and 100% for 30 min. Samples were placed in Histosolve (Thermo Shandon Inc., Pittsburgh, PA) for three 1-hr periods, followed by paraffin for two 4-hr periods. Samples were soaked in a glycerin softening solution (96% alcohol:water:glycerin, 1:1:3 by vol.) for 3 hr (Schoch et al. 2004). Tissue samples were embedded in paraffin and sectioned with a Shandon Finesse paraffin microtome (Thermo Electron Corporation, Waltham, Massachusetts) (Xi and Burnett 1997). Sections were stained with Chloraxol black E (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) in lactoglycerol (lactic acid:glycerol:water, 1:1:1 by vol.) (Chen et al. 2004, Heinz et al. 1998, Newcombe and Robb 1989). Sections were placed into 3 changes of xylene substitute (5 min each), distilled water wash (5 min), chlorazol black E in lactoglycerol (3 hr), 95% ethanol (5 min), 100% ethanol (5 min), and 3 additional changes of xylene substitute (5 min each). Stained sections were mounted in Shandon xylene substitute mountant (Thermo Electron Corp. Pittsburgh, PA) and observed using a Olympus BX51 fluorescence light microscope (Olympus Imaging America Inc., Center Valley, PA). Cross and radial sections of Ailanthus seedling stems were observed for \( V. \) albo-atrum hyphae, and conidia, as well as host tyloses, within vessel elements.

**RESULTS**

**Detection of Verticillium using nested PCR.**

Primers FVD and RVD, developed by Karajeh and Masoud (2006), Karajeh (2006), and Masoud (2002) were modified resulting in FVAL6 and RVA for \( V. \) albo-atrum, and modified forward primer FVD (Masoud 2002) to FVAL5 for use with reverse
primer RVD in assays of *V. dahliae*. Final primers developed and used are shown in Table 1.

For *V. albo-atrum* primer set FVAL6/RVA, the optimal PCR amplification was determined to be at primer concentrations of 1mM, 2.5mM MgCl₂, and an annealing temperature of 61°C (Fig. 3). For *V. dahliae* primer set FVDA5/RVD optimal PCR amplification was at primer concentrations of 10 mM, 2.5mM MgCl₂, and an annealing temperature of 64 °C (Fig. 4). To increase sensitivity, in addition to the end of the 30-cycle protocol at optimum Ta, 10 additional cycles were added in which the annealing temperatures were 3 °C lower than optimized Ta (“touchdown PCR”) to the second round of the nested-PCR.

Primer sets were tested using varying concentrations of conidia (2x10⁴, 2x10³, 200, 20, and 2 conidia/μl) as DNA template in the PCR reactions (Karajeh 2006). After the second amplification with FVAL6/RVA and FVDA5/RVD, DNA fragments of the correct size were observed in each positive reaction (Fig. 5). After the first round using NESF18S/NESR28S, a faint band was observed, but only at a conidial concentration of 2x10⁴/μl. These results revealed that our nested PCR primers adapted from Karajeh (2006) were sensitive and could detect conidia concentrations as low as 2 conidia/μl.

To ensure that primers developed for *V. albo-atrum* and *V. dahliae* could detect more than one isolate of each *Verticillium* species, primers were tested with pure fungal DNA extractions from our isolate library of 14 *V. albo-atrum* isolates and 8 *V. dahliae* isolates which were all collected from Pennsylvania. As expected, the first round of nested PCR amplified all isolates. The second round produced optimum results for both
primer sets FVAL6/RVA and FVDA5/RVD. Both second-round primer sets amplified only DNA of the intended *Verticillium* species (*V. albo-atrum* or *V. dahliae*) and both second-round primer sets amplified all isolates of each species (Fig. 6) (Karahjeh 2006).

**Survival (overwintering)**

*Within Ailanthus trees.* We have evidence that *V. dahliae* can overwinter within infected *Ailanthus* trees, since trees inoculated with *V. dahliae* were still alive and symptomatic a year after inoculation (Chapter II).

Two lines of evidence indicate that *V. albo-atrum* can also overwinter in infected trees. First, naturally infected *Ailanthus* trees with mild symptoms in late August 2006, were sampled and *V. albo-atrum* was detected. In the following spring in 2007, the same *Ailanthus* trees were still alive, produced leaves, and began to decline. Second, several naturally infected *Ailanthus* trees with mild symptoms during summer 2006 (Fig. A4) were still alive in the spring of 2007. However, we did not isolate from these trees. Nevertheless, symptom characteristics and location indicated that *V. albo-atrum* was the pathogen involved. Thus it is likely that *Verticillium* can survive within infected *Ailanthus* trees for one or more winters, depending on which *Verticillium* species is involved. In addition to surviving in *Ailanthus* xylem, *Verticillium* can also survive within fallen leaves from infected trees.

Tree species other than *Ailanthus altissima* may also serve as overwintering reservoirs of *Verticillium* (Fig. 1, 2). Our results (chapter III) implicate only striped maple as possible overwintering host. Stem injection inoculation of *V. albo-atrum* into striped maple resulted in mortality, but isolations from naturally infected wilting striped maple trees in the field recovered only *V. albo-atrum* at a very low rate.
Soils could be another major site of survival. However, *Verticillium* spp. were not recovered using soil plating assays. All subcultures of fungi with microsclerotia-like morphology were neither *V. albo-atrum* nor *V. dahliae*, as determined by on cultural morphology on PEA. Based on these measurements *Verticillium* soil inoculum appears to be extremely low in the study area, even within severely infested portions of the stand.

**Dissemination**

**Patterns of spread.** Patterns of spread were evaluated using two methods. A third method was established, but data will not be taken until 2009. First, dendrochronology was used to evaluate temporal and spatial patterns of the naturally occurring *Ailanthus* wilt epidemic, based on mortality (Fig. 8). The natural epidemic within the forest of the main study area apparently originated at a single location. During the initial 3 years (2000 to 2002) of the epidemic, mortality was limited to approximately 2.19 ha, and expanded linearly at a rate of 27.8 m/yr. Since then, there has been a significant increase in mortality both downwind (to the northeast) and upwind (to the southwest) (Fig. 9). From 2003 to 2007, the average spread of mortality was approximately 116.05 m/yr to the northeast, and approximately 64.56 m/yr to the southwest. The pattern of downwind spread following establishment from 2003 to 2007 was significantly linear (p=0.011, R²=91.4%). The downwind model developed to illustrate rate of mortality was M = 23.4(yr), where M = mortality spread in m downwind and yr = years after introduction. The upwind (to the southwest) pattern of spread followed a polynomial trend, but was not significant (p=0.132).

Second, artificial inoculation within two small stands also illustrated rapid disease spread. In one small stand containing only 39 *Ailanthus* trees, there was a
dramatic increase in disease within 1 year following inoculation of 5 trees with \textit{V. albo-atrum}. Approximately 94.9\% of the non-inoculated trees in the stand were symptomatic (33.3\% dead, 61.5\% wilting) within 1 year (Fig. 10, 11). In the second small stand containing 95 \textit{Ailanthus} trees, 86.3\% of the non-inoculated trees were symptomatic in 1 year (76.3\% dead and 9.5\% wilting) (Fig. 10, 12). The most severe disease symptoms in both stands were near inoculated trees.

Third, 45 canopy \textit{Ailanthus} trees have been inoculated with \textit{V. albo-atrum} isolate PSU 140, within 9 forest stands in south-central Pennsylvania. Spread from inoculated \textit{Ailanthus} trees will be evident by summer 2009. GPS coordinates of all inoculated stands have been established. Patterns of spread from inoculated trees should be evaluated in 2009 using remote sensing and digital images taken from satellite or fixed-wing aircraft. Remote sensing offers a much more efficient way to monitor annual changes in disease and mortality development within the rugged terrain of south-central Pennsylvania.

\textbf{Agents of dissemination.} Potential agents of dissemination of \textit{Verticillium} that were studied included infected leaves, infected or infested seeds, and insects. Root grafts are also likely involved, but were not studied. \textit{Verticillium} was isolated from leaves on all 10 greenhouse seedlings inoculated with \textit{V. albo-atrum} (PSU 140), as well as from all 10 plants inoculated with \textit{V. dahliae} (PSU 154). \textit{Verticillium} was recovered during the same seedlings as the \textit{Ailanthus} seedlings were inoculated, and developed typical \textit{Verticillium} symptoms.

\textit{Verticillium} was not isolated or identified, using culture morphology from seeds collected from trees naturally infected with \textit{V. albo-atrum} or \textit{V. dahliae}, due to fungal contamination in most seed isolations. However, using nested PCR products and
sequencing, associations from naturally infected *Ailanthus* seeds were confirmed to be *V. albo-atrum* or *V. dahliae*. *Verticillium albo-atrum* was detected on or in approximately 12% of sampled seeds (Fig. 13), and *V. dahliae* was found on or in approximately 6% of sampled seeds (Fig. 14).

**Dissemination by insects.**

The most common insect associated with dead and dying *Ailanthus* trees in the study area were ambrosial beetles. Ambrosial beetles were collected from two stands. The non-native *Euwallacea validus* (Eichhoff) (Figs. 15, A22) was most common in one stand, whereas the non-native *Xylosandrus germanus* (Blandford) (Haack 2001) (Fig. A23) was most common in the second stand. Throughout the entire study area, *Euwallacea validus* was most common, comprising approximately 98.2% of all ambrosial beetles collected (Fig. 15). All beetles collected from 2-mm diameter beetle holes were *E. validus*.

Our counts indicate that *E. validus* produced nearly a thousand ambrosial brood entry holes/m² stem surface area on *Ailanthus* trees in the severely infested study area, assuming all 2-mm diameter holes were caused by *E. validus*. Beetle holes <2 mm diameter were difficult to sample and assess for beetle species due to their infrequency. Only one 0.75-mm diameter hole and one 1.0-mm diameter hole was recorded. Four 1.5-mm diameter holes were recorded. If hole diameter is indicative of ambrosial beetle species, two or three other species of ambrosial beetles may be lightly infesting *Ailanthus* trees in the study area. Due to the low incidence of 1.5, 1.0, 0.75-mm diameter holes, no ambrosial beetle samples were collected for identification other than from the 2-mm diameter holes.
The fungus most commonly associated, 72% (16 isolates), with *E. validus* in our study area was *Fusarium solani* (D. Geiser, personal communication). This *Fusarium* was isolated from brood chambers, as well as on ambrosial beetle exoskeletons.

The initial beetle wash experiments, in which beetles were washed in sterile distilled water and plated onto PEA, were designed to recover *Verticillium*. However, the main organisms cultured were fast-growing fungal contaminates such as *Fusarium*, *Aspergillus*, and *Penicillium*. These fungi may have obscured any *Verticillium* colonies that developed on the plates. Additional studies wherein the beetles were surface sterilized with 70% ethanol and plated onto PEA, yielded two colonies on 21 plates that had colony morphology resembling *Verticillium*. Subcultures from these produced melanized hyphae, indicative of *V. albo-atrum*, indicating that small amounts of viable *V. albo-atrum* propagules are present on beetles.

However, the more sensitive nested PCR analysis revealed *V. albo-atrum* on or in 28% of *E. validus* beetles collected throughout the study area (Fig. 16).

**Inoculation and infection**

*Verticillium* inoculation and infection were evaluated primarily using root wounding experiments on potted seedlings. Root wounding increased the incidence of wilt in seedlings inoculated with *V. albo-atrum*. Wounded seedlings began to show disease symptoms at week 4, whereas non-wounded seedlings did not exhibit symptoms until week 8 (Fig. 17). Non-wounded, inoculated seedlings wilted and died, indicating that *V. albo-atrum* penetrated non-wounded roots. However, wounding did not influence symptom expression on seedlings inoculated with *V. dahliae* (Fig. 17).
Statistical analysis was conducted at the end (15 weeks) of the wounding experiments. Mean treatment values are presented in table 4. ANOVA tables and Tukey’s multiple comparison data are presented in Appendices B and C. Data among replicates were not significantly different and were combined (n=27). Final height increase (HT) data were not normally distributed and were normalized using square root transformations. The ANOVA tables revealed that significant differences exist among treatments for disease (DIS) and height increase (HT).

Tukey’s multiple comparison test revealed location of significant differences. Wounding, followed by inoculation with \textit{V. albo-atrum}, caused a significant 85.8\% increase in disease severity index, and a significant 71.1\% decrease in average height increase (HT), as compared to non-wounded, inoculated seedlings (Figs. 17, 18). However, wounding was not necessary for \textit{V. albo-atrum} to cause disease. \textit{Verticillium albo-atrum} treatments without wounding (NW) caused a significant 73.5\% increase in disease severity index as compared to the non-inoculated, non-wounded seedlings.

There was a 35.9 \% decrease in HT between \textit{V. albo-atrum} NW treatments and wounded controls, but the difference was not significant (p = 0.1745). The non-wounded controls versus the \textit{V. albo-atrum} W treatments showed a 92.3\% increase in disease severity index, and a 77.5\% decrease in HT. \textit{Verticillium albo-atrum} NW treatments showed a 80.0\% increase and severity index in addition to a 50.2\% decrease in HT (Fig. 17, 18). Among the \textit{V. albo-atrum} NW treatments and \textit{V. albo-atrum} W treatments, there was a significant difference in HT among treatments, with \textit{V. albo-atrum} W treatments showing a 54.9\% decrease in height compared to \textit{V. albo-atrum} NW treatments.
Ailanthus seedlings inoculated with *V. albo-atrum* exhibited significantly more disease than any other treatment (Fig. 17). Wounded seedlings, followed by inoculation with *V. albo-atrum*, developed disease more quickly than did non-wounded seedlings (Fig. 17). Most seedlings inoculated with *V. albo-atrum* were dead by week 15.

Wounded or non-wounded *Ailanthus* seedlings inoculated with *V. dahliae* showed no significant difference in final disease severity rating (DIS) or HT as compared to both control W and control NW treatments. Most seedlings inoculated with *V. dahliae* were still alive at the end of the growing season, and remained so until the following growing season.

Seedlings inoculated with *V. albo-atrum* exhibited significantly less height growth at 15 weeks as compared to the other four treatments (Fig. 18). Seedlings wounded prior to inoculation with *V. albo-atrum* showed significantly less height growth than non-wounded seedlings inoculated with *V. albo-atrum*. Height growth of seedlings inoculated with *V. dahliae* was not significantly different from to that of controls, whether wounded or not.

**Soil nematodes.** We found no *Pratylenchus* nematodes in our soil samples. However, very high numbers of dagger nematodes (*Xiphenema* spp.) occurred in soil samples taken beneath wilting *Ailanthus* trees (Table 3, Fig. A30). *Xiphenema* numbers (219.5/100 cc soil) were greatest at the edge of the wilting epidemic. Samples taken under non-wilting *Ailanthus* trees immediately outside of the epidemic area had fewer (89.5/100 cc soil) *Xiphenema* nematodes. No *Xiphenema* spp. was recovered from soils under northern red oaks outside the infested area.
**Colonization.**

Colonization was evaluated by using histology to observe hyphal development in xylem vessel elements within _Ailanthus_ seedlings inoculated with _V. albo-atrum_. _Ailanthus_ xylem vessel elements ranged in dia from 25-60 um, and dia of bordered pits within xylem vessels ranged from 2-5 um.

