DETECTION AND ANALYSIS OF *XYLELLA FASTIDIOSA* IN GLASSY-WINGED SHARPSHOOTER POPULATIONS IN TEXAS

Principal Investigator:
Blake Bextine
Department of Biology
University of Texas
Tyler, TX 75799
bbextine@uttyler.edu

Researcher:
Daymon Hail
Department of Biology
University of Texas
Tyler, TX 75799

Cooperator:
Forrest Mitchell
Texas AgriLife Research
Stephenville, TX 76401

Isabelle Lauzière
Texas PD Res. & Ed. Program
Texas AgriLife Research
Fredericksburg, TX 78624

Jeff Brady
Texas AgriLife Research
Stephenville, TX 76401

Reporting Period: The results reported here are from work conducted October 2007 to September 2008.

ABSTRACT
The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) is a xylophagous insect that is an endemic pest of several economically important plants in Texas. GWSS is the main vector of *Xylella fastidiosa* (*Xf*; Wells), the bacterium that causes Pierce's disease (PD) of grapevine, and can travel long distances putting much of the Texas grape production at risk. Understanding the movement of GWSS populations capable of transmitting *Xf* into PD-free areas is critical for developing a management program for PD. To that end, the USDA-APHIS has developed a program to sample vineyards across Texas to monitor populations of GWSS. From this sampling, GWSS collected during 2005 and 2006 over the months of May, June, and July from eight vineyards in different regions of Texas were recovered from yellow sticky traps and tested for the presence of *Xf*. The foregut contents were vacuum extracted and analyzed using Quantitative Real-Time PCR (QRT-PCR) to determine the percentage of GWSS within each population that harbor *Xf* and have the potential to transmit this pathogen. GWSS from vineyards known to have PD routinely tested positive for the presence of *Xf*. While almost all GWSS collected from vineyards with no history of PD tested negative for the presence of the pathogen, three individual insects tested positive. Furthermore, all three insects were determined to be carrying the PD-strain of the pathogen through DNA sequencing, signifying them as a risk factor for new *Xf* infections.

INTRODUCTION
With the ability to travel long distances, the glassy-winged sharpshooter [GWSS; *Homalodisca vitripennis* (Germar)] can spread quickly once established and have recently been found in French Polynesia, Tahiti, and Hawaii (Hoddle et al. 2003). GWSS have the ability to ingest in excess of 100 times their weight in xylem fluid in a day (Purcell 1999). They have been reported to feed on host plants from at least 35 families including both woody and herbaceous types (Hoddle et al. 2003). GWSS feeding can impact plant health directly by depriving the plant of nutrients and damaging the xylem sufficiently to preclude vascular flow. Indirectly, plant damage is done by the transmission of the xylem-limited bacterium *Xylella fastidiosa* (*Xf*) (Purcell 1999).

Xf infection in grapevines may result in Pierce's disease (PD), which has caused major losses in both wine and table grape production in the US (Davis et al. 1978). In the grapevine (*Vitis* sp.), PD symptoms include marginal leaf scorch, chlorosis, necrosis, stunted growth, leaf loss and dieback, all of which result from occlusion of the xylem tissue by polymeric matrix enclosed bacterial aggregates attached to the inner xylem wall (Hopkins 1989). *Xf* can cause systemic failure of a grapevine within one to five years of initial infection and previous studies have shown that as few as 100 cells (Hill and Purcell 1995) can initiate an infection. As there is currently no cure for PD (Pooler et al. 1997), grapevines showing characteristic symptoms must be uprooted and replanted, usually resulting in a two or three year loss of individual plant productivity.

