

**Role of an Ambrosia Beetle (*Euwallacea validus*) in Dissemination  
of the Fungus *Verticillium albo-atrum*, Causal Agent of Verticillium Wilt  
of Tree-of-Heaven (*Ailanthus altissima*)**

**Submitted by**

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**Cooperators**

The technical contact for this proposal is Dr. Donald D. Davis, representing the Department of Plant Pathology, The Pennsylvania State University, University Park, PA. Dr. Thomas Hall, representing the Pennsylvania Department of Conservation and Natural Resources, Bureau of Forestry, Harrisburg, PA, and Dr. Yun Wu representing Forest Service, Forest Health Technology Enterprise Team, Morgantown, WV will serve as consultants. The BCIP contact person for the proposed project is Dr. Richard Reardon representing Forest Service, Forest Health Technology Enterprise Team, Morgantown, WV.

**Amount Requested; Leverage is Shown in the Attached Budget**

We request \$37,225 from the USDA Forest Service over a 2-year project period (1 October 2011 – 30 September 2013)

**Project Goal**

The overall goal of the proposed project is to develop an effective biological control for management of the highly invasive, non-native, tree-of-heaven [*Ailanthus altissima* (Mill.) Swingle], hereafter called *Ailanthus*. The proposed project will expand and supplement existing studies funded by a current Cooperative Agreement between the USDA Forest Service and Penn State. The proposed Principal Investigator (PI, Dr. D.D. Davis) has discussed the proposed project with Dr. Richard Reardon, who finds the proposed expansion not in conflict with the existing Cooperative Agreement.

**Supporting Statements**

During 2002-2003, the proposed PI discovered a severe, naturally occurring disease of unknown etiology killing large numbers of the highly invasive, non-native *Ailanthus* in south-central Pennsylvania. We determined that the common soil-borne fungus *Verticillium albo-atrum* Reinke & Berthier was causing the disease, which we termed Verticillium Wilt of *Ailanthus* (Schall and Davis 2009a). We made numerous isolations of *V. albo-atrum* from dying *Ailanthus* throughout the area and randomly selected isolate PSU140 to conduct initial host range studies involving a small number of cohort forest species (Schall and Davis 2009b). We have since expanded this initial host range study, and have (as of fall 2010) evaluated the susceptibility of approximately 75 plant species

to *V. albo-atrum* isolate PSU140, including TAG species recommended by the Technical Advisory Group (TAG).

Our initial studies indicate that the form of *V. albo-atrum* present within the forests of south-central Pennsylvania has become host-adapted to *Ailanthus*. From a pathological standpoint, but not in terms of rigid taxonomy, a fungus that has become adapted to a specific host plant species is termed *forma specialis* (i.e., *Verticillium albo-atrum forma specialis ailanthi*). We now know the cause of *Ailanthus* wilt and the approximate host range of the pathogen. The next logical step in pursuing *V. albo-atrum* as a potential biocontrol agent for *Ailanthus* is to gain a more thorough understanding of the *Verticillium-Ailanthus* disease cycle. It is especially important to ascertain how the pathogen moves so rapidly (i.e., the dissemination phase of the disease cycle) within the forest setting.

### Project Justification

**Background.** *Ailanthus altissima* was first introduced into the U.S. from China (via Europe) in 1784, as an ornamental for an arboretum in Philadelphia. Chinese immigrants on the West Coast, who had come to the U.S. to work in the gold fields and build the transcontinental railroad, made further introductions in the mid-1850s. *Ailanthus* has since spread across most of the U.S. within urban, agricultural, and forested areas (Dirr 1998, Miller 1990) and has become one of the most invasive plants in our country. Successful biocontrol of the non-native, highly invasive *Ailanthus* would have national implications.

The invasive *Ailanthus* is an early-successional pioneer tree species, intolerant of shade, and relatively short-lived. However, the species is fast growing and can reach 27 m in height and 100 cm in diameter (Dirr 1998, Miller 1990). State records for the tallest and largest diameter *Ailanthus* trees in Pennsylvania are: height = 31.8 m (104.3 feet) and diameter = 144.3 cm (56.8 inches); tree ages are unknown for these record trees ([http://www.pabigtrees.com/trees/species/ailanthus\\_tree\\_of\\_heaven.htm](http://www.pabigtrees.com/trees/species/ailanthus_tree_of_heaven.htm)).