Within 1 week following inoculation, _V. albo-atrum_ had colonized the entire circumference of the outer xylem at the point of inoculation, primarily in the largest vessels (Fig. A27). Hyphae were visible in outer xylem vessels, and xylem parenchyma cells were stained dark with phenolic deposits, indicating some infection in and surrounding those cells (Heinz et al. 1998). At this time, fungal colonization was also visible in vessels 20 cm apically (upward, with the xylem flow) from point of inoculation, but only 5 cm basally (downward, against xylem flow).

By 2 weeks following inoculation, _Verticillium_ hyphae had colonized to the plant apex (10 cm from point of inoculation) but had progressed no farther downward, remaining at 5 cm below inoculation point. However, colonization at this time appeared to be progressing into the smaller xylem vessels. By 3 weeks after inoculation, the inoculated seedling began to wilt and defoliate.

At 4 weeks following inoculation, the plant was in severe decline, showing 100% wilting and 70% defoliation, and began to die. At this point, the large vessels had some degree of fungal colonization (Fig. 19, A27), and _V. albo-atrum_ hyphae were observed in vessels 10 cm below the inoculation point. During the 4 week period _V. albo-atrum_ hyphae were spreading at the rate of 3.57 mm/day downward and 7.14 mm/day upward.
DISCUSSION

Survival

Within *Ailanthus* trees. Occasional *Ailanthus* trees infected with *V. albo-astrum*, and many trees infected with *V. dahliae*, survived the winter and produced leaves during the following spring. This observation reveals that both species of *Verticillium* can overwinter within infected *Ailanthus* trees, as reported for other perennial hosts (Fig. A4) (Fradin and Thomma 2006).

Within non-*Ailanthus*. Weed hosts have been documented to serve as reservoirs for *Verticillium* species, including resting structures, even if the weed hosts remain symptomless (Pegg and Brady 2002, Vallad 2005). Among understory weed species that commonly grow among dead and dying *Ailanthus* trees in our study area are American pokeweed (*Phytolacca americana* L.), common blackberry (*Rubus allegheniensis* Porter ex L.H.Bailey), garlic mustard (*Alliaria petiolata* Bieb.), hay scented fern (*Dennstaedtia punctilobula* Michx.), nettle (*Urtica* spp.), Pennsylvania smartweed (*Polygonum pennsylvanicum* L.), and Virginia creeper (*Parthenocissus quinquefolia* L.). Of these understory plants, blackberry, nettle, and Pennsylvania smartweed are potential hosts or symptomless carriers of *Verticillium* (Pegg and Brady 2002, Vallad 2005). In addition, Japanese creeper (*Parthenocissus tricuspidata* (Siebold & Zucc.) Planch.) which is in the same genus as Virginia creeper, is a known host for *V. dahliae* (Pegg and Brady, 2002). Thus, weed hosts could assist in survival of *V. albo-astrum* in forest ecosystems of south-central Pennsylvania. In addition, other cohort tree species such as striped maple may be susceptible to *V. albo-astrum* (Fig. 1, Chapter III), or may be symptomless carriers. Species apparently tolerant to *Verticillium*, based on visual symptoms, can be colonized
systemically and serve as reservoirs of inoculum (Pennypacker et al. 1985, Robb 2007). Additional research is needed to determine susceptibility of striped maple in the field, and presence of this and other symptomless carriers in forest stands that may aid persistence of *V. albo-atrum*.

**Soil.** We did not recover *Verticillium* from any soil samples collected in our study area. Survival of *Verticillium* species outside of host plants usually occurs as microsclerotia (*V. dahliae*) and melanized hyphae (*V. albo-atrum*) (Fig. A6) in the soil (DeVay and Pullman 1984). Soil is a complicating factor in our study area, with regard to survival of *Verticillium* in the soil. Soil characteristics of our study area, such as low pH (4.16) and high extractable aluminum (1011 μg/g) may be related to the death of *Verticillium* propagules in the soils. Baard and Pauer (1982) and Guba (1934) reported that a soil pH < 5.5 reduced microsclerotial production and survival of *V. dahliae*. Soluble aluminum has been reported to cause decreased pathogenicity, pathogen occurrence, and pigment development of *Verticillium* resting structures (Orellana et al. 1974). Soluble aluminum concentrations increase exponentially as pH in forest soils decrease < pH 5 (Reuss et al. 1990, Mulder et al. 1989). Soluble aluminum concentrations, as well as the very low pH, in soils of our study area may have a detrimental effect on *V. albo-atrum* resting structures and may help explain our inability to recover *Verticillium* resting structures in the preliminary soil plating assay.

Once incorporated into the soil, *Verticillium* propagules and hyphae may spread in various ways. Otten and Gilligan (2006) concluded that soilborne fungi spread predominantly through air-filled soil pores, and the most rapid spread occurs in coarse soil media, due to their larger pore sizes. *Verticillium* wilts have been shown to be more
severe in loam soils, similar to the sandy loams in our study area. *Verticillium albo-atrum* and *V. dahliae* are not strong soil residents and grow only a few mm from their germinated resting structures. They rely primarily on host roots growing towards the resting structures, rather than mycelium growing to the host roots (Devay and Pullman 1984). The sandy loam soils of the study area have uncompacted pores that are conducive to plant root elongation, as well as rapid spread of soilborne fungal pathogens (other than *Verticillium*) (Bengough et al. 2006).

Inoculum densities of *V. albo-atrum* melanized hyphae needed to initiate disease on *Ailanthus* are unknown. However an inoculum density of *V. albo-atrum* of approximately 17 and 23 propagules/g of soil are necessary to cause significant yield reductions in potato fields (Nnodu and Harrison 1979). Since we recovered no *Verticillium*, we were not able to calculate inoculum densities within our study area.

Concentrations of other elements in our soil samples were nitrogen 0.82%, phosphate 455 kg/ha, potash 439 kg/ha, magnesium 186 kg/ha, and calcium 1506 kg/ha. However, it is not known if these levels relate to the low levels of *Verticillium* propagules recovered in our assay.

**Dissemination.**

**Leaves and Leaflets.** *Verticillium* spp. can be disseminated in plant materials via movement of resting structures, such as microsclerotia or melanized hyphae, within infected leaves. Defoliated infected petioles and leaves have been reported as an important means of transmission in *V. dahliae* diseases of ash, Norway maple, and yellow-poplar (Pegg and Brady 2002, Rijkers 1992, Morehart and Melchior 1982, Heimstra 1997). Control of *Verticillium* infected leaves is a recognized integrated pest
management approach to reduce inoculum of *Verticillium dahliae* in olive orchards (Tjamos 2008). Petioles and aerial stems contributed to microsclerotial production in potato-*V. dahliae* pathosystems (Mol and Scholte 1995). Since we found both *V. albo-astrum* and *V. dahliae* in petioles and leaflets of *Ailanthus*, it is likely that resting structures formed in *Ailanthus* leaf materials provide inoculum contributing to localized spread of both pathogens in forests of south-central Pennsylvania. However, Gomez and Canham (2008) studied spatial variation in *Ailanthus* leaf litterfall biomass, and found that annual, modal distance of leaf litter deposition was only 3.28 m downwind and 1.28 m upwind from *Ailanthus* tree stems. Leaf litter deposition seldom occurred beyond 25 m from the tree. We found the spread of *V. albo-astrum* from 2003-2007 to be approximately 116 m/yr downwind and 64 m/yr upwind. These longer distances, although measured for four years, weaken the hypothesis that windblown infected leaves and leaflets are the major means of dissemination of *Verticillium* in *Ailanthus* (Fradin and Thomma 2006). Other means of dissemination are likely involved in spreading *Verticillium* to distances beyond that of windblown leaves.

**Seeds.** Many plant pathogens exist on or in seeds. Infected or infested host seeds ensure that a susceptible host is present upon germination of the host seed (Elmer 2001). Whether *Verticillium* is internal or external *Ailanthus* the seed, its presence provides a logical means of pathogen dispersal in areas containing *Ailanthus*. The light, winged *Ailanthus* seeds are readily dispersed to distances of 100 m or more upon falling from the tree (Landenberger et al. 2007). We found 12% of *Ailanthus* seeds from infected trees were either infested or infected with *V. albo-astrum*. We also found that 28% of *E. validus* ambrosial beetles within our study contained *V. albo-astrum*. Infected or infested seeds, or
beetles, could help explain the longer distances that \textit{V. albo-atrum} spread in a few short years. Seed dissemination of \textit{Verticillium} has been reported for several host plants. Percentage of seeds infested or infected by \textit{Verticillium} range from 0.3 to 84.8\% in spinach (du Toit et al 2005), 79\% in peanut (Pegg and Brady 2002), 66 to 90\% in lettuce (Vallad 2005), 0.7\% in cotton (Pegg and Brady, 2002), 2\% in alfalfa (Christen 1983), and up to 35\% in olive (Karajeh 2006). \textit{Verticillium} also has been associated with seeds of other hosts such as chickpea (\textit{Cicer arietinum} L.), safflower (\textit{Carthamus tinctorius} L.), \textit{Capsicum} spp., sunflower (\textit{Helianthus annuus} L.), and various weeds (Pegg and Brady 2002). In terms of infection (internal) or infestation (external) of seeds, it is simpler for a pathogen such as \textit{Verticillium} to be attached to the outside of a seed, as opposed to being incorporated into the reproductive seed tissues within seed, and thus seed infestation may be more common.

Our initial attempts to culture \textit{V. albo-atrum} and \textit{V. dahliae} on or in seeds collected from infected \textit{Ailanthus} trees were thwarted by contaminants. Therefore, we modified a nested PRC based assay, originally developed to detect of \textit{V. dahliae} in olive seeds (Karajeh 2006), for assessing presence of \textit{Verticillium} on or in \textit{Ailanthus} seeds. Percentage seed infestation by \textit{V. albo-atrum} (12\%) and \textit{V. dahliae} (6\%) confirmed the potential for dispersal and persistence of both \textit{V. albo-atrum} and \textit{V. dahliae} in or on \textit{Ailanthus} seeds. These percentages are significant when one considers that a single \textit{Ailanthus} tree is capable of producing more than 300,000 seeds annually (Miller 1990).

\textbf{Insects.} Approximately 28\% of \textit{E. validus} ambrosial beetles collected from declining \textit{Ailanthus} trees were infested with \textit{V. albo-atrum}. Ambrosial beetles could contribute to dissemination of \textit{V. albo-atrum} in \textit{Ailanthus} stands, by vectoring
Verticillium spores from diseased Ailanthus trees to healthy or slightly stressed trees. We calculated that a 25.4-cm dbh (10-inch) Ailanthus tree colonized by E. validus to a height of 4 m, could yield more than 100,000 adult E. validus (Batra 1963). If 28% of the adult beetles emerging from that single tree carried Verticillium, more than 30,000 beetles could vector V. albo-atrum. Our forest stands have hundreds, if not thousands, of dead Ailanthus trees infested with ambrosial beetles. Thus the potential exists for millions of ambrosial beetles to carry Verticillium within our study area. Since ambrosia beetles have been reported to disperse from several meters to nearly 50 (Byers 2000, Rundinsky 1962), infested beetles could vector V. albo-atrum from our infested study area to healthy stands of Ailanthus some distance away.

Primary dispersal of E. validus in our study area occurred in mid-May, when temperatures were 17 to 18°C. Likewise primary dispersal of Scolytidae beetles in South Carolina was reported occur primarily in early spring, when average temperatures reach approximately 15° C (Coyle et al. 2005). These early spring temperatures, apparently correlated with first beetle flight, are concurrent with Verticillium colonization (Johnson et al. 2000). Since temperature ranges for maximum beetle dispersal and Verticillium colonization are similar, this increases the potential for dissemination of Verticillium by ambrosial beetles.

In general, ambrosial beetles are considered to attack only dead, dying, or weakened trees. Ailanthus altissima trees weakened by mild primary pathogens, such as Armillaria or drought stress, could be attractive to E. validus ambrosia beetles carrying V. albo-atrum, resulting in new outbreaks of Verticillium wilt in relatively healthy stands of Ailanthus. However, some beetles can be primary instigator in forest outbreaks
Dr. Rabaglia (U.S. Forest Service, personal communication), stated that *E. validus* is an introduced, non-native ambrosial beetle that may not require a weakened *Ailanthus* host for infestations. If so, this could help explain rapid dissemination of *Verticillium* within our study area. The potential for ambrosial beetles to be the primary vectors of *Verticillium* deserves further study.

Vascular pathogens are closely associated with beetles in other pathosystems. Oak wilt (caused by *Ceratocystis fagacearum* (Bretz) Hunt) and Dutch elm disease (caused by *Ophiostoma ulmi* (Buisman) and *Ophiostoma novo-ulmi* (Moreau)) are two classic cases of wilt fungi vectored by bark beetles (Sinclair and Lyon 2005). In addition to ambrosial beetles have been shown to vector wilt diseases caused by their symbiont fungus (*Raffaelea* sp.) to the oak trees *Quercus serrata* (Thunb.) and *Q. crispula* (Blume), as well as redbay (*Persea borbonia* (L.) Spreng.) (Fraedrich 2008, Kuroda 2001). Wainhouse et al. (1998) implicated a bark beetle (*Cryphalus trypanus* Sampson) in passive transmission of the fungus *Leptographium calophylli* (Wiehe) that causes wilt of tamarack (*Calophyllum inophyllum* L.). This latter host-pathogen-insect system is similar to the potential ambrosia beetle-*Verticillium-Ailanthus* pathosystem within our study area.

The potential for insect transmission has been documented in other *Verticillium* pathosystems, including agricultural crops. Kalb and Millar (1986) reported that *V. albo-atrum* could be transmitted from diseased to healthy alfalfa plants by infested fungus gnats (*Bradysia impatiens* Johannsen) at a transmission rate of 31 to 41%. They also found that 79% of fungus gnats that fed on *V. albo-atrum* infected alfalfa plants were infested with *V. albo-atrum*. *Verticillium* can survive the digestive tracts of insects,
grasshoppers (*Melanoplus sanguinipes* Stal.), alfalfa weevil (*Hypera postica* Gyllenahl), wooly bears (*Apantesis blakei* Grote), and bulb mites (*Rhizoglyphus echinopus* Fumouze & Robin). As these vectors feed on *Verticillium* infected plant material, the pathogen can be dispersed within fecal pellets to new hosts (Huang and Harper 1985, Price 1985).

*Verticillium albo-atrum* can survive in grasshopper feces for 21 months and on aphid exoskeletons for up to 5 months at cool temperatures (15° C) (Harper et al. 1988). Infestations of the exoskeletons of leaf cutter bees and aphids have also been reported for *V. albo-atrum* and are another feasible means of dissemination for *Verticillium*, (Huang and Richards 1983, Huang et al. 1983). We have observed *Ailanthus* webworm (*Atteva punctella* Cramer) feeding on leaves of symptomatic *Ailanthus* trees in our study area. The potential exists for this webworm to vector *Verticillium*, and this possible vectoring system deserves future study.

**Spread.** One of our objectives was to identify spatial patterns of the epidemic within our study area. However, within two small stands, the epidemic expanded less than 20 m from inoculated trees until most *Ailanthus* trees were infected, and gave little insight into dissemination or pattern of the epidemic. The pathogen infected nearly all *Ailanthus* trees in these stands within 1 year. Although these epidemics did illustrate the virulence and aggressiveness of *V. albo-atrum* in dense *Ailanthus* stands, the only pattern appeared to be one of aggregation, with symptomatic trees initially restricted to the area near inoculated trees. This aggregated pattern has been observed with *V. dahliae* infections in olive, mint, and pepper (Navas-Cortes et al. 2008, Johnson et al. 2006, Bhat et al. 2003). Aggregation apparently is often related to a single point source of inoculum.