Many economically important plants including citrus, almond and oleander are affected by separate strains of *Xf* resulting in a multitude of plant diseases such as citrus variegated chlorosis (Chang et al. 1993; Pooler and Hartung 1995), almond leaf scorch (Mircetich et al. 1976) and oleander leaf scorch (Purcell 1999). Many strains of *Xf* are host specific and in transmission studies the strain that causes disease symptoms in oleander will not cause disease symptoms in grape or almond. Additionally, the grape and almond strains were unable to cause disease symptoms in oleander.
Greenhouse studies suggest that between 10% and 20% of GWSS are able to transmit *Xf* (Almeida and Purcell 2006) but there is little data on naturally occurring infectivity (Daane et al. 2007). Many methods have been developed to detect *Xf* in natural and experimental environments including transmission (Purcell and Finlay 1980), insect head culture (Almeida and Purcell 2003), plant tissue culture (Hill and Purcell 1995), chloroform/phenol extraction (Frohme et al. 2000) and PCR-based vacuum extraction (Bextine et al. 2005). Culture based detections methods are difficult and time consuming given the fastidious nature of the bacterium and are inherently less sensitive than PCR based techniques. QRT-PCR can be used to

detect as few as five *Xf* cells in an insect head (Bextine et al. 2005) and is a viable approach even when dealing with dead insects making it a highly valuable procedure when compared to other forms of detection.

In *Xf*, *gyrase B* is conserved in all strains and is diverse enough to also be used as a molecular marker for both detection and strain differentiation (Bextine et al. 2005). In this study, eight vineyards from different regions of Texas were surveyed for the presence of GWSS and potential vectors were tested for the presence and strain of *Xf*.

MATERIALS AND METHODS

Sample Preparation. Eight vineyards in different regions of Texas were sampled for the presence of *Xf* vector species. Vineyard A is in Washington County, Vineyard B is in Anderson County, Vineyard C is in Camp County, Vineyard D is in Tarrant County, Vineyard E is in Wichita County, Vineyard F is in Lubbock County, Vineyard G is in Tom Green County and Vineyard H is in Val Verde County (**Figure 1**). Monitoring of insect populations took place using standard double-sided traps (Seabright Laboratories, Emeryville, CA), each 23 x 14 cm in size, bright yellow in color (Pantone® Matching System (PMS) 102) and coated with Stikem Special® glue. Traps were tightly stapled to a 1.8 m bamboo stake driven into the ground a little lower than grapevine canopy. Between 6 and 13 traps were placed in each vineyard (Lauziere et al. 2008). Upon retrieval from the vineyard, the traps were placed into Ziploc bags and stored at 4° C. The traps were then removed from the bags and GWSS were removed by applying the solvent orange oil (Citrus King, St. Petersburg, FL) around the insect to dissolve the adhesive and remove the insect from the trap. Each insect was then washed in 95% ethanol and then in deionized water to remove any residual orange oil. Insect heads were removed (Bextine et al. 2004) and a novel silica-based DNA extraction was performed to test for the presence of *Xf*.

DNA Extraction. Each head was placed into a well of a 96-well plate (VWR International – North American, West Chester, PA) and submerged in 100 µL of PBS buffer. Vacuum pressure was applied to the plate four times for two minutes each (Bextine 2004). With *Xf* cells dislodged during vacuum extraction, the heads were discarded and the vacuum solution was retained. To each well, 100 µL of Lysis Buffer L6 [50 samples - 18.6 g Guanadine Thiocyanate, 1.5 ml Tris-HCL (1M, 6.8 pH), 1.2 ml EDTA (0.5M, 8.0 pH), 390 µl Triton X-100, 26.9 ml ddH_2O] was added to lyse bacterial cells. The contents were mixed by pipetting and the mixture was centrifuged for 5 min at 5000 rpm to separate DNA from the cellular debris. The contents of each well were then transferred into the corresponding wells of 0.2 mL eight-well strips and 53 µL of silica slurry (molecular grade H_2O and silicon dioxide) were added and mixed by pipetting. The eight-well strips were then returned to their corresponding rows in a 96-well plate. The plate was incubated at RT for 5 min and centrifuged at 2000 rpm for 5 min. The supernatant was then discarded and the DNA pellet was retained. DNA pellets were washed four times by resuspending the silica in 200 µL wash buffer [40% vol/vol 100 mM Tris-Cl (pH 7.5), 20 mM EDTA (pH 8.0), 0.4 M NaCl and 60% vol/vol 100% EtOH] and centrifuging for 5 min at 2000 rpm. After the wash buffer was removed, the resulting pellets were dried in an incubator at 60º C for 10 min. The silica was resuspended in 100 µL of TE Buffer and incubated again for 5 min at 60º C, followed by a final centrifuge for 5 min at 5000 rpm. Seventy µL of the resulting DNA elution were then saved for PCR analysis.