*Ailanthus* is a very prolific seeder, capable of producing more than 300,000 seeds/tree/year by age 12. Seeds can be dispersed immediately after ripening in early fall, or can persist on the tree through winter and be dispersed in the spring. Seeds require no stratification, but rather germinate immediately upon contact with soil. Following seed germination, resultant seedlings can reach 1-2 m in height within the first growing season (Dirr 1998, Wyman 1990).

The invasive *Ailanthus* has many negative features. The species produces several quassinoid chemicals, including ailanthone (Heisey 1996), that possess allelopathic properties (Lin *et al.* 1995). Ailanthone has strong herbicidal activity against 70 hardwood and coniferous tree species (Mergen 1959). These phytotoxic, allelopathic compounds give *Ailanthus* a competitive advantage over native plant species, which they supplant by killing them outright or by preventing their establishment. *Ailanthus* pollen is a known human allergen, and the leaves and stems cause contact dermatitis (Derrick and

Darley 1994). *Ailanthus* wood is light and weak, splitting and breaking in wind, snow, and ice storms. The brittle wood makes the species especially hazardous along highways and in public areas. Control of *Ailanthus* along major roadways is a major expense for many state highway departments, which currently use repeated applications of chemical herbicides in attempts to control the species. However, use of chemicals in public places is coming under increasingly rigid public scrutiny and restrictions (TNC 1995). *Ailanthus* also invades forests, especially at the edges and within canopy gaps, where herbicidal applications may not be practical.

There is an urgent need to develop biocontrol components within existing IPM programs (TeBeest *et al.* 1992) for invasive species such as *Ailanthus*. Farr *et al.* (1989) listed 44 fungi that attack *Ailanthus*, causing leafspots, twig and branch dieback, decay, cankers, and root rot, most of which are not lethal. Ding *et al.* (2006) assessed potential biocontrol agents of *Ailanthus*, including arthropods, 68 species of fungi, and one potyvirus, some of which showed promise as biocontrol agents. However, until our studies at Penn State, biocontrol research for *Ailanthus* had not progressed beyond the theoretical stage, due to a lack of a highly lethal pathogen.

**Previous Research at Penn State.** In the early 2000s, the proposed PI observed wilting *Ailanthus* within a single canopy gap in the Tuscarora State Forest in south-central Pennsylvania. Within 2 years, all *Ailanthus* in the canopy gap were dead. Pennsylvania DCNR Bureau of Forestry personnel, alerted to this fact, soon reported a large stand that contained thousands of dead and dying *Ailanthus* seedlings, root sprouts, saplings, and canopy trees. Intermingled oaks, maples, and other cohort species appeared to be unharmed. (We later established permanent study plots, confirming that intermingled non-*Ailanthus* trees (oaks, maples, etc.) were not exhibiting wilt symptoms).

Upon visiting the stand, we observed that the dying *Ailanthus* displayed wilting and vascular discoloration, symptoms implicating a fungal wilt pathogen such as *Fusarium* or *Verticillium* (Agrios 2005, Sinclair and Lyon 2005). However, a literature review conducted at the time revealed found no *Fusarium* or *Verticillium* species capable of quickly killing such large numbers of *Ailanthus* within a forest setting and on forest soils.

The cause (etiology) of the wilt disease of *Ailanthus* was determined by fulfilling Koch's Postulates (Agrios 2005). We isolated from diseased *Ailanthus* trees, cultured the suspected pathogen, re-inoculated the suspected pathogen into potted *Ailanthus* seedlings in the greenhouse and *Ailanthus* canopy trees in the field, produced classic symptoms, and re-isolated the pathogen (Schall and Davis 2009a). As previously stated, the pathogen proved to be the common, indigenous, soil-borne fungus *Verticillium albo-atrum*. Initial pathogen identification was based on culture morphology, later confirmed using PCR and DNA sequencing.