**Inoculation and Infection.**
**Wounding.** Results from stem-inoculation experiments proved that both *V. albo-atrum* and *V. dahliae* were pathogenic to *Ailanthus* seedlings and canopy trees. However, *V. albo-atrum* and *V. dahliae* are soil-borne pathogens that often infect through the roots (Fradin and Thomma 2006), and stem injection may have bypassed root defense mechanisms (Talboys 1972). Therefore, potted *Ailanthus* seedlings were inoculated using a soil drench of both *V. albo-atrum* and *V. dahliae* conidia. Seedlings inoculated by soil drench of *V. albo-atrum* developed symptoms approximately 4 weeks later than those inoculated using stem injections. The 4-week lag in disease symptom development and inhibition of height growth on non-wounded seedlings indicates that it takes *V. albo-atrum* approximately 4 weeks to overcome defenses in the root cortex and establish a vascular infection. However, when *V. dahliae* was used as a soil drench, this *Verticillium* species did not cause significant disease in *Ailanthus* seedlings, so the comparison between soil drench and stem inoculations using *V. dahliae* could not be made. Perhaps *V. dahliae* conidia did not survive well in the potting mix, inoculum density was not high enough (Nnodu and Harrison 1979, Xiao and Subbarao 1998), or intra- and extra-vascular root defenses compartmentalized and eliminated *V. dahliae* (Robb 2007). With regard to the last possibility, Griffiths (1971) reported that cortical lignification and vesicular materials accumulated in cells adjacent to infected cells within 12 hours after *Verticillium* hyphae entered host cells. Subsequent infections resulted in formation of lignitubers, which eventually compartmentalized and eliminated the infection (Griffiths 1971). *Verticillium dahliae* has been reported to induce cortical lignification and the development of lignitubers on infected tomato roots (Griffiths and Isaac 1966). It is not known if such a system could be active in *Ailanthus* roots, and very little is understood
about the biological mechanisms of plant tolerance to *Verticillium* infections (Robb 2007).

Root wounding favors infection of vascular tissues by *Verticillium* (Talboys 1958, Selman and Buckley 1959). The root cortex plays a vital role in delaying vascular wilt fungi ingress into the root stele and subsequent systemic infection (Huisman 1988). However, the success rate of shoot infections developing from root infections in some plant species is surprisingly small, ranging from only 0.02% to 0.5% (Bejarano-Alc’azar et al. 1999).

In contrast, root wounding followed with *V. albo-astrum* resulted in greater disease and less height growth (Fig. 18), as compared to non-wounded seedlings. In addition, wounded seedlings developed disease symptoms 4 weeks earlier than non-wounded plants (Fig. 17). The 4-week lag in symptom development in non-wounded *V. albo-astrum* treatments was likely due to the time needed for *V. albo-astrum* to traverse the non-wounded extra-vascular root defenses, such as lignitubers and phytoalexins induced in root cells by initial infection (Daayf et al. 1997). Once the defenses in the root cortex were overcome, *V. albo-astrum* likely entered the vascular system and began to inhibit xylem flow. However, host defense responses are not restricted to the root cortex. Upon initial infection, tyloses, suberin, and “gums” begin to coat the walls of the vessel elements and tracheids in the infected host (Robb and Powelson 1989, Benhamou 1995). Pathogenesis-related proteins, phenolic compounds, phenylalanine ammonia-lyase, and deposition of sulfur and terpenoid phytoalexins, contribute to *Verticillium* compartmentalization and elimination when the infection has reached the vascular system (Pegg and Brady 2002, Hahlbrock and Scheel 1989, Williams et al. 2002, Mace et al.)
The ability of *V. albo-atrum* to suppress these defense responses, both intra- and extra-vascularly, defines its ability to be an aggressive and virulent pathogen. In contrast root wounding with *V. dahliae* had little influence on disease severity or average seedling height increase. Although our *V. dahliae* isolates were proven pathogens, they apparently are not very virulent or aggressive against *Ailanthus*.

**Root grafts.** In 2007 several *Ailanthus* trees growing near inoculated trees developed symptoms of *Verticillium* wilt approximately 2 weeks after the inoculated trees became symptomatic. However, our previous inoculation experiments showed that the latent period from inoculation to expression of wilting symptoms was approximately 4 weeks. The 2-week time period is likely not enough time for newly infected trees to disperse spores, infected leaves, or other propagules and cause new infections on adjacent *Ailanthus* trees. It is more likely that intraspecific root grafts play a role in short distance spread of the pathogen. However, intraspecific root grafts have not been reported for *Ailanthus*, and root grafts as a means of short-distance spread of *Verticillium* among *Ailanthus* trees has not been tested.

*Verticillium* transmission by root grafts in *Ailanthus* is a possibility, since *Verticillium albo-atrum* transmission via root grafts have been reported in potato (McKay 1926). In addition, Isaac (1953) reported that spread of *V. albo-atrum* and *V. dahliae* in tomato and *Antirrhinum* was likely due to root contact (not grafts). Similar high incidences of *Verticillium* infection have been associated with close proximity of infected and healthy plants within raspberry, maple, mint, olive, and pepper (Bhat et al. 2003, Epstein 1978, Johnson et al. 2006, Navas-Cortes et al. 2008). Other xylem-limited vascular wilt diseases including bacterial leaf scorch, Dutch elm disease, and oak wilt
involve root grafts in transmission of the wilt pathogen to adjacent trees by root grafts (Epstien 1978, Sanderlin 2005, Sinclair and Lyon 2005).

**Nematodes.** The presence of high populations of *Xiphenema* spp. nematode within soils of severely infested areas was surprising and interesting, although based only on six samples. Certain species of phytonematodes play a synergistic role with *Verticillium* in wilt diseases of maple, potato, mint, tomato, cotton, strawberry, and others (Pegg and Brady 2002, Rowe and Powelson 2002, Bowers et al. 1996, Francl et al. 1987, Santamour 1992). Also, *Verticillium* wilt has been reported to stimulate increased *Pratylenchus* spp. reproduction in eggplant, tomato, and peppermint (Mountian and McKeen 1960, Mountain and McKeen 1962, Faulkner and Skotland 1965).

However, it is difficult to envision on a possible nematode association with *Verticillium* wilt of *Ailanthus*. Nematode feeding sites are not likely entrance wounds (Bowers et al. 1996) for *Verticillium* infections in *Ailanthus*. The direct association between nematode populations and *Verticillium* wilt may simply be due to increased root exudation from infected *Ailanthus* plants, or from root decay as infected hosts die, that stimulates both nematode populations and microsclerotia or melanized hyphae germination. *Verticillium* can also be stimulated when nematodes feed and stimulate host root branching, which in effect results in more host root tips available for infection. An increase in root tips has been reported to increase the potential infection points for *Verticillium* in a root, but most research has been done with a *Pratylenchus*-*V. dahliae* pathosystem (Bowers et al 1996, Rowe and Powelson 2002).

In addition, the wounds we induced using a soil knife were mechanical and not similar to those produced by nematode feeding. In some *Verticillium*-nematode
interactions it is likely that the nematodes initiate a general host defense response at phytonematode feeding sites, making nematode wounds undesirable to fungal infection, due to accumulated plant defense enzymes and phytoalexins. Nematode feeding induces increases of \( \beta \)-1,3-glucanase and chitinase activity as well as increases in initial isoflavonoid phytoalexin levels. These substances are known to be factors in anti-fungal resistance of host plants (Baldridge et al. 1998). Efficient plant tolerance to \textit{V. dahliae} has been shown to be a function of plant ability to quickly initiate defense responses, such as production of \( \beta \)-1,3-glucanase, chitinase, and phytoalexins (Ying-Zhang et al. 2003, McFadden et al. 2001). Mechanical wounding by soil knife would not stimulate defense mechanisms such as those induced by nematodes. And, we have proven that \textit{V. albo-atrum} is a virulent and aggressive pathogen of \textit{Ailanthus} in the absence of nematodes or root wounding. Therefore, it is likely that soil nematodes are involved in the \textit{Verticillium-Ailanthus} pathosystem.

**Colonization.** Histology provided insights into colonization of \textit{Ailanthus} xylem by \textit{V. albo-atrum}. \textit{Verticillium} wilt fungi are usually limited to xylem vessels until plant mortality, at which time they invade other tissues (Pegg and Brady 2002). Histology revealed that xylem parenchyma cells of inoculated seedlings were not colonized until 4 weeks after inoculation. By that time, inoculated seedlings were in severe decline. The presence of \textit{V. albo-atrum} in vessels 10 cm below the inoculation point by week 4 indicates that the pathogen was spreading downward at approximately 3.57 mm/day, a rate downward similar to other \textit{Verticillium} spp. (Sinclair et al. 1981). However the initial downward movement observed 1 week after inoculation was very fast (5cm), indicating that the colonization rate during 0 to 4 weeks after inoculation was not linear. This initial
rapid rate of colonization may have been due to the disruption of the xylem fluid column, causing inoculum to be drawn downward in the xylem vessel elements under negative tension (Tattar and Tattar 1999). Downward movement of *V. albo-atrum* could lead to rapid colonization and death of the root system.

At 1 week following inoculation, upward colonization was visible at 20 cm above the inoculation point, indicating an initial upward spread in the xylem of 28.6 mm/day. This rapid upward movement is attributed to movement of water within the xylem of the transpiration stream. Upward movement is theoretically proportional to rate of plant transpiration. In addition to the continuous, end-to-end nature of xylem elements, pits in the vessel end walls may also play a role. Pits within *Ailanthus* xylem vessels could also facilitate the rapid upward movement of conidia, since the pits are larger than the conidia of *V. albo-atrum* (Pegg and Brady 2002). If larger trees had greater rates of transpiration, the upward movement of *V. albo-atrum* could be much greater than 28.6 mm/day.

Banfield (1941) showed that released *V. dahliae* conidia within the vascular tissue could move 1.5 m up an elm tree in 8 to 15 seconds (Harris and Heimstra 1998).

The peaks in fungal colonization in vessels at -5, 2.5, and 10 cm from the inoculation point (Fig. 19, week 4), are likely related to cyclical systemic colonization, as related to the time for colonization to reach these points. Heinz et al. (1998) reported that systemic colonization of *V. albo-atrum* in tomato cycled between periods of fungal increases, peaking at 2 to 4 and 12 to 15 days, and periods of fungal inactivity. Thus the spatial patterns observed within inoculated *Ailanthus* seedlings could actually be related to temporal cycles (Chen et al. 2004, Heinz et al. 1998).
Literature Cited


Chen, P., Lee, B., and Robb, J. 2004. Tolerance to a non-host isolate of *Verticillium*


Fradin E.F. and Thomma, B.P.H.J. 2006. Pathogen Profile: Physiology and molecular
aspects of *Verticillium* wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology 7:71-86.


Navas-Cortes, J.A., Landa, B.B., Mercado-Blanco, J., Trapero-Casas, J.L., Rodriguez-


Sanderlin, R.S. 2005. Cultivar and seedling susceptibility to pecan bacterial leaf scorch caused by *Xylella fastidiosa* and graft transmission of the pathogen. Plant Disease 89:446-449.


Sewell, G.W.F., and Wilson, J.F. 1964. Occurance and dispersal of *Verticillium conidia*
in xylem sap of the hop (*Humulus lupulus* L.). Nature 204:901.


FIGURES AND TABLES

Figure 1. Average disease severity index chart of *Ailanthus*, striped maple, red maple, sugar maple, white ash, yellow-poplar, red oak, and chestnut oak field inoculations of *V. albo-atrum*. Only *Ailanthus* and striped maple exhibited significant susceptibility. Red maple, sugar maple, white ash, yellow-poplar, red oak, and chestnut oak did not develop significant disease symptoms. DSI = (I x S)/M, where I = is the percentage of diseased plants, S = mean severity of foliar symptoms in diseased plants, and M = maximum severity value (M = 4 in our experiments).

Figure 2. Percent of tree species that were dead, wilting, or healthy trees in permanent plots.

Figure 3. MgCl₂ and annealing temperature (Ta) optimization, for *V. albo-atrum* primer set, FVAL6 / RVA. Columns, C = negative (water) control, and gradient of Ta (°C). In the positive reactions, *V. albo-atrum #PSU140* DNA template was used, in negative reactions, *V. dahliae #PSU154* DNA template was used. Rows, A = 1.5 mM MgCl₂ concentration, B = 2 mM MgCl₂ concentration, and C = 2.5 mM MgCl₂ concentration.

Figure 4. Ta optimization for *V. dahliae* primer set, FVDA5 / RVD. Rows: L = ladder marker (1 kb), C = negative control, 1 = 55.6, 2 = 58.3, 3 = 59.2, 4 = 60.0, 5 = 60.0, 6 = 60.8, 7 = 61.7, 8 = 62.9, 9 = 64.3, 10 = 66.0, 11 = 67.5, 12 = 68.5, 13 = 69.3, 14 = 70.0 °C. Columns: A, positive reaction (*V. dahliae #PSU154*) DNA template, B, negative reaction (*V. albo-atrum #PSU140*) DNA template.

Figure 5. *V. albo-atrum* conidial sensitivity, of *V. albo-atrum* primer set FVAL6 / RVA. Columns: L = DNA ladder marker (1 kb), C = negative (water) control, Conidia per reaction (20,000 – 2). Rows: A, second round of nested PCR using FVAL6 / RVA primer set, B, first round PCR using NESF18S / NESR28S primer set.

Figure 6. Agarose gel showing an isolate screen of *V. albo-atrum* isolates used in a PCR reaction with FVAL6 and RVA primer set. Columns: L = DNA ladder marker (1 kb), C = negative (water) control, followed by *V. albo-atrum* isolate number. Rows: A, second round nested PCR using specific *V. albo-atrum* primer pair FVAL6 / RVA, B, first round PCR using general fungal primer set NESF18S / NESR28S.

Figure 7. Striped maple canker survey showing percent of striped maples in the study area in four health ratings contrasting trees with cankers vs. trees without cankers.
Figure 8. Estimated *V. albo-atrum* epidemic spread throughout the study area starting around the year 2000 and spreading at a significant rate to encompass 19.5 ha by 2007. Numbers refer to year of death of felled trees, based on tree-ring analysis.

Figure 9. Average mortality spread, upwind and downwind, following establishment of the *V. albo-atrum* from years 2003-2007.

Figure 10. Percent disease spread from 5 *Ailanthus* trees inoculated in each plot during May 2007, until time of evaluation on June 2008. Stand 1 had 39 total *Ailanthus* trees stand 2 had 95 *Ailanthus* trees.

Figure 11. Inoculated Stand 1 with *V. albo-atrum* ~1 year following inoculation, showing the spread from inoculated trees to adjacent *Ailanthus* trees. Circles illustrate mortality occurring initially near inoculated trees.

Figure 12. Inoculated Stand 2 with *V. albo-atrum* ~1 year following inoculation, showing the spread from inoculated trees to adjacent *Ailanthus* trees. Circles illustrate mortality occurring initially near inoculated trees.

Figure 13. Image of 1% agarose gel showing amplified PCR products of the nested PCR using *V. albo-atrum* specific FVAL6/RVA primer pair, used for detection of *V. albo-atrum* in *Ailanthus* seeds. L = 1 kb ladder marker, 1 – 50 seed samples, and negative (water) controls (C1, C2, C3, C4, C5, C6).