Xf Detection. A SYBR-green based quantitative real-time PCR (QRT-PCR) was performed on the subsequent elutions using *Xf* specific primers (Bextine and Child 2007). A master mix was made using 10 µL of IQ Supermix (BioRad, Hercules, CA), 0.8 µL of both primers (at a concentration of 10 µM), 5.4 µL of autoclaved molecular grade water, 1 µL of 10 µM Sybr Green (Invitrogen, Carlsbad, CA) and 2 µL DNA template per reaction. The run conditions for the PCR were 95° C for 3 min then 40 cycles of 95° C for 20 sec, 55° C for 30 sec and 72° C for 60 sec followed by DNA melting temperature curve analysis which ramped from 77-90° C by 0.5° C each step.

Xf Strain Differentiation. Another SYBR-Green based QRT-PCR was performed on the GWSS testing positive for *Xf* using GyrBLONG primers. A master mix was made using 10 µL of IQ Supermix (BioRad, Hercules, CA), 0.8 µL of both primers (at a concentration of 10 µM), 5.4 µL of autoclaved molecular grade water, 1 µL of 10 µM Sybr Green (Invitrogen, Carlsbad, CA) and 2 µL DNA template per reaction. The run conditions for the PCR were 95° C for 3 min then 40 cycles of 95° C for 30 sec, 53° C for 60 sec and 72° C for 120 sec followed by DNA melting temperature curve analysis which ramped from 70-99° C by 0.1° C each step.

The positive PCR products were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. A DNA-sequencing PCR was performed in a 10-µl reaction containing 4 µl of DTCS Quick Start Master Mix (Beckman Coulter, Fullerton, CA, USA), 2 µl of either the forward or reverse primer, 2 µl of autoclaved nanopure water, and 2 µl of a DNA template. The sequencing PCR (30 cycles) was conducted under the following conditions: 95°C for 20 s, 50°C for 20 s, and 60°C for 4 min, with the product then held at 4°C until removal from the machine. The DNA product was purified using standard ethanol precipitation, which resulted in samples re-suspended in 40 µl of sample-loading solution (Beckman Coulter).
The resuspended samples were transferred to the appropriate Beckman Coulter 96-well microplates, centrifuged at 300 rpm at 2°C for 30 s, and then overlaid with one drop of mineral oil. Samples then were sequenced in a CEQ 8000 Genetic Analysis System (Beckman Coulter) using the manufacturer's protocol. Sequences were processed using BioEdit (Ibis Biosciences, Carlsbad, CA, USA) and matched to known sequences using basic local alignment search tool (BLAST) (www.ncbi.nih.gov).

RESULTS

As expected, some vineyards were more heavily infested with GWSS than others. Vineyards such as H and A had as many as two-hundred or more GWSS recovered from their yellow-sticky cards whereas other vineyards like D and G had around one-hundred. More northern vineyards (E and C) had as few as three and as many as a few dozen individuals while the Vineyard F, the only vineyard sampled on the High Plains had no GWSS at all. While this trend may seem concordant with the presence or absence of PD in a vineyard, there were exceptions and irregular sampling at various sites. Vineyard B was collected from only once in 2005 which prevented statistical analysis of this site.

Of those GWSS collected, many tested negative for the presence of *Xf*. Taking all vineyards into account, the statewide percentage positive was 6.16 (79/1283), this is a lower percentage than estimation given by Almeida and Purcell (2003**).** The highest percentage positive occurred in the same vineyard as the highest number of individuals positive (Vineyard A, 11.96%, 25/209). Other vineyards such as B and D had similar percentage positives (9.09 and 9.33 respectively) but lower GWSS counts. The northern-most vineyards (Vineyard F, E and C) tended to have not only the lowest numbers of individuals but also the lowest percentages positive for both years sampled (0/0, 0/17 and 1/85 respectively). Almost all samples that tested positive for the presence of *Xf* contained the PD strain of the pathogen.