#### **Related Current Research at Penn State**

**Host Range Studies and *Ailanthus* Inoculations.** As part of their dissertation research, funded by our current USDA Forest Service Cooperative Agreement, Dr. Davis and his PhD graduate students (Mark Schall and Matthew Kasson) have inoculated

canopy *Ailanthus* trees with *V. albo-atrum* isolate PSU140 within 15 sites within south-central Pennsylvania. These inoculations have yielded a large dataset regarding the high level of pathogenicity of this form of *V. albo-atrum* against *Ailanthus*. The following data from 4 of the 15 inoculation sites illustrate this high level of pathogenicity, as well as the rapidity at which the epidemic progresses.

1. Blue Mountain: we inoculated 20 *Ailanthus* trees in late April 2006. As of November 2009, there were 2,663 *Ailanthus* trees that were dead and dying at the site. The disease has spread rapidly outward from the original point of inoculation. Intermingled oaks, maples, and other cohort species appear unharmed.

2. Canoe Creek State Park: we inoculated 20 *Ailanthus* trees in June 2009 within the state park, as a demonstration site in an area suffering from *Ailanthus* invasions. As of July 2010, there were 503 *Ailanthus* trees that were dead and dying at the site as a result of natural spread from the point of inoculation. Intermingled oaks, maples, and other cohort species appear unharmed.

3. Buchanan State Forest: we inoculated 10 *Ailanthus* trees in May 2008. As of August 2010, there were 941 *Ailanthus* trees that were dead and dying at the site as a result of natural spread from point of inoculation. Intermingled oaks, maples, and other cohort species appear unharmed.

4. PA State Game Lands No. 211: we inoculated 10 *Ailanthus* trees in May 2008. As of August 2010, there were 701 *Ailanthus* trees that were dead and dying at the site as a result of natural spread from point of inoculation. Intermingled oaks, maples, and other cohort species appear unharmed.

All sites were within mixed hardwood stands that contained a substantial component of canopy *Ailanthus* trees. *Verticillium albo-atrum* appears to spread and incite wilt until all *Ailanthus* are killed within a site (site size not yet determined). Again, intermingled oaks, maples, and other non-*Ailanthus* cohort species appear unharmed, even within the most severely infested areas. We are cautiously optimistic about this last result, and are still investigating the host range.

Nevertheless, our results to date indicate that the form (*forma specialis*) of *V. albo-atrum* causing Verticillium wilt of *Ailanthus* on south-central Pennsylvania is likely host-adapted. However, it is unknown why this form of the common, soil-borne, *V. albo-atrum* has begun to attack *Ailanthus* in recent years, a condition that had not occurred previously to the best of our knowledge. Possibly some change occurred in one of the triads of the classic disease triangle: host, pathogen, and environment (Agrios 2005). First, with regard to host, perhaps the *A. altissima* plants (hosts) in south-central Pennsylvania are genetically different (*i.e.*, more susceptible) compared to *A. altissima* from other parts of the U.S. Secondly, it is possible that this form of *V. albo-atrum* (pathogen) has mutated in recent years to become host-adapted to *Ailanthus*, or an adapted form was introduced. Thirdly, some factor of the environment (*i.e.*, climate change, introduction of an exotic insect vector, etc.) may have occurred that is more conducive to Verticillium wilt development. Regardless, we now estimate that at least 10,000 *Ailanthus* trees have died from Verticillium wilt in south-central Pennsylvania. In addition, two outbreaks of Verticillium wilt of *Ailanthus* were recently discovered in

Virginia by Dr. Scott Salom's group at Virginia Tech, and we found one new outbreak in west-central Pennsylvania near the Ohio border. Dr. Salom's group isolated *V. albo-atrum* from the dead and dying *Ailanthus* trees in Virginia. We visited both the Virginia and west-central Pennsylvania sites, sampled symptomatic *Ailanthus* trees, and also recovered *V. albo-atrum* from dead and dying trees. However, it is unknown if these are the same form of *V. albo-atrum* (see next paragraph).