Figure 14. Image of 1% agarose gel showing amplified PCR products of the nested PCR using *V. dahliae* specific FVDA5/RVD primer pair, used for detection of *V. dahliae* in *Ailanthus* seeds. L = 1 kb ladder marker, 1 – 50 seed samples, and negative (water) controls (1-1, 1-2, 2-1, 2-2, 3-1, 3-2).

Figure 15. Frequency of ambrosial beetle hole diameter in infested *Ailanthus*. The 2-mm beetle holes were caused primarily by *E. validus* ambrosial beetles.

Figure 16. Image of 1% agarose gel showing amplified PCR products of the nested PCR using *V. albo-atrum* specific FVAL6/RVA primer pair, used for detection of *V. albo-atrum on E. validus* ambrosial beetles. L = 1 kb ladder marker, 1 – 50 seed samples, + = positive controls, and negative (water) controls (C1, C2, C3, C4, C5, C6).

Figure 17. Disease severity index of *Ailanthus* potted greenhouse seedlings at various times after soil drench inoculation of wounded and non-wounded roots with 20 ml (10^7 spores / ml) *V. dahliae* and *V. albo-atrum* conidia. Control plants were injected with distilled water. Index of 0 to 100, where 0 = all plants were healthy and 100 = all plants were dead. DSI = (I x S)/M, where I = is the percentage of diseased plants, S = mean severity of foliar
symptoms in diseased plants, and $M = \text{maximum severity value (M = 4 in our experiments)}$.

**Figure 18.** Average cumulative height (cm) of *Ailanthus* potted greenhouse seedlings at various times after soil drench inoculation of wounded and non-wounded roots with 20 ml ($10^7$ spores / ml) *V. dahliae* and *V. albo-atrum* conidia. Control plants were injected with distilled water.

**Figure 19.** Percent of *Ailanthus* seedling large xylem vessels colonized by *V. albo-atrum*, 4 weeks following stem-inoculation with *V. albo-atrum* (PSU 140).

**Table 1.** Primers, annealing temperature (Ta), species specificity, and reference of primers used in the nested PCR protocol

**Table 2.** Percent striped maple in 4 different health classes, for striped maple in permanent plots with and without stem cankers.

**Table 3.** *Xiphenema* spp. nematode numbers/100 cc of soil at different locations within and outside the study area. Under wilting *Ailanthus* in the study area, Under healthy *Ailanthus* in the study area, Under healthy *Ailanthus* outside study area, Oak stand outside of study area.

**Table 4.** Disease severity index (0 to 100) and cumulative height growth (cm) of *Ailanthus* seedlings 15 weeks after treatments; *V. albo-atrum, V. dahliae*, and non-inoculated with wounded and non-wounded roots. Index of 0 to 100, where 0 = all plants were healthy and 100 = all plants were dead.
Fig. 1. Average disease severity index chart of *Ailanthus*, striped maple, red maple, sugar maple, white ash, yellow-poplar, red oak, and chestnut oak field inoculations of *V. albo-atrum*. Only *Ailanthus* and striped maple exhibited significant susceptibility. Red maple, sugar maple, white ash, yellow-poplar, red oak, and chestnut oak did not develop significant disease symptoms. DSI = (I x S)/M, where I = is the percentage of diseased plants, S = mean severity of foliar symptoms in diseased plants, and M = maximum severity value (M = 4 in our experiments).

Fig. 2. Percent of tree species that were dead, wilting, or healthy trees in permanent plots.
Fig. 3. MgCl₂ and annealing temperature (Ta) optimization, for *V. albo-atrum* primer set, FVAL6 / RVA. Columns, C = negative (water) control, and gradient of Ta (°C). In the positive reactions, *V. albo-atrum* #PSU140 DNA template was used, in negative reactions, *V. dahliae* #PSU154 DNA template was used. Rows, A = 1.5 mM MgCl₂ concentration, B = 2 mM MgCl₂ concentration, and C = 2.5 mM MgCl₂ concentration.
Fig. 4. Ta optimization for *V. dahliae* primer set, FVDA5 / RVD. Rows: L = ladder marker (1 kb), C = negative control, 1 = 55.6, 2 = 58.3, 3 = 59.2, 4 = 60.0, 5 = 60.0, 6 = 60.8, 7 = 61.7, 8 = 62.9, 9 = 64.3, 10 = 66.0, 11 = 67.5, 12 = 68.5, 13 = 69.3, 14 = 70.0 °C. Columns: A, positive reaction (*V. dahliae* #PSU154) DNA template, B, negative reaction (*V. albo-atrum* #PSU140) DNA template.
**Fig. 5.** *V. albo-atrum* conidial sensitivity, of *V. albo-atrum* primer set FVAL6 / RVA. Columns: L = DNA ladder marker (1 kb), C = negative (water) control, Conidia per reaction (20,000 – 2). Rows: A, second round of nested PCR using FVAL6 / RVA primer set, B, first round PCR using NESF18S / NESR28S primer set.
Fig. 6. Agarose gel showing an isolate screen of *V. albo-atrum* isolates used in a PCR reaction with FVAL6 and RVA primer set. Columns: L = DNA ladder marker (1 kb), C = negative (water) control, followed by *V. albo-atrum* isolate number. Rows: A, second round nested PCR using specific *V. albo-atrum* primer pair FVAL6 / RVA, B, first round PCR using general fungal primer set NESF18S / NESR28S.
Fig. 7. Striped maple canker survey showing percent of striped maples in the study area in four health ratings contrasting trees with cankers vs. trees without cankers.
Fig. 8. Estimated *V. albo-atrum* epidemic spread throughout the study area starting around the year 2000 and spreading at a significant rate to encompass 19.5 ha by 2007. Numbers refer to year of death of felled trees, based on tree-ring analysis.
Fig. 9. Average mortality spread, upwind and downwind, following establishment of the *V. albo-astrum* from years 2003-2007.

Fig. 10. Percent disease spread from 5 *Ailanthus* trees inoculated in each plot during May 2007, until time of evaluation on June 2008. Stand 1 had 39 total *Ailanthus* trees stand 2 had 95 *Ailanthus* trees.
Fig. 11. Inoculated Stand 1 with *V. albo-atrum* ~1 year following inoculation, showing the spread from inoculated trees to adjacent *Ailanthus* trees. Circles illustrate mortality occurring initially near inoculated trees.

Fig. 12. Inoculated Stand 2 with *V. albo-atrum* ~1 year following inoculation, showing the spread from inoculated trees to adjacent *Ailanthus* trees. Circles illustrate mortality occurring initially near inoculated trees.
**Fig. 13.** Image of 1% agarose gel showing amplified PCR products of the nested PCR using *V. albo-astrum* specific FVAL6/RVA primer pair, used for detection of *V. albo-astrum* in *Ailanthus* seeds. L = 1 kb ladder marker, 1 – 50 seed samples, and negative (water) controls (C1, C2, C3, C4, C5, C6).
Fig. 14. Image of 1% agarose gel showing amplified PCR products of the nested PCR using *V. dahliae* specific FVDA5/RVD primer pair, used for detection of *V. dahliae* in *Ailanthus* seeds. L = 1 kb ladder marker, 1 – 50 seed samples, and negative (water) controls (1-1, 1-2, 2-1, 2-2, 3-1, 3-2).
Fig. 15. Frequency of ambrosial beetle hole diameter in infested *Ailanthus*. The 2-mm beetle holes were caused primarily by *E. validus* ambrosial beetles.
Fig. 16. Image of 1% agarose gel showing amplified PCR products of the nested PCR using *V. albo-atrum* specific FVAL6/RVA primer pair, used for detection of *V. albo-atrum* on *E. validus* ambrosial beetles. L = 1 kb ladder marker, 1 – 50 seed samples, + = positive controls, and negative (water) controls (C1, C2, C3, C4, C5, C6).
Fig. 17. Disease severity index of *Ailanthus* potted greenhouse seedlings at various times after soil drench inoculation of wounded and non-wounded roots with 20 ml ($10^7$ spores / ml) *V. dahliae* and *V. albo-atrum* conidia. Control plants were injected with distilled water. Index of 0 to 100, where 0 = all plants were healthy and 100 = all plants were dead. DSI = (I x S)/M, where I = is the percentage of diseased plants, S = mean severity of foliar symptoms in diseased plants, and M = maximum severity value (M = 4 in our experiments).
Fig. 18. Average cumulative height (cm) of *Ailanthus* potted greenhouse seedlings at various times after soil drench inoculation of wounded and non-wounded roots with 20 ml ($10^7$ spores / ml) *V. dahliae* and *V. albo-atrum* conidia. Control plants were injected with distilled water.
Fig. 19. Percent of *Ailanthus* seedling large xylem vessels colonized by *V. albo-atrum*, 4 weeks following stem-inoculation with *V. albo-atrum* (PSU 140).

Table 1. Primers, annealing temperature (Ta), species specificity, and reference of primers used in the nested PCR protocol

<table>
<thead>
<tr>
<th>Primer</th>
<th>5' - 3'</th>
<th>Ta</th>
<th>Species Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVAL6</td>
<td>CGGTACATCAGTCTCTTTATTC</td>
<td>61 °C</td>
<td><em>V. albo-atrum</em></td>
<td>adapted from Masoud 2002</td>
</tr>
<tr>
<td>RVA</td>
<td>TCCGATGCGAGCTGTAAT</td>
<td>61 °C</td>
<td><em>V. albo-atrum</em></td>
<td>adapted from Masoud 2002</td>
</tr>
<tr>
<td>FVDA5</td>
<td>CGTCCATCAGTCTCTGTTTT</td>
<td>64 °C</td>
<td><em>V. dahliae</em></td>
<td>adapted from Masoud 2002</td>
</tr>
<tr>
<td>RVD</td>
<td>TCCGATGCGAGCTGTAAC</td>
<td>64 °C</td>
<td><em>V. dahliae</em></td>
<td>Masoud 2002</td>
</tr>
<tr>
<td>NESF18S</td>
<td>CCTCATAACCCCTTTGTAACC</td>
<td>56 °C</td>
<td>-----</td>
<td>Volossiouk et al. 1995</td>
</tr>
<tr>
<td>NESR28S</td>
<td>CCGAGGTCAACCGTTGCCG</td>
<td>56 °C</td>
<td>-----</td>
<td>Volossiouk et al. 1995</td>
</tr>
</tbody>
</table>
Table 2. Percent striped maple in 4 different health classes, for striped maple in permanent plots with and without stem cankers.

<table>
<thead>
<tr>
<th></th>
<th>Striped maple without cankers</th>
<th>Striped maple with cankers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>29.1%</td>
<td>29.6%</td>
</tr>
<tr>
<td>Dieback</td>
<td>1.5%</td>
<td>17.6%</td>
</tr>
<tr>
<td>Wilting</td>
<td>0.0%</td>
<td>1.5%</td>
</tr>
<tr>
<td>Dead</td>
<td>2.0%</td>
<td>18.6%</td>
</tr>
</tbody>
</table>

Table 3. *Xiphenema* spp. nematode numbers/100 cc of soil at different locations within and outside the study area. Under wilting *Ailanthus* in the study area, Under healthy *Ailanthus* in the study area, Under healthy *Ailanthus* outside study area, Oak stand outside of study area.

<table>
<thead>
<tr>
<th>Sample location</th>
<th># <em>Xiphenema</em> spp. / 100 cc soil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under wilting <em>A. altissima</em> in the study area</td>
<td>219.5</td>
</tr>
<tr>
<td>Under healthy <em>A. altissima</em> in the study area</td>
<td>89.5</td>
</tr>
<tr>
<td>Under healthy <em>A. altissima</em> outside study area</td>
<td>1</td>
</tr>
<tr>
<td>Oak stand outside of study area</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 4. Disease severity index (0 to 100) and cumulative height growth (cm) of *Ailanthus* seedlings 15 weeks after treatments; *V. albo-atrum*, *V. dahliae*, and non-inoculated with wounded and non-wounded roots. Index of 0 to 100, where 0 = all plants were healthy and 100 = all plants were dead.

<table>
<thead>
<tr>
<th></th>
<th>Disease Severity Index</th>
<th>Height Growth (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wounded</td>
<td>Non-wounded</td>
</tr>
<tr>
<td><em>V. albo-atrum</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wounded</td>
<td>96.3</td>
<td>84</td>
</tr>
<tr>
<td>Non-wounded</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>V. dahliae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wounded</td>
<td>12.1</td>
<td>5.8</td>
</tr>
<tr>
<td>Non-wounded</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No inoculation</td>
<td>10.5</td>
<td>4</td>
</tr>
</tbody>
</table>
Chapter V. *Verticillium* wilt of *Ailanthus altissima*: effect on forest composition.

Mark J. Schall, and Donald D. Davis Department of Plant Pathology, The Pennsylvania State University, University Park 16802

ABSTRACT

*Ailanthus altissima* (tree-of-heaven) is an important invasive tree species in forests of Pennsylvania and much of North America. However, this species is attacked by a virulent and aggressive pathogen, *Verticillium albo-atrum*, that is currently causing significant wilt and mortality in south-central Pennsylvania. During the early 1980s, *Ailanthus* invaded an oak-dominated forest that had been logged. *Ailanthus* subsequently dominated stand openings, encompassing nearly 40% of stand basal area. In 2000 the stand was attacked by *V. albo-atrum* from an unknown source. By summer 2007, the pathogen had killed more than 7000 canopy *Ailanthus* trees in that stand. In severely affected parts of the stand, the entire overstory component of *Ailanthus* was removed and approximately half of the understory *Ailanthus* seedlings or sprouts were killed.

Following decline of the invasive *Ailanthus*, the canopy composition is beginning to revert to a native state, as residual oak trees left from the salvage harvest expanded their crowns and young red maple and black birch trees were recruited into the overstory. As of 2008, the large openings caused by dead and dying *Ailanthus* are being filled by seedlings of red maple, sweet birch, and striped maple.
Invasive plants and weeds result in approximately $20 billion loss annually in the United States, including cost of damage and control and encompass approximately 8 to 47% of the total flora of most states (Van Driesche et al. 2002). Exotic plant species, such as *Ailanthus altissima* [(Mill.) Swingle], are capable of quickly invading and replacing portions of native ecosystems. *Ailanthus* is a successful invader due to several characteristics including drought resistance (Trifilo et al. 2004), rapid growth, seed proliferation, aggressive root sprouting (Miller 1990), and production of allelopathic chemicals such as ailanthone (Heisey 1996). Northern red oak (*Quercus rubra* L.), red maple (*Acer rubrum* L.), and sugar maple (*Acer saccharum* Marsh.), seedlings grown in the presence of ailanthone under *Ailanthus* trees, exhibited net decrease in shoot extension, shoot extension biomass, and leaf biomass as compared to seedlings growing at a site where ailanthone was inactivated by addition of activated charcoal (Gómez-Aparicio and Canham 2008b). Heisey (1996) reported that ailanthone at rates as low as 0.3 kg/ha had strong herbicidal effects on 13 of 17 species tested, but had noted herbicidal effects of *Ailanthus* bark extract, containing ailanthone, decreased with time during a growing season.