Samples that tested positive for the presence of *Xf* from vineyards that had no history of PD were reanalyzed to determine strain through DNA sequencing. Sequence data from the *gyrB* and *mopB* genes were analyzed to determine the strain of *Xf* that was detected within the vector insects (Morano et al. 2008). The GWSS that were collected from vineyards that were considered PD-free (E, G and C) were found to be positive for a Temecula-like PD-strain.

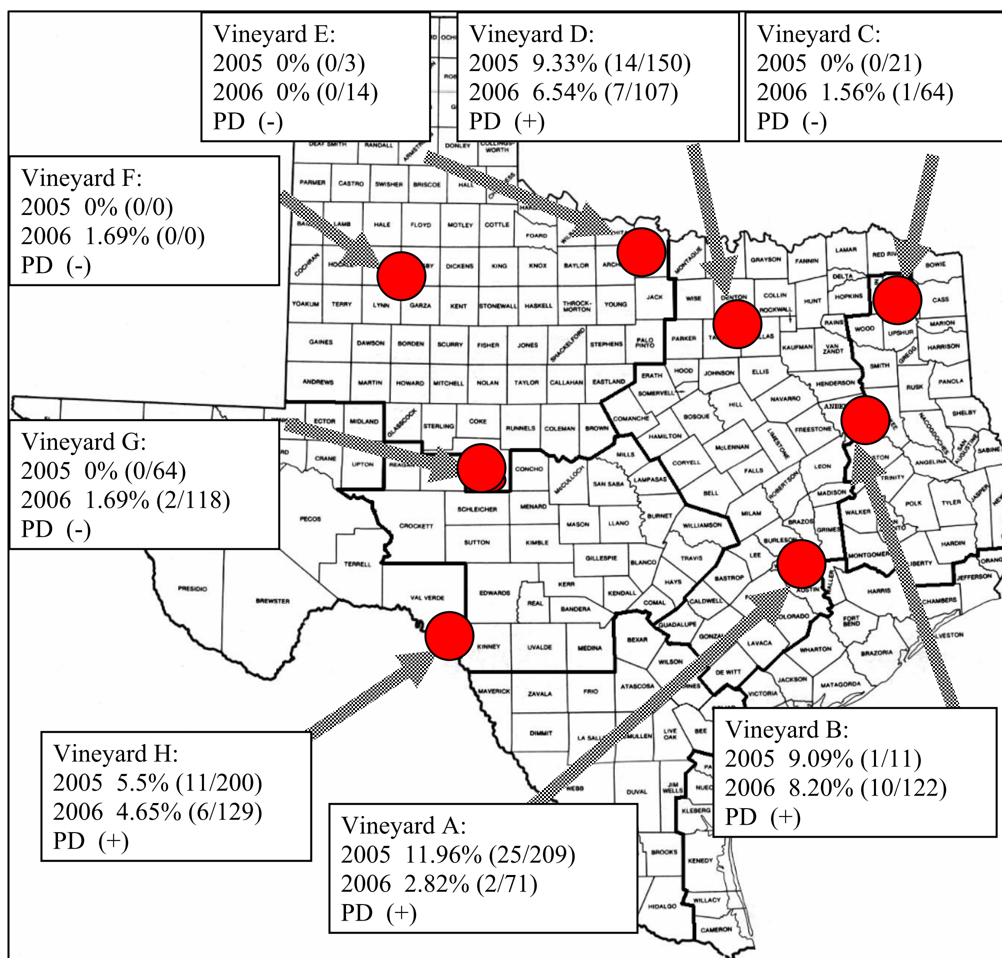

Figure 1. Regional map of Texas showing collection locations in counties sampled. Percentages shown reflect *Xf* infectivity for 2005 and 2006. Also shown are independent results of PD known for each vineyard.

