Dr. Donald D. Davis – Penn State
Final Report
"Biological Control of Non-native, Highly Invasive Tree-of-Heaven"
Forest Health (FHTET) Program

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Progress Achieve in Accomplishing Project Goals & Objectives: The over-arching goal of the project was to develop an effective biocontrol for the highly invasive tree-of-heaven (*Ailanthus altissima)*. We definitely met this goal, as we have discovered a very effective biocontrol agent again *Ailanthus*. Our progress for specific objectives is described below.

Objective: Maintain cultures of *Verticillium nonalfalfae*
Planned: to develop and maintain a reservoir of virulent, sporulating cultures of our potential biocontrol agent (*Verticillium nonalfalfae*) for potential use by cooperators.
Actual: We have adapted techniques to isolate, grow, and maintain *V. nonalfalfae* on plum extract agar (PEA; 900 ml distilled water, 20 g agar, 100 ml concentrated plum extract, 1 g yeast, 5 g lactose, pH 5.6 to 6.0), a semi-selective medium for *Verticillium*, that we amend with streptomycin and neomycin antibiotics to reduce bacterial contamination. *Verticillium nonalfalfae* conidia readily form within Petri plates on the amended PEA and spores are easily harvested. We wash conidia from the PEA and fill 4 L tanks of spore suspensions containing 3 x 10^7 conidia/ml (*ca.* 4 x 10^{10} conidia/tank of solution). We can now maintain a reservoir of virulent, sporulating cultures of *V nonalfalfae* for our use and for potential use by cooperators. In addition, we have studied various delivery systems for applying the biocontrol agent.

Objective: Simulate weevil feeding wounds
Planned: to determine if simulated weevil feeding wounds on *A. altissima* seedlings can be successfully colonized by *V. nonalfalfae*, resulting in seedling mortality.
Actual: This goal was addressed by our cooperator, Dr. Scott Salom of Virginia Tech University, Blacksburg, VA, and his graduate student, Ms. Amy Snyder. The first step to meet this objective was for Dr. Salom's entomology laboratory to become familiar with handling *V. nonalfalfae* and to obtain cultures for use within his containment facility. Dr. Salom sent his graduate student Ms. Amy Snyder to Penn State to visit our laboratory for several days. Ms. Snyder learned how to make PEA media, pour plates, inoculate PEA with *V. nonalfalfae*, grow the fungus, and wash conidia off the plates, all under laboratory hoods using sterile techniques. This was a very successful cooperative endeavor. In fact, the knowledge gained at Penn State

helped Ms. Snyder with her field work, in which she found *V. nonalfalfae* killing *Ailanthus* trees at several locations in Virginia, a very exciting and important find. Dr. Davis (PI) served as a member of Ms. Snyder's M.S. thesis committee at Virginia Tech.

Objective: Determine susceptibility of additional hosts *V. nonalfalfae* isolate PSU140
Planned: as part of our risk analyses, to determine if non-target plant species are susceptible to *V. nonalfalfae*
Actual: We first compiled a list of more than 80 native and non-native plant species, for which we wanted to test susceptibility to *V. nonalfalfae.* Species were selected based on several criteria: 1) reported susceptibility to *V. nonalfalfae* based a thorough scientific literature review, 2) species that are ecologically important and occur naturally in Pennsylvania, 3) species that are economically important in Pennsylvania, and/or 4) TAG species on a list furnished by Dr. Scott Salom, our cooperator at Virginia Tech. We inoculated potted seedlings in the greenhouse, as well as native species in the field with *V. nonalfalfae*, and collected bi-weekly data regarding symptom development up to morality. In addition, we evaluated symptom development on those non-*Ailanthus* species that did not develop symptoms following previous years' inoculations. The purpose of these follow-up studies was to determine if latent symptoms develop years after inoculation on species thought to be resistant. Isolations were made from all previously inoculated species. We also established permanent plots within stands where *V. nonalfalfae* was killing *Ailanthus* and rated the susceptibility of non-*Ailanthus* species (*e.g.,* maples and oaks) that were growing intermingled with dead and dying *Ailanthus* trees. All results revealed that *V. nonalfalfae* poses little or no threat to non-*Ailanthus* plant species. It appears likely that *V. nonalfalfae* has become host-adapted to *Ailanthus*.