In contrast, forest sites dominated *Ailanthus* that decline and die, are primed for reforestation by native plant species after degradation of ailanthone in site soils, due to an increase in site soil fertility related to *Ailanthus* (Ehrenfeld 2003, Gómez-Aparicio and Canham 2008a). The presence of *Ailanthus* on a site increases soil pH, nitrogen cycling, and the availability of essential soil cations such as Ca, Mg, and K, likely due to the increased base cation concentrations in leaf litter (Gómez-Aparicio and Canham 2008a). Red maple, a survivor capable of flourishing in varied environmental situations and
common in our study area, appears to benefit the greatest from sites modified by *Ailanthus* (Gómez-Aparicio and Canham 2008b). Due to its extreme generalism, red maple has begun to dominate eastern North American forests, and will likely become more common in future forests (Abrams 1998). We hypothesize that red maple will become a more important component in those forest stands that contain a significant proportion of *Ailanthus*, as *Ailanthus* wilt causes significant mortality.

Control of exotic invasive plants such as *Ailanthus* has been difficult, and has been accomplished mainly by use of traditional chemical herbicides. Biological controls, including use of fungal mycoherbicides, can provide an efficient, inexpensive, and sustainable control of a wide range of invasive plants (Templeton et al. 1979) from ferns to trees (Van Driesche et al. 2002). However, there are concerns regarding use of biological controls as a means of eliminating or suppressing invasive weed species. Effective pathogens of exotic invasive plants may themselves be exotic, non-native organisms. The risk involved through intentional introduction of exotic insects or pathogens may outweigh benefits of such controls. Risk analysis, or assessment of biocontrols with regard to the risk to other economic and ecologically important species, is of utmost importance when using biocontrols (Van Driesche et al. 2002).

The ability of exotic weeds to become naturalized and thrive in new geographic origins is often related to escape from pathogens in their native range. Likewise, plants introduced to new geographic regions may be attacked by pathogens native or endemic to the new region. Endemic pathogens, with respect to an exotic host, play an integral role in determining the success of an invasive plant, since the host has not evolved tolerance
to this “new” pathogen. As such, native, endemic pathogens can serve as effective biological controls for non-native, exotic plant species (Mitchell and Power 2003).

Exotic pests and pathogens affecting native trees, or conversely, native pests and pathogens affecting exotic trees, have the potential to significantly change forest composition, especially when a devastating disease occurs to a dominant forest species. Well known examples of such devastating forest diseases by exotic pathogens in eastern hardwood forests include chestnut blight, Dutch elm disease, dogwood anthracnose, and butternut canker (Leibhold 1995, Lovett et al. 2006, Sinclair and Lyon 2005). As forests become more open due to loss of susceptible trees, trees are increasingly replaced by early successional, pioneer tree species like *Ailanthus* (Knapp and Canham 2000). The new forest may be comprised of very few species, or in drastic cases, become nearly a monoculture (Silveri et al. 2001). As species diversity decreases, the potential for devastating diseases to significantly and adversely affect remaining species increases (Shipton 1977). Based on six features of a pathogen/host relationship, Lovett et al. (2006) proposed that “…the most severe long-term impacts (of a pathogen on a forest ecosystem) would be expected for a virulent, host specific pest attacking a dominant or unique tree species that grows in nearly pure stands.”

*Ailanthus* wilt and mortality in south-central Pennsylvania is caused by *Verticillium albo-atrum* (Reinke & Berthold), and to a lesser degree by *V. dahliae* (Klebahn) (Chapter II). It is unknown if isolates causing the wilt are native or exotic. *Verticillium albo-atrum* is more virulent and aggressive to *Ailanthus* than is *V. dahliae* (Chapter II). Forest stands that had been invaded by *Ailanthus* in our study area are rapidly changing character as large numbers of *Ailanthus* canopy trees and reproduction...
are killed. However, the effect of *Ailanthus* wilt and mortality on stand composition is unknown. Historical records allow us to approximate the composition of the forest stand prior to invasion by *Ailanthus*. Taking data on permanent plots allows us to determine the influence of *Ailanthus* wilt on stand composition.

The objectives of this chapter are to 1) determine the effect that invasive *Ailanthus* has on forest composition, and 2) the effect that Verticillium wilt of *Ailanthus* and mortality has on forest stand composition.

**MATERIALS AND METHODS**

**Study Area.** The study area, which has been described in detail (Chapter II), is in south-central Pennsylvania within the Tuscarora State Forest in Perry and Franklin counties. The stand encompasses approximately 56 ha, with approximately 17.4 ha (31.0%) containing wilting and dead *Ailanthus* (in 2007). As of 2007, we estimated more than 7000 canopy *Ailanthus* trees and more than 500,000 seedlings, root sprouts, and saplings had succumbed to *Ailanthus* wilt. The study stand is an oak-dominated, mixed-hardwood stand containing American basswood (*Tilia americana* L.), black locust (*Robinia pseudoacacia* L.), black oak (*Quercus velutina* Lam.), chestnut oak (*Quercus montana* Willd.), northern red oak, red maple, shellbark hickory (*Carya laciniosa* (F. Michx.) Luodon), striped maple (*Acer pensylvanicum* L.), sugar maple, yellow-poplar (*Liriodendron tulipifera* L.), sweet birch (*Betula lenta* L.), and white ash (*Fraxinus americana* L.), as well as an extensive acreage of *Ailanthus* within more open portions of the stand. No species of trees has exhibited obvious wilting to the degree that *Ailanthus* has, with the exception of the occasional striped maple. Also, occasional dead striped maple and black locust trees occur throughout the stand. Understory vegetation consists

**Characterization of the forest before *Ailanthus* invasion**

*Ailanthus* likely became established in the study area following gypsy moth-induced stand mortality and harvesting in the early 1980s (Chapter II). Stand inventory data obtained from the Pennsylvania Bureau of Forestry for 1982-1983 revealed that canopy tree (mainly oak) mortality from gypsy moth infestations had occurred within the study area in 1982 and 1983. Inventory data was intended to estimate numbers of dead or alive trees within a 54-ha salvage harvest, which contained much of our study area, and provided us a general description of the pre-1982 forest composition. We assumed that dead trees, as listed in the inventory, died from gypsy moth defoliations and were alive prior to 1982. Inventory sheets listed relative tree size [small tree (5 - 15.2 cm dia.), pole (15.2 - 30.4 cm dia.), immature sawtimber, mature sawtimber] and whether each tree was dead or alive at time of survey. Based on these data, we calculated basal area (BA) of various tree species in the stand prior to *Ailanthus* invasion. Regeneration data was not listed on the inventory sheets, but general observations were recorded regarding understory species and were used in data interpretation.

The 54-ha harvest was conducted in 1983 to salvage canopy trees (mainly oak) killed by gypsy moth larval feeding. Tree mortality and subsequent harvesting created large openings in the forest, ideal for invasion by *Ailanthus*. Although stand records
indicate that *Ailanthus* was not present in the stand prior to the early 1980s, the open stand quickly became heavily invaded by this species. This rapid invasion indicates a seed source in the immediate vicinity, perhaps on the ridgetops.

**Characterization of the forest after *Ailanthus* invasion**

In 2005, we categorized the study area, based on general observations, into three sub-areas regarding amount of Ailanthus wilt: control (no disease), moderate disease, and severe disease. To characterize our study area after establishment of *Ailanthus* trees, and following the *Ailanthus* wilt epidemic, we sampled both overstory and understory vegetation within these three sub-areas.

**Overstory.** Within each sub-area, we established two parallel transects at a compass bearing of N 56° 45’ E. Two plots were established at 62 m apart on each transect. Point sampling data was collected using a 10-factor prism (Avery and Burkhart 1994). Diameters of all plot trees >5 cm dbh (diameter breast height, 1.37m) were measured and recorded. Basal area (BA) was calculated as: \( BA = \left( \frac{T}{p} \right) \times BAF \), where \( T \) is total number of trees counted, \( p \) is the number of points where data was collected, and \( BAF \) is the basal area factor of the prism. Trees per hectare (T/ha) was calculated as: \( T/ha = \left( \frac{N \times Cf}{p} \right) \), where \( N \) is the number of trees counted in a diameter class, \( Cf \) is the per acre conversion factor, and \( p \) is the number of data collecting points. The per acre conversion factor was calculated using: \( Cf = \frac{43,560}{\pi \times (dbh \times 2.75)^2} \) or \( BAF/BA \) per tree. Data were converted to metric units. Since many of the dead *Ailanthus* trees in the severely infested area had fallen, we estimated the original number of *Ailanthus* trees/ha from plots in the moderately diseased and non-diseased sub-stands (Fig. A14). All *Ailanthus*
trees, healthy and diseased, were still standing in these areas, allowing an accurate count of number of *Ailanthus* stems and dbh for each tree.

To estimate the impact of the wilt on future composition of canopy tree species, we used data only from the severe disease sub-stand and excluded standing dead trees, since these trees would no longer be in the future stand. The number of dead *Ailanthus* canopy trees/ha was determined by calculating the number of living *Ailanthus* canopy trees/ha in the severe disease sub-stand as compared to numbers of *Ailanthus* stems in the moderate disease and control sub-stands. By knowing the number of *Ailanthus* trees/ha and the approximate percent mortality, we estimated number of dead *Ailanthus* stems/ha throughout the severely infested study area.

**Understory.** To determine the effect of Ailanthus wilt on understory vegetation, we counted the number of seedlings/species in a 1 m² plot at each plot center (Fig. 4). Using this data, we estimated the number of *Ailanthus* seedlings/ha in the moderate disease and control sub-stands, as well as the number of living *Ailanthus* seedlings/ha in the original infected study (Fig. A14). For post-*Ailanthus* wilt calculations, we estimated the number of living, non-defoliated *Ailanthus* seedlings/ha in the severe disease sub-stand, and the number of seedlings killed by *Ailanthus* wilt.

General notes were taken as to the presence of plants other than tree seedlings, such as grasses and ferns, on the 1 m² plots.

**Ailanthus wilt survey.** At each sample point on our transect (Fig. A14), we rated the severity of *Ailanthus* wilt using a 0 – 3 scale, where 0 = healthy tree, 1 = chlorotic foliage, 2 = wilting foliage, and 3 = dead/defoliated tree. Using this data, we were able to
estimate the incidence and severity of wilt throughout out the three study stands (control, moderate disease, and severe disease).

RESULTS

Characterization of the forest before *Ailanthus* invasion

The forest in the study area prior to the 1982 gypsy moth defoliation and resulting 1984 salvage harvest was a mixed-hardwood stand comprised mainly of oak, hickory, and maple, with a few associated hardwood species such as American basswood, and black locust (PA BF District 5 records) (Fig. 1). The two primary species in the overstory were northern red oak, with a BA of approximately 8.2 m²/ha, and chestnut oak with a BA of 5.9 m²/ha. The composition of understory vegetation was not reported, except for a notation of the presence of oak and striped maple seedlings.

Characterization of the forest after *Ailanthus* invasion

Mortality due to gypsy moth larval feeding and resultant salvage harvesting in 1984 resulted in large stand openings. We approximated the overstory species composition present during 1984 to 2003 by evaluating control portions of the salvage cut not yet affected by Ailanthus wilt (Fig. A14). Following the salvage harvest, the stand was apparently dominated by early successional species such as *Ailanthus*, black locust, and red maple. These three species accounted for BA of 8.90, 3.44, 2.87 m³/ha respectively (Fig. 2). *Ailanthus altissima* dominated the overstory species composition, comprising nearly 40% of the canopy BA (Fig. 3)

The understory of the stand was also dominated by *Ailanthus*, which comprised 62% of seedlings (67,500 seedlings/ha). Red maple seedlings were second in abundance (16,250 seedlings/ha), followed by sweet birch (12,500 seedlings/ha), and striped maple
(8,750 seedlings/ha) (Fig. 4, Tables 2 and 3). Other understory vegetation included Pennsylvania smartweed, nettle, common blackberry, Virginia creeper, garlic mustard, American pokeweed, and hay-scented fern.

**Characterization of the forest following Ailanthus wilt.**

Before Ailanthus wilt, *Ailanthus* comprised about 40% of the overstory basal area and more than 60% of the understory in the forest stand. However, as *Ailanthus* canopy trees died from *Verticillium*, other fast-growing, early successional pioneer species in the stand, such as red maple, striped maple, and sweet birch began to dominate. In addition, the occasional canopy oaks that had not been killed by gypsy moth, and therefore not harvested, became more prevalent in the canopy. Ailanthus wilt reduced the basal area of large living *Ailanthus* trees within the affected stands from 8.9 m²/ha in the pre-wilt stand to zero following the wilt epidemic. Overstory species including red maple, striped maple, and black birch generally retained their original basal area (Fig. 5, Table 1).

The incidence and severity of Ailanthus wilt progressed outward from what appeared to be a central starting point (Fig. 7, Table 4). In the portion of the stand where the wilt likely started in 2001, all overstory *Ailanthus* canopy trees were dead as of fall 2006. By 2006, approximately 50% of the canopy *Ailanthus* trees in the more recently infected 2.1 ha moderate disease sub-stand (Fig. A14) were dead and 20% were wilting or chlorotic. Approximately 30% of the trees appeared healthy, showing no visual foliar symptoms. In the stand not yet infected in 2006 (Fig. A14), the majority (81.8%) of the *Ailanthus* trees appeared healthy, showing no foliar symptoms. However, approximately 18.2% of the *Ailanthus* exhibited chlorotic foliage (Fig. 6, Table 4), possibly indicating early stages of disease.
Mortality of *Ailanthus* seedlings and sprouts was reflected in drastic changes in understory composition. Verticillium wilt reduced the proportion of *Ailanthus* as compared to other seedling species. Following the wilt epidemic, there were only 35,000 *Ailanthus* seedlings/ha (25% of total seedling composition), a reduction of nearly half as compared to pre-wilt estimates (Fig. 4, Table 2 and 3). The primary species replacing *Ailanthus* understory was sweet birch, numbering 65,000 seedlings/ha (46% of total seedlings) (Fig. 4, Table 2 and 3).

**DISCUSSION**

*Ailanthus altissima* is one of the most destructive, invasive plant species in forests of North America. However, the negative effects of *Ailanthus* invasions on forest ecosystems may be mitigated by the forest pathogen *V. albo-atrum*. As canopy *Ailanthus* die, they are replaced by existing species in the canopy, such as red maple and sweet birch that will become the dominant canopy in the next generation of forest trees (Pacala et al. 1996). However, while canopy *Ailanthus* die from wilt, the resultant canopy gaps are filled with *Ailanthus* seedlings. Fortunately these seedlings live for only 1-2 years, before they in turn are killed by soil-borne *V. albo-atrum*. Black birch, red maple and striped maple seedlings will likely fill the niche left by the dying *Ailanthus* seedlings. In time, species composition will likely revert to forest comprised primarily of native species. After eradication of *Ailanthus*, and the subsequent rapid environment degradation of ailanthone, many poor sites may actually be better for establishment of pioneer species such as red maple (Abrams 1998, Gomez and Canham 2008b).

Soilborne diseases caused by fungi such as *Verticillium* can be especially destructive, or helpful in terms of biocontrol, since survival propagules may persist in the
soil for many years. Forest stands are generally managed on long rotations, growing on the same soil for decades or centuries. This long time period increases the potential for soil-borne pathogens to interact with the tree species (Shipton 1977). Since *Ailanthus* tends to grow in large dense stands, and suppresses competing vegetation, virulent epidemics could sweep through nearly pure stands of *Ailanthus*. Our research to date (Chapter III) indicates that *V. albo-atrum* is attacking *Ailanthus* with relative specificity. If so, this *Verticillium-Ailanthus* pathosystem has great potential to have drastic long-term effects on forest composition in this invaded forest.