**Genetic Studies with *Verticillium albo-atrum*.** Under our current USDA Forest Service Cooperative Agreement, we are also sequencing the complete genome of PSU140 to compare its DNA sequence with a *V. albo-atrum* isolate from alfalfa in Pennsylvania. The alfalfa isolate is the only *V. albo-atrum* isolate whose genome has been completely sequenced (see [http://www.broadinstitute.org/annotation/genome/verticillium\\_dahliae/GenomeDescriptions.html#VA\\_VaMs.102](http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/GenomeDescriptions.html#VA_VaMs.102)) and represents a Vegetative Compatibility (VC) group of *V. albo-atrum* that is different from the VC group of isolate PSU140. (There are only two VC groups of *V. albo-atrum*, but this area is greatly under-researched, and there may be many more groups). As stated in the above link, "The genomic variation identified through such comparative analysis will help elucidate the molecular mechanisms that underly pathogenicity, differentiation, and host-adapted virulence." Such bioinformatic comparisons of the complete, known genome from the alfalfa isolate vs. PSU140, may shed light on the origin of isolate PSU140 and reasons for its host adaptation to *Ailanthus*. We also plan to compare four selected genes from different isolates of *V. albo-atrum* cultured from *Ailanthus*, to determine if all isolates are of the same genetic structure (as can be determined from four genes). These genetic studies will allow us to explore phylogenetic relationships among different isolates of *V. albo-atrum*, including isolates from the new *Ailanthus* wilt outbreak in extreme west-central Pennsylvania, the outbreak in Virginia, and from numerous cultures isolated from *Ailanthus* in Pennsylvania.

### **Proposed FY 2011 Cooperative Agreement Studies**

We now know the etiology and approximate host range of Verticillium wilt, but the method of *V. albo-atrum* spread (dissemination) in the forest is unknown. Although root grafts and root sprouts are likely involved in slow, short-distance, tree-to-adjacent tree spread, the method(s) by which the pathogen moves so rapidly (dissemination) within the forest setting needs to be determined, if *V. albo-atrum* is to become a valid biocontrol agent for *Ailanthus*.

The speed at which the epidemic has progressed (and is still progressing) implicates a very efficient means of pathogen dissemination. In this regard, we have consistently observed large numbers of ambrosia beetles attacking *Ailanthus* trees that were dead or dying from Verticillium wilt throughout our study area. Other insects, such as bark beetles or the *Ailanthus* webworm, have not been routinely associated with the disease. We hypothesize that ambrosia beetles serve as agents of dissemination (vectors) of *V. albo-atrum*. In this Proposed Cooperative Agreement, we propose to study the relationship between ambrosia beetles and *V. albo-atrum* within the *Verticillium-Ailanthus* pathosystem?

To collect preliminary data, we have begun trapping beetles at selected *Ailanthus* wilt research sites under our current USDA Forest Service Cooperative Agreement. Our trapping results to date indicate that *Euwallacea validus* (identified by Dr. Robert Rabaglia, USDA Forest Service, Washington, DC) is the most common ambrosia beetle associated with symptomatic *Ailanthus*. *Euwallacea validus* was introduced into the United States from China several decades ago, but has only recently reached south-central Pennsylvania, coincidentally at approximately the same time that *Verticillium* wilt of *Ailanthus* was first discovered in the area. We propose to examine possible relationships between *E. validus* and *V. albo-atrum* infections of *Ailanthus*, and to determine if infested beetles can successfully inoculate healthy or stressed *Ailanthus* trees, inducing *Verticillium* wilt.

It is also important to identify the fungal symbiont that *E. validus* carries within its mycangia. If the symbiont proves to be a *Verticillium* sp., or if the beetle mycangia contain another species plus *Verticillium* sp., this could have significant implications in the *Ailanthus-Verticillium* pathosystem.

## **Objectives**

The proposed objectives of this proposed 2-year Cooperative Agreement are to determine 1) if *E. validus* can disseminate *V. albo-atrum* and induce infection in *Ailanthus* trees; and 2) identify the species of fungal symbiont that *E. validus* is carrying within its mycangia. Proposed funding will complement our existing *Ailanthus* biocontrol project that is funded under our current USDA Forest Service Cooperative Agreement. Funding will be used primarily to fund Mr. Kasson's (PhD candidate) dissertation research.