Objective: Determine susceptibility of *Ailanthus* to other isolates of *V. nonalfalfae*
Planned: to collect diverse isolates of *V. nonalfalfae* from non-*Ailanthus* hosts and test their pathogenicity to *A. altissima*
Actual: We collected *V. nonalfalfae* isolates from *Ailanthus* infections of in in various regions of PA. These surveys furnished us with additional PA isolates of *V. nonalfalfae* that were used in comparative pathogenicity tests. Isolates were very similar and there appeared to be little difference among isolates. In addition to *Ailanthus*, we also inoculated several agricultural host species (alfalfa, eggplant, hops, potato, and tomato) with the various isolates. Again, results revealed that *V. nonalfalfae* poses little or no threat to non-*Ailanthus* agricultural species and is likely host-adapted to *Ailanthus*.

Objective: Characterize the genetic diversity and genotypic susceptibility of *Ailanthus altissima* within the United States
Planned: to determine if *A. altissima* trees from various parts of the United States are equally susceptible to *V. nonalfalfae*
Actual: We conducted a thorough literature review to document multiple introductions of *A. altissima* into the United States from 1784-2010. We then collected *Ailanthus* seed and/or leaf tissue for DNA analysis from most states where *Ailanthus* was reported to occur. To characterize *Ailanthus* diversity in a more geographically local area, we also collected *Ailanthus* seed and/or leaf tissue for DNA analysis from most counties in Pennsylvania where *Ailanthus* grows. We conducted micro-satellite analyses on the samples; data are being analyzed.

Objective: Molecular characterization of *V. nonalfalfae*
Planned: To sequence the entire genome of *V. nonalfalfae isolate*
Actual: Complete sequencing of the DNA of *V. nonalfalfae isolate* allow us to identify this isolate if it is collected from other states, and may allow us to determine why it is so pathogenic to *Ailanthus* and not to other plant species. It will also help us determine if the isolate is actually a new species of *Verticillium*. We initiated Illumina genome sequencing of one randomly selected *V. nonalfalfae* isolate, using isolate VaMS.102 from alfalfa as reference sequence to align our short-read sequence data. (VaMS.102 from alfalfa is the only isolate of *V. nonalfalfae* that has been completely sequenced). Using Illumina high throughput sequencer, a total 20 million reads were assembled into 45,381 contigs, whose length ranged from 100 bp to 6,610 bp. The total size of these contigs was 13Mb. The 45,381 contigs were blasted against nr db and shown with MEGAN. Bioinformatics data are being interpreted and additional sequencing is being conducted.

Objective: Determine means of dissemination
Planned: to determine means of dissemination *V. nonalfalfae*
Actual: We isolated *Verticillium* from 100% of wind-blown infected *Ailanthus* leaflets, indicating that infected leaflets are one possible means of short-range dissemination. In a preliminary study, we detected *Verticillium* on 6 - 12% of seeds collected from infected canopy female trees. In later studies, we recovered *V. nonalfalfae* from 100% of seeds sampled from infected female trees. Seeds are capable of flight even further than leaflets, and represent a likely means of moderate range dissemination. In terms of long-range dissemination, we have detected *Verticillium* on exoskeletons of *Euwallaceae validus*, an ambrosia beetle that we had observed on *Ailanthus* trees dying from Verticillium wilt. This ambrosia beetle is a non-native species introduced from China. We trapped insects within infected stands using funnel traps baited with Ultra-High-Release ethanol and alpha-pinene at five locations in southern Pennsylvania. We separated and identified the trapped *E. validus* ambrosia beetles, disarticulated them, and isolated their associated fungi. Results indicated that *V. nonalfalfae* is present on the exoskeleton of the beetles, but not in the mycangea, and any dissemination of *V. nonalfalfae* by the beetles is likely passive.

Objective: Aerial Photography.
Planned: to conduct aerial surveys and take digital photographs of the portion of southern Pennsylvania to detect new *Verticillium* wilt centers
Actual: We conducted several aerial surveys, but abandoned this type of survey as it was not efficient, and was very expensive.

Objective: Develop and Test Various Inoculum Formulations.
Planned: to develop various formulations of our PSU140 isolate of *V. nonalfalfae* inoculum for inoculating canopy *Ailanthus* trees in the field
Actual: We compared our standard inoculum (conidia in water) against: 1) reconstituted, lyophilized (freeze-dried), pulverized rye grain containing *V. nonalfalfae* resting structures (melanized hyphae), 2) reconstituted, lyophilized, pulverized *V. nonalfalfae* hyphal fragments and conidia that had been grown in various culture media, and 3) hyphae mixed with soil and

refrigerated for up to one year. However, these were preliminary studies; we are currently conducting additional analyses of these, as well as other formulations, and delivery systems.