Mortality of tree species other than *Ailanthus* was also observed in the study area. For example, one striped maple, one black birch, and five black locust trees were dead in one stand. However, these few trees likely died from causes other than *Verticillium*, such as cankers, natural competition, and locust borer respectively (Boring and Swank 1984). This level of mortality appeared in the control stand (Table 5), and throughout the area. No trees species, other than *Ailanthus*, were obviously wilting due to *Verticillium* within the severely infested portions of the study area.

Approximately 420 overstory *Ailanthus* trees/ha had died in the earliest *Verticillium* infection center in the early 2000’s (Table 6). By 2006, approximately 7,270 overstory *Ailanthus* trees had died throughout the study area. The rate of spread following establishment of *Verticillium* is rapid and will lead to additional forest composition changes when *V. albo-atrum* becomes established in the non-infected parts of the stand. At the current rate of spread, all *Ailanthus* canopy trees (23,600) will be dead in the study area by the year 2021. As larger *Ailanthus* canopy trees die, they would normally be replaced in the resultant canopy gaps by thousands of *Ailanthus* seedlings, dramatically
changing the composition of the understory. However, with the onset of Verticillium wilt, this change will likely be temporary, or may not occur at all, as seedlings may be killed very quickly. As of 2007, approximately 32,500 Ailanthus seedlings/ha have been killed by Verticillium throughout the original infected area, indicating that more than 500,000 seedlings have died throughout the infected area (Table 6). As Ailanthus seedlings are killed, the composition of the understory will likely revert to pre-Ailanthus composition and levels, and canopy gaps will be dominated by birch and maple seedlings.

Since V. albo-astrum is capable of killing large trees in only 2-3 months after inoculation, the pathogen can also quickly remove large Ailanthus seed sources. Each female Ailanthus canopy tree is capable of producing more than 325,000 seeds/tree/year at sexual maturity, which may occur as early as 12 years of age (Fig. A28) (Dirr 1998, Miller 1990). Ailanthus seeds have a very high germination capacity, and do not require stratification (Fig. A29) (Schopmeyer 1974). Each female Ailanthus tree in the canopy may produce 243,750 viable seedlings in an ideal environment. Obviously, mortality of these female Ailanthus seed trees will have a great impact on potential forest composition.
LITERATURE CITED


LIST OF FIGURES AND TABLES

Figure. 1. Overstory Species composition change due to gypsy moth defoliation/salvage harvest in 1984 and Ailanthus wilt in 2003.

Figure. 2. Percent trees per hectare of tree species following A. altissima invasion, and before and after the Ailanthus wilt epidemic.

Figure. 3. Percent basal area of species within the pre wilt (1984-2003) study area.

Figure. 4. Estimated number seedlings / ha, before and after Verticillium wilt.

Figure. 5. Post Ailanthus wilt basal area (m²/ha) composition of overstory tree species.

Figure. 6. Percent basal area of Ailanthus in each tree health class as the pathogen expanded from the original infested stand (wilt as of 2005), to the newly infested stand (new wilt in 2006), to the healthy stand (“non infected”).

Figure. 7. Yearly Ailanthus wilt epidemic spread, mapped by year of death of felled dead trees, showing progression of disease mortality through study area.

Table 1. Percent basal area by tree species in the study area, illustrating effect of the Verticillium wilt epidemic on Ailanthus on percent basal area before and after the wilt.

Table 2. Estimated percent seedlings/ha of understory species before and after the Verticillium wilt epidemic on Ailanthus.

Table 3. Estimated species composition of seedlings/ha, before and after the Verticillium wilt epidemic on Ailanthus.

Table 4. Ailanthus altissima tree health (% basal area) within three sub-stands in study area, used to estimate pre and post wilt species composition.

Table 5. Number of dead trees observed within overstory plots, illustrating that species observed other than Ailanthus were small and likely died due to natural competition.

Table 6. Estimated number Ailanthus seedlings and canopy trees/ha, before and after the wilt epidemic had altered the forest. These estimations illustrate approximate numbers of trees and seedling killed by the pathogen throughout entire study area.
Fig. 1. Overstory Species composition change due to gypsy moth defoliation/salvage harvest in 1984 and Ailanthus wilt in 2003.
Fig. 2. Percent trees per hectare of tree species following *A. altissima* invasion, and before and after the *Ailanthus* wilt epidemic.
Fig. 3. Percent basal area of species within the pre wilt (1984-2003) study area.

Fig. 4. Estimated number seedlings / ha, before and after *Verticillium* wilt.
**Fig. 5.** Post *Ailanthus* wilt basal area (m$^2$/ha) composition of overstory tree species.

**Fig. 6.** Percent basal area of *Ailanthus* in each tree health class as the pathogen expanded from the original infested stand (wilt as of 2005), to the newly infested stand (new wilt in 2006), to the healthy stand (“non infected”).
Fig. 7. Yearly *Ailanthus* wilt epidemic spread, mapped by year of death of felled dead trees, showing progression of disease mortality through study area.
Table 1. Percent basal area by tree species in the study area, illustrating effect of the *Verticillium* wilt epidemic on *Ailanthus* on percent basal area before and after the wilt.

<table>
<thead>
<tr>
<th>Species</th>
<th>Pre-Wilt</th>
<th>Post-Wilt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ailanthus altissima</td>
<td>39.8%</td>
<td>0.0%</td>
</tr>
<tr>
<td>Acer rubrum</td>
<td>12.8%</td>
<td>26.4%</td>
</tr>
<tr>
<td>Acer saccharum</td>
<td>0%</td>
<td>21.0%</td>
</tr>
<tr>
<td>Betula lenta</td>
<td>12.8%</td>
<td>15.8%</td>
</tr>
<tr>
<td>Tilia americana</td>
<td>1.3%</td>
<td>10.6%</td>
</tr>
<tr>
<td>Acer pensylvanicum</td>
<td>6.4%</td>
<td>10.6%</td>
</tr>
<tr>
<td>Quercus rubra</td>
<td>3.8%</td>
<td>5.2%</td>
</tr>
<tr>
<td>Carya laciniosa</td>
<td>2.5%</td>
<td>5.2%</td>
</tr>
<tr>
<td>Fraxinus americana</td>
<td>1.3%</td>
<td>5.2%</td>
</tr>
<tr>
<td>Robinia pseudoacacia</td>
<td>15.4%</td>
<td>0%</td>
</tr>
<tr>
<td>Quercus montana</td>
<td>2.5%</td>
<td>0%</td>
</tr>
<tr>
<td>Liriodendron tulipifera</td>
<td>1.3%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 2. Estimated percent seedlings/ha of understory species before and after the *Verticillium* wilt epidemic on *Ailanthus*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Pre-Wilt</th>
<th>Post-Wilt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ailanthus altissima</td>
<td>62%</td>
<td>25%</td>
</tr>
<tr>
<td>Acer rubrum</td>
<td>15%</td>
<td>19%</td>
</tr>
<tr>
<td>Betula lenta</td>
<td>11%</td>
<td>46%</td>
</tr>
<tr>
<td>Acer pensylvanicum</td>
<td>8%</td>
<td>11%</td>
</tr>
<tr>
<td>Hamamelis virginiana</td>
<td>3%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 3. Estimated species composition of seedlings/ha, before and after the *Verticillium* wilt epidemic on *Ailanthus*.

<table>
<thead>
<tr>
<th>Species</th>
<th>Pre-Wilt</th>
<th>Post-Wilt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ailanthus altissima</td>
<td>67,500</td>
<td>35,000</td>
</tr>
<tr>
<td>Acer rubrum</td>
<td>16,250</td>
<td>27,500</td>
</tr>
<tr>
<td>Betula lenta</td>
<td>12,500</td>
<td>65,000</td>
</tr>
<tr>
<td>Acer pensylvanicum</td>
<td>8,750</td>
<td>15,000</td>
</tr>
<tr>
<td>Hamamelis virginiana</td>
<td>3,750</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. *Ailanthus altissima* tree health (% basal area) within three sub-stands in study area, used to estimate pre and post wilt species composition.

<table>
<thead>
<tr>
<th></th>
<th>Healthy</th>
<th>Chlorotic</th>
<th>Wilting</th>
<th>Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control area</td>
<td>82</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Moderate disease area</td>
<td>30</td>
<td>5</td>
<td>15</td>
<td>50</td>
</tr>
<tr>
<td>Severe disease area</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 5. Number of dead trees observed within overstory plots, illustrating that species observed other than *Ailanthus* were small and likely died due to natural competition.

* trees located in the control portion of the study

<table>
<thead>
<tr>
<th>Diameter at dbh</th>
<th><em>Ailanthus altissima</em></th>
<th><em>Acer pensylvanicum</em></th>
<th><em>Betula lenta</em></th>
<th><em>Robinia pseudoacacia</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>2*</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>3*</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 6. Estimated number *Ailanthus* seedlings and canopy trees/ha, before and after the wilt epidemic had altered the forest. These estimations illustrate approximate numbers of trees and seedling killed by the pathogen throughout entire study area.

<table>
<thead>
<tr>
<th></th>
<th>seedlings</th>
<th>canopy trees</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post – Wilt (<em>Ailanthus</em> / ha)</td>
<td>67,500</td>
<td>419</td>
</tr>
<tr>
<td>Pre – Wilt (<em>Ailanthus</em> / ha)</td>
<td>35,000</td>
<td>0</td>
</tr>
<tr>
<td>Estimated number of dead <em>Ailanthus</em> / ha</td>
<td>32,500</td>
<td>419</td>
</tr>
<tr>
<td>Estimated number of dead <em>Ailanthus</em>/Study Area (17.4 ha)</td>
<td>565,500</td>
<td>7,291</td>
</tr>
</tbody>
</table>
Chapter VI. Future Work.

- Determine the *Verticillium* inoculum density (melanized hyphae or conidia) in the soil needed to incite disease in *Ailanthus*.

- Determine the rate at which *Ailanthus* seeds, collected from *Verticillium*-infected trees, become infected by seedborne *Verticillium*.

- Determine the role of ambrosial beetles vectoring *Verticillium* to *Ailanthus* hosts.

- Identify if other insects, such as *Ailanthus* webworm can vector *Verticillium* from diseased *Ailanthus* trees to healthy trees.

- Determine if *Verticillium* can be transmitted through *Ailanthus* intraspecific root grafts.

- Identify the potential for *Verticillium* to spread through small streams or springs within infested forest stands.

- Conduct an expanded host range study for *V. albo-astrum* isolate PSU 140.

- Revisit and evaluate permanent plots to continue to evaluate possible symptoms and mortality on other plant species.

- Investigate *Verticillium* colonization in *Ailanthus* by way of quantitative real-time PCR.

- Investigate if *Xiphenema* nematodes play a role in the *V. albo-astrum-Ailanthus* pathosystem.

- Identify optimal temperatures for *V. albo-astrum* colonization in *Ailanthus*.

- Identify weedy host (symptomless) reservoirs of *V. albo-astrum*, concentrating on Pennsylvania smartweed, common blackberry, nettle,
Virginia creeper, and mile-a-minute weeds that are common in the study area.

- Determine the effect of soil pH on survival and production of *V. albo-atrum* melanized hyphae and conidia.

- Investigate the potential for *V. albo-atrum* to overwinter in lightly or late season infected, *Ailanthus*.

- Use remote sensing to monitor and map rates and patterns of disease spread from inoculated *Ailanthus* trees.

- Determine the number of isolates of *V. albo-atrum* and *V. dahliae* present on *Ailanthus* and other hosts in the study area.
APPENDIX A

LIST OF FIGURES

Fig. A1. Location of study area. The yellow dot indicates the location of initial *Ailanthus* wilt observations, The larger red oval indicates the location of first observation of *Ailanthus* wilt epidemic and our primary study area.

Fig. A2. Aerial photograph of stand, county line, showing stand demarcation (arrow) due to salvage cutting. (A) represents uncut stand and (B) indicates the salvage cut area that contains *A. altissima*. Photograph courtesy Pennsylvania DCNR Bureau of Forestry.

Fig. A3. County line stand, showing salvage cut boundaries overlaid with our study area in yellow. Map courtesy Pennsylvania DCNR Bureau of Forestry.

Fig. A4. Field symptoms of *Ailanthus* wilt: (A) canopy tree decline and (B) seedling or sprout wilt.

Fig. A5. Vascular tissue of *Ailanthus altissima*: (A) healthy tree, (B) vascular discoloration within a wilting tree.

Fig. A6. Microscopic features of fungi isolated from wilting *A. altissima*: (A) scanning EM of verticillate phialid (1000x), (B) tip of a phialid and conidia (7500x), (C) conidial masses borne in mucilaginous material at the tips of conidiophores of *V. albo-atrum*, (D) melanized hyphae of *V. albo-atrum* formed in potato dextrose broth, (E) conidial masses borne on conidiophores of *V. dahliae*, (F) typical microsclerotia of *V. dahliae* formed on plum extract agar (PEA).

Fig. A7. Wilting of entire *A. altissima* leaf on potted greenhouse seedlings inoculated with .5 ml of $10^7$ *V. albo-atrum* conidia / ml.

Fig. A8. Defoliating chlorotic *A. altissima* leaflets on potted greenhouse seedlings inoculated with .5 ml of $10^7$ *V. dahliae* conidia / ml.

Fig. A9. Image of non-*A. altissima* host range study conducted in the greenhouse.

Fig. A10. Hypohatchet® used for inoculations of large field trees with *V. albo-atrum*.

Fig. A11. Map of the non-*A. altissima* host range study plots.

Fig. A12. Image of blue *V. albo-atrum* $10^7$ conidial suspension.

Fig. A13. Map of study area permanent plots.
Sampling locations where overstory and understory plot data was recorded within the study area, showing the three sub-stands (control, moderate disease, and severe disease). Also the sampling locations for soil samples used in a soil assay for *Verticillium*

Black locust borer damage on black locust trees throughout the study area

Opportunistic *Botryosphaeria dothidea* canker on striped maple in the study area

*Armillaria* spp. mycelial fans and rhizomorphs on striped maple

Isolation from striped maple cankers yielding *Botryosphaeria dothidea*

Typical *V. dahliae* disease cycle (Berlanger and Powelson, 2000)

Map showing the location within the study area where samples were collected for tree-ring-analysis to determine the rate of spread and origin of the *V. albo-atrum* epidemic.

Image of *A. altissima* crossections, collected for estimation of epidemic spread and origin of epidemic.

Scanning EM images of *Euwallacea validus*.

Scanning EM images of *Xylosandrus germanus*.

*A. altissima* bolt infested with ambrosial beetles, showing a fungus associated with beetle hole entrances.

*E. validus* ambrosial beetle hole colonized by a fungal associate on *A. altissima*.

Fungal isolation from *E. validus* beetles following a wash in 70% ethanol, and plated on PEA.

Hyphae of *V. albo-atrum* in *A. altissima* xylem vessels stained with Chlorazol Black E

Female *A. altissima* trees showing seeds produced in clusters of twisted samaras.

*A. altissima* seedlings dominating a canopy gap in the forest understory

Map showing the location of nematode samples in relation to the epidemic study area.
Fig. A1. Location of study area. The red oval indicates the location of first observation of *Ailanthus* wilt epidemic and our primary study area.
Fig. A2. Aerial photograph of stand, county line, showing stand demarcation (arrow) due to salvage cutting. (A) represents uncut stand and (B) indicates the salvage cut area that contains *A. altissima*. Photograph courtesy Pennsylvania DCNR Bureau of Forestry.
Fig. A3. County line stand, showing salvage cut boundaries overlaid with our study area in yellow. Map courtesy Pennsylvania DCNR Bureau of Forestry.
Fig. A4. Field symptoms of *Ailanthus* wilt: (A) canopy tree decline and (B) seedling or sprout wilt.