## **Research Approach**

### **Objective 1): Dissemination of *V. albo-atrum* by *E. validus***

To meet this objective, a mixed hardwood stand containing at least 130 *Ailanthus* trees will be located in Pennsylvania outside of areas known to contain *Verticillium* wilt of *Ailanthus*. During April 2011, 10 *Ailanthus* trees will be numbered and inoculated with *Verticillium albo-atrum* isolate PSU140 in the approximate geographic center of the stand. Permanent plots will be established in two directions along a SW (upwind from point of inoculation) to NE (downwind from point of inoculation) transect at 10, 100, and 1000 m from the inoculation point in each direction, resulting in a 2-km gradient with the inoculation point in the center. Within each plot along the gradient, 10 *Ailanthus* trees will be wounded (total n=60) and 10 *Ailanthus* trees will remain non-wounded (total n=60) as control trees. Trees will be numbered using paint. This experimental design will result in a total of 10 inoculated *Ailanthus* trees and 120 evaluation trees. If additional suitable sites and *Ailanthus* trees are located, the study will be replicated.

Based on our previous observations, ambrosia beetles (mainly *Euwallaceae validus*) will be quickly attracted to the dead and dying inoculated *Ailanthus* trees in the center of the

transect. We hypothesize that *E. validus* will pick up *V. albo-atrum* PSU140 from the inoculated trees, fly to the wounded *Ailanthus* trees, and inoculate them with *V. albo-atrum* PSU140 when making galleries. Wounded trees within plots located upwind from the inoculation center will produce stress compounds (*i.e.*, ethanol) that will travel downwind to the inoculation center, possibly attracting the beetles, which will have to then fly upwind to reach the wounded trees. The converse is true from plots located downwind from the inoculation center. Our data will reveal such spatiotemporal trends, if the *Verticillium* wilt epidemic is introduced to *Ailanthus*.

Beginning 2 weeks after inoculation, and at biweekly intervals, all inoculated *Ailanthus* trees and all *Ailanthus* trees at each of the 6 permanent plots will be evaluated. During each plot visit, numbers of *E. validus* present will be counted on the outside bark of *Ailanthus* trees. Our previous research shows that this usually occurs twice a year, in May and July. We can make putative beetle identifications, based on 2 years of beetle trapping. Beetle subsamples from stems and galleries (a small number of infested stems will be sacrificed and beetles removed from galleries) will be mailed to Dr. Robert Rabaglia, USDA Forest Service, for species verification. Beetle subsamples will be cultured onto plum-extract agar amended with antibiotics (Schall and Davis 2007a,b) to detect presence of *V. albo-atrum*.

Each plot tree will be examined for 2 mm-dia gallery holes, which we have associated with *E. validus*. Number of holes will be counted and expressed as number holes/m<sup>2</sup> of stem. Macerated wood tubes extruding from the holes will be collected, returned to the laboratory, and cultured onto plum-extract agar amended with antibiotics for *V. albo-atrum*.

Data summarization and statistical analyses is not part of this proposal.

### **Objective 2): Identification of fungi in mycangia of *E. validus***

This research is part of Mr. Kasson's PhD dissertation. Initial work by Mark Schall and Matthew Kasson revealed the following fungi associated with the ambrosia beetle *E. validus*: *Fusarium*, *Graphium*, *Penicillium*, *Sporothrix*, *Verticillium*, and unknown yeasts. If *Verticillium* proves to be the *E. validus* fungal symbiont, in a manner similar to the bay laurel wilt disease complex where the fungal symbiont also causes wilt in the host tree (Fraedrich *et al.* 2008), this would have important consequences in the *Verticillium* wilt of *Ailanthus* disease cycle. We propose to determine the fungal species carried by *E. validus* as mycangial symbiont(s). Our working hypothesis is that the symbiont fungus is not *V. albo-atrum*.

The proposed research will be conducted using only female *E. validus*, since the males are very scarce on infested *Ailanthus* trees. Thirty adult females will be collected in July from infested bolts of *Ailanthus* within three geographically separated *Ailanthus* stands (10 beetles/stand). Live beetles will be surface sterilized and individually transferred to PCR tubes containing 100% ethanol for 1-2 minutes. Beetles will be aseptically disarticulated into head, thorax, and abdomen sections to relate presence of fungal

propagules to respective body parts (Fraedrich et al. 2008). Since *E. validus* has mandibular mycangia (Goto 1998), we expect the fungal symbiont to be associated with the head region.