DISCUSSION

Screening of samples was conducted using the INF2 and INR1 primer set. These primers were originally designed to differentiate between strains of *Xf* through melt curve analysis; however, the results were difficult to interpret due to the influence of background noise that caused overlapping melt temperatures between strains. Using GyrBLONG primers (another primer set that is being developed for *Xf* strain identification), we determined that while the majority of the samples tested positive for the PD strain of *Xf* (about 99%), some of the GWSS that tested positive for *Xf* contained an ornamental strain (*Xf* multiplex) (Schaad et al., 2004) of the bacterium. Strains of *Xf* are specific in respect to their role in pathogenicity and as such colonization by ornamental strain *Xf* multiplex or *Xf* sandyi) (Schaad et al., 2004) will be benign and not result in the overt symptomology typical among PD infections (Almeida and Purcell 2003).

Although the results reported here are reasonably consistent with greenhouse infectivity estimates published by Almeida and Purcell (2003), there is always the possibility of false negatives or inconclusive "positives" due to environmental background. Some potential false negative could be the results of trap exposure to the elements. The two-week period in which the traps were exposed to cycles of mid-day heating and nighttime cooling could have lead to degradation of *Xf* DNA. However, in previous studies DNA was recoverable and detection by PCR was competent from insects on traps that were exposed to the elements in southern California as long as 10 days (the longest period tested). We feel confident that false negatives did not impact the integrity of the studies.

Most GWSS collected within vineyards with a documented history (B, D and A) tested positive for the PD strain of *Xf*. This was not surprising, given the presence of this bacterial strain in the immediate plant community. However, the detection of the non-PD strain in insects collected from vineyards with a known history of PD was not expected and suggests a level of migration between vineyard and non-vineyard wild populations. This is an interesting finding, given the availability of the PD strain of *Xf*.

Overall, the majority of GWSS collected from multiple locations in Texas over two years in this study (more then 93%) tested negative for the presence of *Xf*. Yet, GWSS is considered one of the greatest risk factors in relation to the epidemiology of *Xf* spread in the grape-growing regions of the US (Hoddle et al. 2003). The specifics of *Xf* spread are not simple; in fact, two modes of spread can be involved in PD epidemiology. Primary spread, or the movement of the pathogen into an area from an outside source, is of major concern in non-PD vineyards. Understanding where new infections come from is of paramount importance when attempting to control the problem through area-wide management programs.

REFERENCES CITED

Almeida, R. P. P. and A. H. Purcell. 2003. Transmission of *Xylella fastidiosa* to grapevines by *Homalodisca coagulata*. Journal of Economic Entomology 96, 264-271.

Almeida, R. P. P. and A. H. Purcell. 2006. Patterns of *Xylella fastidiosa* colonization of the precibarium of sharpshooter vectors relative to transmission to plants. *Annals of the Entomological Society of America* 99, 884-890.

Bextine, B., S. J. Tuan, H. Shaikh, M. Blua and T. Miller. 2004. Evaluation of Methods for Extracting *Xylella fastidiosa* DNA from the Glassy-Winged Sharpshooter. *Journal of Economic Entomology.* Vol. 97, No. 3 pp. 757–763.

Bextine, B., M. Blua, D. Harshman and T. Miller. 2005. A SYBR Green-Based Real-Time Polymerase Chain Reaction Protocol and Novel DNA Extraction Technique to Detect *Xylella fastidiosa* in *Homalodisca coagulata. Journal of Economic Entomology*: Vol. 98, No. 3 pp. 667–672.

Bextine, B., and B. Child. 2007. *Xylella fastidiosa* genotype differentiation by SYBR® Green-based QRT-PCR. *FEMS Microbiology Letters* 276 (1), 48–54.

Chang, C. J., M. Garnier, L. Zreik, V. Rossetti, J. M. Bove. 1993. Culture and serological detection of xylem-limiting bacterium causing citrus variegated chlorosis and its identification as a strain of *Xylella fastidiosa*. *Current Microbiology* 27, 137-142.

Costa H.S., Raetz E., Pinckard T., Gispert C., Hernandez-Martinez R., Dumenyo C.K. & Cooksey D.A. 2004. Plant hosts of *Xylella fastidiosa* in and near southern California vineyards. Plant Dis. 88: 1255–1261.