Fig. A5. Vascular tissue of *Ailanthus altissima*: (A) healthy tree, (B) vascular discoloration within a wilting tree.
Fig. A6. Microscopic features of fungi isolated from wilting *A. altissima*: (A) scanning EM of verticillate phialid (1000x), (B) tip of a phialid and conidia (7500x), (C) conidial masses borne in mucilaginous material at the tips of conidiophores of *V. albo-atrum*, (D) melanized hyphae of *V. albo-atrum* formed in potato dextrose broth, (E) typical microsclerotia of *V. dahliae* formed on plum extract agar (PEA).
Fig. A7. Wilting of entire *A. altissima* leaf on potted greenhouse seedlings inoculated with .5 ml of $10^7$ *V. albo-atrum* conidia / ml.

Fig. A8. Defoliating chlorotic *A. altissima* leaflets on potted greenhouse seedlings inoculated with .5 ml of $10^7$ *V. dahliae* conidia / ml.
**Fig. A9.** Image of non- *A. altissima* host range study conducted in the greenhouse. White ash, sugar maple, and red oak were stem inoculated with *V. albo-astrum* (PSU140).

**Fig. A10.** Hypohatchet® used for inoculations of large field trees with *V. albo-astrum* (PSU 140), which injects 1 ml of suspension into hatchet wounds.
Fig. A11. Map of the non-*A. altissima* host range study plots, at each sample plot five trees of one species was inoculated with *V. albo-atrum* (PSU #140), along with one control inoculated with sterile distilled water. (CO= chestnut oak, RO= red oak, SM= sugar maple, RM= red maple, St M= striped maple, YP= yellow-poplar, WA= white ash)
Fig. A12. Image of blue *V. albo-astrum* $10^7$ conidial suspension made for use with the hypohatchet. Blue food coloring was added to conidial suspensions to visualize the inoculum and confirm that the hypohatchet was working correctly.
Fig. A13. 0.081 ha (1/5 acre) Permanent plots through the infested study area. Outlined in red is the diseased study area, outlined in black is the entire *A. altissima* stand.
Fig. A14. Sampling locations where overstory and understory plot data was recorded within the study area, showing the three sub-stands (control, moderate disease, and severe disease). Also the sampling locations for soil samples used in a soil assay for *Verticillium*.
Fig. A15. Black locust borer damage on black locust trees throughout the study area.

Fig. A16. Opportunistic *Botryosphaeria dothidea* canker on striped maple in the study area.
Fig. A17. Armillaria spp. mycelial fans and rhizomorphs on striped maple roots and lower stems of trees collected in our study area
Fig. A18. Isolation from striped maple cankers yielding *Botryosphaeria dothidea*.

In *V. albo-atrum*, resting structures are melanized hyphae, not microsclerotia.

Fig. A19. Typical *V. dahliae* disease cycle (Berlanger and Powelson, 2000)
Fig. A20. Map showing the location within the study area where samples were collected for tree-ring-analysis to determine the rate of spread and origin of the *V. albo-atrum* epidemic.
Fig. A21. Image of *A. altissima* crossections, collected for estimation of epidemic spread and origin of epidemic.

Fig. A22. Scanning EM images of *Euwallacea validus*. 
Fig. A23. Scanning EM images of *Xylosandrus germanus*.

Fig. A24. *A. altissima* bolt infested with ambrosial beetles, showing a fungi associated with beetle hole entrances.
Fig. A25. *E. validus* ambrosial beetle hole colonized by a fungal associate on *A. altissima*.

Fig. A26. Fungal isolation from *E. validus* beetles following a wash in 70% ethanol, and plated on PEA.
Fig. A27. Hyphae of *V. albo-atrum* in *A. altissima* xylem vessels stained with Chlorazol Black E.

Fig. A28. Female *A. altissima* trees showing seeds produced in clusters of twisted samaras.
Fig. A29. *A. altissima* seedlings dominating a canopy gap in the forest understory
Fig. A30. Map showing the location of nematode samples in relation to the epidemic study area.
Appendix B.  Chapter III, Root wounding Disease severity rating (DIS) Minitab output

General Linear Model: V. a-a Final Rating versus Treatment

Factor     Type   Levels  Values
Treatment  fixed       6  Control (Non-W), Control (W), V. albo-atrum (Non-W), V. albo-atrum (W), V. dahliae (Non-W), V. dahliae (W)

Analysis of Variance for V. a-a Final Rating, using Adjusted SS for Tests

Source      DF   Seq SS   Adj SS  Adj MS      F      P
Treatment    5  306.481  306.481  61.296  99.68  0.000
Error      122   75.019   75.019   0.615
Total      127  381.500

S = 0.784159   R-Sq = 80.34%   R-Sq(adj) = 79.53%

Unusual Observations for V. a-a Final Rating

<table>
<thead>
<tr>
<th>Rating</th>
<th>Final Rating</th>
<th>Final</th>
<th>SE Fit</th>
<th>Residual</th>
<th>St Resid</th>
</tr>
</thead>
<tbody>
<tr>
<td>28</td>
<td>0.00000</td>
<td>3.62963</td>
<td>0.15091</td>
<td>-3.62963</td>
<td>-4.72 R</td>
</tr>
<tr>
<td>35</td>
<td>0.00000</td>
<td>3.62963</td>
<td>0.15091</td>
<td>-3.62963</td>
<td>-4.72 R</td>
</tr>
<tr>
<td>73</td>
<td>1.00000</td>
<td>3.85185</td>
<td>0.15091</td>
<td>-2.85185</td>
<td>-3.71 R</td>
</tr>
<tr>
<td>102</td>
<td>4.00000</td>
<td>0.81481</td>
<td>0.15091</td>
<td>3.18519</td>
<td>4.14 R</td>
</tr>
</tbody>
</table>

R denotes an observation with a large standardized residual.

Tukey Simultaneous Tests
Response Variable V. a-a Final Rating
All Pairwise Comparisons among Levels of Treatment

Treatment = Control (Non-W) subtracted from:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Difference</th>
<th>SE of Difference</th>
<th>T-Value</th>
<th>Adjusted P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (W)</td>
<td>0.30000</td>
<td>0.3507</td>
<td>0.8555</td>
<td>0.9561</td>
</tr>
<tr>
<td>V. albo-atrum (Non-W)</td>
<td>3.22963</td>
<td>0.2903</td>
<td>11.1258</td>
<td>0.0000</td>
</tr>
<tr>
<td>V. albo-atrum (W)</td>
<td>3.45185</td>
<td>0.2903</td>
<td>11.8913</td>
<td>0.0000</td>
</tr>
<tr>
<td>V. dahliae (Non-W)</td>
<td>0.08148</td>
<td>0.2903</td>
<td>0.2807</td>
<td>0.9998</td>
</tr>
<tr>
<td>V. dahliae (W)</td>
<td>0.41481</td>
<td>0.2903</td>
<td>1.4290</td>
<td>0.7094</td>
</tr>
</tbody>
</table>

Treatment = Control (W) subtracted from:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Difference</th>
<th>SE of Difference</th>
<th>T-Value</th>
<th>Adjusted P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. albo-atrum (Non-W)</td>
<td>2.9296</td>
<td>0.2903</td>
<td>10.923</td>
<td>0.0000</td>
</tr>
<tr>
<td>V. albo-atrum (W)</td>
<td>3.1519</td>
<td>0.2903</td>
<td>10.8578</td>
<td>0.0000</td>
</tr>
<tr>
<td>V. dahliae (Non-W)</td>
<td>-0.2185</td>
<td>0.2903</td>
<td>-0.7528</td>
<td>0.9747</td>
</tr>
<tr>
<td>V. dahliae (W)</td>
<td>0.1148</td>
<td>0.2903</td>
<td>0.3955</td>
<td>0.9987</td>
</tr>
</tbody>
</table>

Treatment = V. albo-atrum (Non-W) subtracted from:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Difference</th>
<th>SE of Difference</th>
<th>T-Value</th>
<th>Adjusted P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. albo-atrum (W)</td>
<td>2.9296</td>
<td>0.2903</td>
<td>10.923</td>
<td>0.0000</td>
</tr>
<tr>
<td>V. dahliae (Non-W)</td>
<td>-0.2185</td>
<td>0.2903</td>
<td>-0.7528</td>
<td>0.9747</td>
</tr>
<tr>
<td>V. dahliae (W)</td>
<td>0.1148</td>
<td>0.2903</td>
<td>0.3955</td>
<td>0.9987</td>
</tr>
<tr>
<td>Treatment</td>
<td>Difference</td>
<td>SE of Difference</td>
<td>Adjusted Difference</td>
<td>T-Value</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>------------</td>
<td>-----------------</td>
<td>---------------------</td>
<td>---------</td>
</tr>
<tr>
<td>V. albo-atrum (W)</td>
<td>0.222</td>
<td>0.2134</td>
<td>1.04</td>
<td>0.9030</td>
</tr>
<tr>
<td>V. dahliae (Non-W)</td>
<td>-3.148</td>
<td>0.2134</td>
<td>-14.75</td>
<td>0.0000</td>
</tr>
<tr>
<td>V. dahliae (W)</td>
<td>-2.815</td>
<td>0.2134</td>
<td>-13.19</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

Treatment = V. albo-atrum (W) subtracted from:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Difference</th>
<th>SE of Difference</th>
<th>Adjusted Difference</th>
<th>T-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. dahliae (Non-W)</td>
<td>-3.370</td>
<td>0.2134</td>
<td>-15.79</td>
<td>0.0000</td>
<td></td>
</tr>
<tr>
<td>V. dahliae (W)</td>
<td>-3.037</td>
<td>0.2134</td>
<td>-14.23</td>
<td>0.0000</td>
<td></td>
</tr>
</tbody>
</table>

Treatment = V. dahliae (Non-W) subtracted from:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Difference</th>
<th>SE of Difference</th>
<th>Adjusted Difference</th>
<th>T-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. dahliae (W)</td>
<td>0.3333</td>
<td>0.2134</td>
<td>1.562</td>
<td>0.6251</td>
<td></td>
</tr>
</tbody>
</table>
Appendix C. Chapter III, Root wounding height growth (HT) Minitab output

General Linear Model: SqRtHeight versus Treatment

<table>
<thead>
<tr>
<th>Factor</th>
<th>Type</th>
<th>Levels</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>fixed</td>
<td>6</td>
<td>Control (Non-W), Control (W), V. albo-atrum (Non-W), V. albo-atrum (W), V. dahliae (Non-W), V. dahliae (W)</td>
</tr>
</tbody>
</table>

Analysis of Variance for SqRtHeight, using Adjusted SS for Tests

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Seq SS</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>5</td>
<td>213.349</td>
<td>213.349</td>
<td>42.670</td>
<td>24.66</td>
<td>0.000</td>
</tr>
<tr>
<td>Error</td>
<td>122</td>
<td>211.088</td>
<td>211.088</td>
<td>1.730</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>127</td>
<td>424.437</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S = 1.31538   R-Sq = 50.27%   R-Sq(adj) = 48.23%

Unusual Observations for SqRtHeight

<table>
<thead>
<tr>
<th>Obs</th>
<th>SqRtHeight</th>
<th>Fit</th>
<th>SE Fit</th>
<th>Residual</th>
<th>St Resid</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>0.00000</td>
<td>2.82623</td>
<td>0.25315</td>
<td>-2.82623</td>
<td>-2.19 R</td>
</tr>
<tr>
<td>64</td>
<td>6.70820</td>
<td>2.82623</td>
<td>0.25315</td>
<td>3.88197</td>
<td>3.01 R</td>
</tr>
<tr>
<td>78</td>
<td>3.00000</td>
<td>5.76378</td>
<td>0.25315</td>
<td>-2.76378</td>
<td>-2.14 R</td>
</tr>
<tr>
<td>87</td>
<td>1.41421</td>
<td>5.76378</td>
<td>0.25315</td>
<td>-4.34956</td>
<td>-3.37 R</td>
</tr>
<tr>
<td>124</td>
<td>3.46410</td>
<td>6.13172</td>
<td>0.25315</td>
<td>-2.66762</td>
<td>-2.07 R</td>
</tr>
</tbody>
</table>

R denotes an observation with a large standardized residual.

Tukey Simultaneous Tests

Response Variable SqRtHeight

All Pairwise Comparisons among Levels of Treatment

Treatment = Control (Non-W) subtracted from: 0.5362
V. dahliae (W)       -0.459    0.4869   -0.942   0.9348

Treatment = Control (W) subtracted from:

<table>
<thead>
<tr>
<th>Difference</th>
<th>SE of Difference</th>
<th>Adjusted Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment of Means</td>
<td>T-Value</td>
<td>P-Value</td>
</tr>
<tr>
<td>V. albo-atrum (Non-W)</td>
<td>-1.155</td>
<td>0.4869</td>
</tr>
<tr>
<td>V. albo-atrum (W)</td>
<td>-2.896</td>
<td>0.4869</td>
</tr>
<tr>
<td>V. dahliae (Non-W)</td>
<td>0.041</td>
<td>0.4869</td>
</tr>
<tr>
<td>V. dahliae (W)</td>
<td>0.409</td>
<td>0.4869</td>
</tr>
</tbody>
</table>

Treatment = V. albo-atrum (Non-W) subtracted from:

<table>
<thead>
<tr>
<th>Difference</th>
<th>SE of Difference</th>
<th>Adjusted Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment of Means</td>
<td>T-Value</td>
<td>P-Value</td>
</tr>
<tr>
<td>V. albo-atrum (W)</td>
<td>-1.741</td>
<td>0.3580</td>
</tr>
<tr>
<td>V. dahliae (Non-W)</td>
<td>1.196</td>
<td>0.3580</td>
</tr>
<tr>
<td>V. dahliae (W)</td>
<td>1.564</td>
<td>0.3580</td>
</tr>
</tbody>
</table>

Treatment = V. albo-atrum (W) subtracted from:

<table>
<thead>
<tr>
<th>Difference</th>
<th>SE of Difference</th>
<th>Adjusted Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>of Means</td>
<td>Difference</td>
</tr>
<tr>
<td>-----------</td>
<td>----------</td>
<td>------------</td>
</tr>
<tr>
<td>V. dahliae (Non-W)</td>
<td>2.938</td>
<td>0.3580</td>
</tr>
<tr>
<td>V. dahliae (W)</td>
<td>3.305</td>
<td>0.3580</td>
</tr>
</tbody>
</table>

Treatment = V. dahliae (Non-W) subtracted from:

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Difference</th>
<th>SE of</th>
<th>Adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. dahliae (W)</td>
<td>0.3679</td>
<td>0.3580</td>
<td>1.028</td>
</tr>
</tbody>
</table>
Appendix D. ITS sequence of *Verticillium* isolates used in inoculations.