After separation, body parts will be placed individually into 1.5 mL eppendorf tubes; 800 µL of sterilized MilliQ water will be added to each tube. Contents will be macerated using disposable micropestels; 40 µL of sterile milliQ water will be added to a 40-µL aliquot of the suspension. Aliquots of the 80 µL suspension will be placed onto several types of agar media, including media specific to expected fungi genera, using a flame-sterilized glass rod used to spread the suspension evenly across each plate. Plates will be placed into a controlled environment chamber and evaluated 4-7 days after plating. Fungi will be characterized morphologically and assigned to a putative genus. Number of fungal units/genus will be quantified with regard to associated body part. Emphasis will involve fungal genera associated with beetle head regions.

Representative isolates from the most common fungal genera will be sub-cultured and maintained within controlled-environment chambers on PDB (Potato dextrose broth) for 7 days until mycelial growth plugs are apparent. Plugs will be aseptically removed, dried, and transferred to individual 1.5 mL eppendorf tubes for DNA extraction. The Internally Transcribed Spacer (ITS) region will be amplified using a polymerase chain reaction (PCR) on extracted DNA. Primers ITS4 and ITS5 will be used to amplify approximately 500 bases of the ITS gene region. Once products are confirmed via agarose gel electrophoresis, products will be sequenced and blasted against NCBI's BLAST database to determine species within each genus. Most ambrosia beetles have only one fungal symbiont. We anticipate that this procedure will clearly identify a single fungal species associated with *E. validus* mandibular mycangia.

### **Time Line and Expected Outcomes**

All studies will be initiated in Year 1 and completed in Year 2, as described above. Upon completion, the proposed studies will yield valuable information regarding dissemination of *V. albo-atrum*, a potential biocontrol agent for *Ailanthus*. Identification of the fungal symbiont will actually be performed first, in Year 1, since it involves considerable laboratory work, once samples have been collected, that can be conducted during the winter. The field research involving dissemination of *V. albo-atrum* by *E. validus* will be set up in Year 1 and completed in Year 2. Scientific papers, outreach materials, and presentations will be prepared to distribute the findings to the scientific community and to the public as the project progresses. Part of the proposed budget is for attendance at one international meeting per year by both D.D. Davis (PI) and Matthew Kasson (PhD candidate). We have made considerable effort to publicize this biocontrol project in the U.S., but have not had funds to travel abroad to international meetings and present papers on our results. We propose to attend a International Union of Forestry Researchers (IUFRO) meeting and an international Biocontrol Meeting in FY 2011 and FY 2012.



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## Curriculum Vitae: Donald D. Davis

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### Degrees Awarded:

Ph.D., 1970 - Plant (Forest) Pathology

M.S., 1966 - Forestry (Silviculture/Ecology)

B.S., 1964 - Forestry

### Professional Experience:

January 1972-present: Assistant Professor, Associate Professor, Professor, Department of Plant Pathology, The Pennsylvania State University

January 1970-December 1971: Forest Pathologist, USDA Forest Service, Pineville LA and Asheville NC

Fulbright Senior Research Scholar to The Netherlands

Fulbright Senior Research Scholar to Australia

### Related University Activities

#### Teaching:

My current teaching activities involve a graduate-level summer course entitled "Plant Disease Diagnosis", which I developed for our Plant Pathology Graduate Students in 1981. In 2008, I taught the course in Costa Rica, where I plan to teach it again in 2011. The course involves teaching the students the techniques and thought processes necessary to diagnose disorders of various types of plants, with emphasis on fungi. During the Spring semester I teach a lecture/lab course entitled "Diseases of Forest and Shade Trees", that involves the diagnosis and control of diseases that affect trees, emphasizing tree diseases of Pennsylvania.

#### Research:

My studies focus on the health of northeastern hardwood forests and forest mycology, emphasizing etiology of hardwood diseases. Past and present relevant projects include biocontrol of artillery fungi (*Sphaerobolus* spp.), biocontrol of tree-of-heaven (*Ailanthus altissima*), spatiotemporal progression of tree diseases using tree-ring analysis, and identification of decay fungi following fire.

**Publications:** More than 100 technical papers -- available upon request.