Daane, K., C. Wistrom, J. Hashim, G. Yokota, M. Pryor and R. Almeida. 2007. Host plant preferences and natural infectivity of insect vectors of *Xylella fastidiosa* on common weeds and crop plants, pp 3-6. In: Proceedings, 2007 Pierce's Disease Research Symposium. California Department of Food and Agriculture, Sacramento, CA.

Davis, M. J., A. H. Purcell and S. V. Thomson. 1978. Pierce's disease of grapevines: isolation of the causal bacterium. *Science* 199, 75-77.

Frohm, M., A. A. Camargo, S. Hebber, C. Czink, A. J. D. Simpson, J. D. Hoheisel and A. Pereira de Souza. 2000. Mapping analysis of the *Xylella fastidiosa* genome. *Nucleic Acids Research* 28, 3100-3104

Hoddle, M. S., S. V. Triapitsyn and D. J. W. Morgan. 2003. Distribution and plant association records for *Homalodisca coagulata* (Hemiptera: Cicadellidae) in Florida. *Florida Entomology* 86, 89–91.

Hopkins, D. L. 1989. *Xylella fastidiosa*: A xylem-limited bacterial pathogen of plants. *Annual Reviews - Phytopathology* 27:271-290.

Hill, B. L., and A. H. Purcell. 1995. Acquisition and retention of *Xylella fastidiosa* by an efficient vector, *Graphocephala atropunctata*. *Phytopathology* 85, 209-212.

Hill, B. L., and Purcell, A. H. 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. *Phytopathology* 85:1368-1372.
Hunnicutt, L., W. B. Hunter, R. D. Cave, C. A. Powell and J. J. Mozoruk. 2006. Genome sequence and molecular characterization of *Homalodisca coagulate* virus-1, a novel virus discovered in the glassy-winged sharpshooter (Hemiptera: Cicadellidae). *Virology* 350: 67-78.
Mircetich, S. M., S. K. Lowe, W. J. Moller and G. Nyland. 1976. Etiology of almond leaf scorch disease and transmission of the causal agent. *Phytopathology* 66,1-24.
Morano, L.D., Bextine, B.R., Garcia, D.A., Maddox, S.V., Gunawan, S., Vitovsky, N.J., Black, M.C. 2008. Initial Genetic Analysis of *Xylella fastidiosa* in Texas. Current Microbiol. 56, 346-351.
Pooler, M. R. and J. S. Hartung. 1995. Specific PCR detection and identification of *Xylella fastidiosa* strains causing citrus variegated chlorosis. *Current Microbiology* 31, 377-381.
Pooler, M. R., I. S. Myung, J. Bentz, J. Sherald and J. S. Hartung. 1997. Detection of *Xylella fastidiosa* in potential insect vectors by immunomagnetic separation and nested polymerase chain reaction. *Letters in Applied Microbiology* 25, 123-126.
Purcell, A. H. and A. Finlay. 1980. Evidence for noncirculative transmission of Pierce's disease bacterium by sharpshooter leafhoppers. *Phytopathology* 69, 393-395
Purcell, A. H., S. Saunders, M. Hendson, M. Grebus and M. Henry. 1999. Causal role of *Xylella fastidiosa* in oleander leaf scorch disease. *Phytopathology* 89, 53-58.
Turner, W., and H. Pollard. 1959. Life histories and behavior of five insect vectors of phony peach disease. *Technical Bulletin of the United States Department of Agriculture* 1188, 28.
Schaad, N. W., E. Pastnikova, G. Lacey, M. Fatmi, and C. J. Chang. 2004. *Xylella fastidiosa* subspecies: *X. fastidiosa* subsp. *piercei,* subsp. nov., *X. fastidiosa* subsp. multiplex subsp. nov., and *X. fastidiosa* subsp. pauca subsp. nov. Syst. Appl. Microbiol. 27:290–300.
Young, D.A.. 1958. A synopsis of the species of *Homalodisca* in the United States. *Bulletin of the Brooklyn Entomological Society* 53 (1), 7–13.

FUNDING AGENCIES
Funding for this project was provided by the Texas Pierce's Disease Research and Education Program, and the USDA Animal and Plant Health Inspection Service.