*V. albo-astrum* isolate PSU 140 (GenBank accession # FJ424082):

ACCGAGTATCTACTCATAAACCCCTTTTGTGAAACCATATTGTGGTTCGCCG
CTCGTTCTGCGAGCCCGGCCGTACCATCAGTCTCTTTATCTCATAACCAACGA
TACTTCTGAGTGTCTTATTGAAGACTATTAAAAACTTTTAACAAACGGGATCTC
TGGTCTCTAGCATCGATGAAGAGCAGCAACGCGAATCTGATGTTGTA
ATTGCAGAAATTTCAGTGGAATCATCGAATTCTTTGAACGCACATGGCGCCTTC
CAGTATCCTGGGAGGCAATGCTGTCCCTGAGCCTCGTTTTCAACCCCTCGAGCC
CCAGTGGGCCCCTGTGGGAGATCTACGTCTGTAGGCCCCCTAAAAGCAGTG
GCGAACCCGCTGCGCCCTCTCCTTGCCTAGTTAATTACAGCTCGCATCGGAG
TCGCCAGGCACTTGCCTCTAAACCCCTAACAAGGCCGCTCGTGCAGGCA
ACGGTGGACCTCGGATCAAGTGGCTAATACCGCTGAACTTAAAGCATATCA
ATAAGCGGAGGAA

*V. dahliae* isolate PSU 154 (GenBank accession # FJ424083):

TCCTCCGCTTTATTGATATGCTTAAGTTCAGGCGGTATTCCCTACCTGATCC
GAGGTCAACGGTGCCGCACGAGGCCTTAGGGGTTTAGAGGAAGGCA
GGCCTGCGGACTCCGAGCAGCTGAATTACGCTACGCAAGGAGGCCC
ACGGGCGGTCCGCCACTGCTTTTAAAGGCCCTACAGACGTAGATCCCAACA
CCGGGACACTGCGGCTGCGAGGGTTGAAACGACGCTCGGAGAGCATGCCCT
CCAGGATACGGAAGGCGGAGGCGCAGTGCGTTAAAGATCGATGATTCAT
GAATTCTGCAATTACACATTACATACGCTGTTTCGCTCGTCTTCCATCA
TGCTAGAACAGATCTGCTTGAATAGTTAATAGGTCCCTGCTAGGAA
CAGTGAAAGTATCGGTGTTATAAACAGAGAGACTGATGGACCAGCCGCGG
TCGCAGAAACGAGCCGCGAAGCAAATAATGTGGTTCAAAAGGTTATGAG
TAGATACTCGGTAAATGATCCCTCGCTGTT
Appendix E. NCBI BLASTN Results (National Center for Biotechnology Information, Bethesda, MD) of isolates used in inoculations

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query coverage</th>
<th>E value</th>
<th>Max ident</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB458830.1</td>
<td><em>Verticillium albo-atrum</em> genes for ITS1, 5.8S rRNA, ITS2, complete sequence, strain: VA001</td>
<td></td>
<td>941</td>
<td>99%</td>
<td>0</td>
<td>99%</td>
</tr>
<tr>
<td>AF108476.1</td>
<td><em>Verticillium albo-atrum</em> isolate UAMH 5393 internal transcribed spacer 1, complete sequence</td>
<td>933</td>
<td>933</td>
<td>99%</td>
<td>0</td>
<td>99%</td>
</tr>
<tr>
<td>AF104926.1</td>
<td><em>Verticillium dahliae</em> 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence</td>
<td>915</td>
<td>915</td>
<td>100%</td>
<td>0</td>
<td>98%</td>
</tr>
<tr>
<td>EU835817.1</td>
<td><em>Verticillium dahliae</em> isolate cotton001 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence</td>
<td>913</td>
<td>913</td>
<td>99%</td>
<td>0</td>
<td>98%</td>
</tr>
<tr>
<td>AF108478.1</td>
<td><em>Verticillium dahliae</em> isolate UAMH 5360 internal transcribed spacer 1, complete sequence</td>
<td>909</td>
<td>909</td>
<td>99%</td>
<td>0</td>
<td>98%</td>
</tr>
<tr>
<td>AB370339.1</td>
<td><em>Verticillium dahliae</em> genes for ITS1, 5.8S rRNA, ITS2, 28S rRNA, complete and partial sequence, isolate: VD</td>
<td>904</td>
<td>904</td>
<td>98%</td>
<td>0</td>
<td>98%</td>
</tr>
<tr>
<td>EF015891.1</td>
<td><em>Verticillium dahliae</em> isolate DB 24102006 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence</td>
<td>900</td>
<td>900</td>
<td>99%</td>
<td>0</td>
<td>98%</td>
</tr>
<tr>
<td>AJ970308.1</td>
<td><em>Verticillium dahliae</em> 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, isolate 813</td>
<td>898</td>
<td>898</td>
<td>98%</td>
<td>0</td>
<td>98%</td>
</tr>
<tr>
<td>AJ865691.1</td>
<td><em>Verticillium dahliae</em> 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, strain MAT-756</td>
<td>887</td>
<td>887</td>
<td>97%</td>
<td>0</td>
<td>98%</td>
</tr>
<tr>
<td>AB353342.1</td>
<td><em>Verticillium albo-atrum</em> genes for ITS1, 5.8S rRNA, ITS2, partial and complete sequence, isolate: HP</td>
<td>885</td>
<td>885</td>
<td>93%</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>Accession</td>
<td>Description</td>
<td>Max score</td>
<td>Total score</td>
<td>Query coverage</td>
<td>E value</td>
<td>Max ident</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------------------------------------------------------</td>
<td>-----------</td>
<td>-------------</td>
<td>----------------</td>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>AF104926.1</td>
<td><em>Verticillium dahliae</em> 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, internal transcribed spacer 2, and 28S ribosomal RNA gene, complete sequence</td>
<td>981</td>
<td>981</td>
<td>100%</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>EU835817.1</td>
<td><em>Verticillium dahliae</em> isolate cotton001 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence</td>
<td>976</td>
<td>976</td>
<td>100%</td>
<td>0</td>
<td>99%</td>
</tr>
<tr>
<td>AJ970308.1</td>
<td><em>Verticillium dahliae</em> 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene, ITS1 and ITS2, isolate 813</td>
<td>966</td>
<td>966</td>
<td>98%</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>EF015891.1</td>
<td><em>Verticillium dahliae</em> isolate DB 24102006 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence</td>
<td>952</td>
<td>952</td>
<td>98%</td>
<td>0</td>
<td>99%</td>
</tr>
<tr>
<td>AJ865691.1</td>
<td><em>Verticillium dahliae</em> 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene, strain MAT-756</td>
<td>950</td>
<td>950</td>
<td>96%</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>AF108478.1</td>
<td><em>Verticillium dahliae</em> isolate UAMH 5360 internal transcribed spacer 1, complete sequence</td>
<td>948</td>
<td>948</td>
<td>97%</td>
<td>0</td>
<td>99%</td>
</tr>
<tr>
<td>AB458830.1</td>
<td><em>Verticillium albo-atrum</em> genes for ITS1, 5.8S rRNA, ITS2, complete sequence, strain: VA001</td>
<td>937</td>
<td>937</td>
<td>100%</td>
<td>0</td>
<td>98%</td>
</tr>
<tr>
<td>AB370339.1</td>
<td><em>Verticillium dahliae</em> genes for ITS1, 5.8S rRNA, ITS2, 28S rRNA, complete and partial sequence, isolate: VD</td>
<td>935</td>
<td>935</td>
<td>95%</td>
<td>0</td>
<td>100%</td>
</tr>
<tr>
<td>DQ458769.1</td>
<td><em>Verticillium sp. VS-6</em> 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence</td>
<td>922</td>
<td>922</td>
<td>95%</td>
<td>0</td>
<td>99%</td>
</tr>
<tr>
<td>AF108476.1</td>
<td><em>Verticillium albo-atrum</em> isolate UAMH 5393 internal transcribed spacer 1, complete sequence</td>
<td>917</td>
<td>917</td>
<td>97%</td>
<td>0</td>
<td>98%</td>
</tr>
</tbody>
</table>
### Appendix F. GPS Locations of Plots

#### Locations of naturally infected *Ailanthus* trees with *Verticillium*

<table>
<thead>
<tr>
<th>Location Name</th>
<th>Species found</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>County-Line</td>
<td><em>V. albo-astrum</em></td>
<td>77°35'57.214&quot;W 40°13'22.754&quot;N</td>
</tr>
<tr>
<td>Prince Knob</td>
<td><em>V.dahliae</em></td>
<td>77°36'55.738&quot;W 40°12'41.194&quot;N</td>
</tr>
<tr>
<td>Hemlocks NA</td>
<td><em>V.dahliae</em></td>
<td>77°38'6.984&quot;W 40°15'35.068&quot;N</td>
</tr>
<tr>
<td>Book Farm</td>
<td><em>V.dahliae</em></td>
<td>77°41'58.533&quot;W 40°14'45.026&quot;N</td>
</tr>
<tr>
<td>Blue Mountain</td>
<td><em>V.dahliae</em></td>
<td>77°37'25.424&quot;W 40°11'1.111&quot;N</td>
</tr>
</tbody>
</table>

#### Locations of 2006 and 2007 *V. albo-astrum/Ailanthus* Inoculations

<table>
<thead>
<tr>
<th>Species</th>
<th>Plot Number</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ailanthus</em></td>
<td>Vaa-1-06</td>
<td>77°38'28.845&quot;W 40°10'25.166&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vaa-2-06</td>
<td>77°38'1.966&quot;W 40°10'45.55&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vaa-3-06</td>
<td>77°37'34.415&quot;W 40°11'15.341&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vaa-4-06</td>
<td>77°37'11.568&quot;W 40°11'29.9&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vaa-1-07</td>
<td>77°48'12.901&quot;W 40°21'18.623&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vaa-2-07</td>
<td>77°47'51.193&quot;W 40°21'27.045&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vaa-3-07</td>
<td>77°47'22.874&quot;W 40°21'46.607&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vaa-4-07</td>
<td>77°46'1.532&quot;W 40°22'56.768&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vaa-5-07</td>
<td>77°44'13.389&quot;W 40°24'48.664&quot;N</td>
</tr>
</tbody>
</table>

#### Locations of 2006 *V.dahliae/Ailanthus* Inoculations

<table>
<thead>
<tr>
<th>Species</th>
<th>Plot Number</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ailanthus</em></td>
<td>Vd-1-06</td>
<td>77°39'24.694&quot;W 40°15'18.316&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vd-2-06</td>
<td>77°35'54.971&quot;W 40°13'54.958&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vd-3-06</td>
<td>77°36'55.739&quot;W 40°30'24.777&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vd-4-06</td>
<td>77°37'13.211&quot;W 40°30'7.642&quot;N</td>
</tr>
<tr>
<td><em>Ailanthus</em></td>
<td>Vd-5-06</td>
<td>77°38'25.784&quot;W 40°29'32.027&quot;N</td>
</tr>
<tr>
<td>Locations of 2007 <em>V. albo-astrum</em> Inoculations to Other Species</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Species</strong></td>
<td><strong>Plot Number</strong></td>
<td><strong>Location</strong></td>
</tr>
<tr>
<td>Red maple</td>
<td>RM-1-07</td>
<td>77°36'44.324&quot;W 40°30'34.366&quot;N</td>
</tr>
<tr>
<td>Red maple</td>
<td>RM-2-07</td>
<td>77°37'6.685&quot;W 40°30'12.435&quot;N</td>
</tr>
<tr>
<td>Red maple</td>
<td>RM-3-07</td>
<td>77°37'12.112&quot;W 40°30'3.971&quot;N</td>
</tr>
<tr>
<td>Striped maple</td>
<td>StM-1-07</td>
<td>77°40'3.367&quot;W 40°28'2.659&quot;N</td>
</tr>
<tr>
<td>Striped maple</td>
<td>StM-2-07</td>
<td>77°40'11.526&quot;W 40°27'57.194&quot;N</td>
</tr>
<tr>
<td>Striped maple</td>
<td>StM-3-07</td>
<td>77°41'24.647&quot;W 40°27'9.273&quot;N</td>
</tr>
<tr>
<td>Sugar maple</td>
<td>SM-1-07</td>
<td>77°45'28.777&quot;W 40°23'27.25&quot;N</td>
</tr>
<tr>
<td>Sugar maple</td>
<td>SM-2-07</td>
<td>77°48'2.32&quot;W 40°21'18.066&quot;N</td>
</tr>
<tr>
<td>Yellow-poplar</td>
<td>YP-1-07</td>
<td>77°46'43.214&quot;W 40°22'10.518&quot;N</td>
</tr>
<tr>
<td>Yellow-poplar</td>
<td>YP-2-07</td>
<td>77°46'12.523&quot;W 40°22'39.248&quot;N</td>
</tr>
<tr>
<td>White ash</td>
<td>WA-1-07</td>
<td>77°44'24.398&quot;W 40°24'37.438&quot;N</td>
</tr>
<tr>
<td>White ash</td>
<td>WA-2-07</td>
<td>77°45'51.045&quot;W 40°23'5.275&quot;N</td>
</tr>
<tr>
<td>Red oak</td>
<td>RO-1-07</td>
<td>77°43'45.815&quot;W 40°25'10.348&quot;N</td>
</tr>
<tr>
<td>Red oak</td>
<td>RO-2-07</td>
<td>77°39'34.063&quot;W 40°28'24.971&quot;N</td>
</tr>
<tr>
<td>Chestnut oak</td>
<td>CO-1-07</td>
<td>77°40'20.279&quot;W 40°27'49.18&quot;N</td>
</tr>
<tr>
<td>Chestnut oak</td>
<td>CO-2-07</td>
<td>77°42'18.985&quot;W 40°26'27.995&quot;N</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Locations of Permanent plots</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plot #</strong></td>
</tr>
<tr>
<td>PP1</td>
</tr>
<tr>
<td>PP2</td>
</tr>
<tr>
<td>PP3</td>
</tr>
<tr>
<td>PP4</td>
</tr>
<tr>
<td>PP5</td>
</tr>
<tr>
<td>PP6</td>
</tr>
<tr>
<td>PP7</td>
</tr>
<tr>
<td>PP8</td>
</tr>
<tr>
<td>PP9</td>
</tr>
<tr>
<td>PP10</td>
</tr>
<tr>
<td>PP11</td>
</tr>
<tr>
<td>PP12</td>
</tr>
</tbody>
</table>
VITA

Mark J. Schall

401 Buckhout Laboratory, Penn. State University, University Park, PA 16802 – mjs457@psu.edu

Education:
2004.................................................................B.A. in Forest Science
The Pennsylvania State University

2002.................................................................A.A.S. in Forest Technology
Pennsylvania College of Technology

Professional Experience:
2005-2008 .........................................................Graduate Research Assistant
The Pennsylvania State University

2003-2004 ........................................................Forest Technician
Pennsylvania Game Commission

2000-2002 ........................................................Forest Technician and Surveyor
Beale Forestry and Land Surveying

Teaching Assistant Experience:
2005, 2006, 2007, 2008...........................................Disease of Forest and Shade Trees
Professor- Dr. Donald Davis.............................The Pennsylvania State University

2007.................................................................Biology of Fungi
Associate Professor- Dr. David Geiser..............The Pennsylvania State University

Honors and Awards:
- Student Research Award (Paper Presentation) 2nd place, American Phytopathological Society Northeast Division Meeting, 2008
- Lester P. Nichols Memorial Award, for outstanding research accomplishments as a graduate degree candidate, 2007 – 2008
- Lester P. Nichols Memorial Award, for outstanding research accomplishments as a graduate degree candidate, 2006 – 2007
- Student Research Award (Paper Presentation) 2nd place, American Phytopathological Society Potomac Division Meeting, 2007
- Outstanding Student Presentation 1st place, Northeast and Southwide Forest Disease Workshop, 2006

Memberships:
- Plant Pathology Association at Penn State (Vice-president 2007, 2008)
- American Phytopathological Society
- Society of American Foresters
- The Pennsylvania Forestry Association
- Pennsylvania Farm Bureau