

# Pierce's Disease Control Program



# Symposium Proceedings

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Research Symposium

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San Diego

California Department of Food & Agriculture

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2008 Pierce's Disease  
Research Symposium*

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and  
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# IMMUNOHISTOCHEMISTRY OF $\beta$ -1,4-GLUCANASE, THE MAJOR ENZYMATIC COMPONENT OF GLASSY-WINGED SHARPSHOOTER SALIVA, IN PROBED GRAPE PETIOLES

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**Reporting Period:** The results reported here are from work conducted October 1, 2007 to September 30, 2008.

## ABSTRACT

The overall goal of our project is to determine whether  $\beta$ -1,4 glucanase (EGase), the major enzymatic protein in the saliva of glassy-winged sharpshooter (GWSS), co-localizes via immunocytochemistry with the few 'pioneer' *Xylella fastidiosa* (*Xf*) cells that are inoculated by this vector's probing (stylet penetration) behaviors. If it does, then this suggests that the enzymatic portion of GWSS saliva is a carrier of the bacteria during inoculation. This year, we acquired commercially-produced, polyclonal antibody serum to purified EGase from GWSS salivary glands, and developed methods for separate immunolocalization of: 1) this purified EGase, and 2) Green fluorescent protein (GFP) *Xf* in grape petioles, using commercial *Xf* antibody, to complement the GFP *Xf* detection method devised last year. We also performed three experiments to monitor feeding of inoculative GWSS, via Electrical Penetration Graph (EPG) technology. Feeding was recorded on healthy grape petiole, followed by histological processing of the fed-upon grape tissues. To date, results show that glucanase is found throughout the solid salivary sheath that encases stylets (the piecing-sucking mouthparts of GWSS that penetrate the plant). However, glucanase was not found diffused into adjoining plant cells along the stylet pathway, as was hypothesized. Glucanase was the major constituent of the deep, narrow sheath branches that enter the xylem, indicating that glucanase is injected into xylem during feeding. If our hypothesis on the role of saliva in inoculation is supported, it suggests that future development of salivary antagonists could enable interference with *Xf* inoculation of grape.

## INTRODUCTION

As introduced in detail in Backus and Labavitch (2006, 2007), this goal of this project is to determine whether the major enzyme in glassy-winged sharpshooter (GWSS) saliva histologically co-localizes with *Xylella fastidiosa* (*Xf*). Sheath saliva of hemipterans in fed-upon plants can be routinely imaged histologically (e.g. Leopold et al. 2003, Backus et al. 2005). However, no researcher studying hemipteran feeding has ever *directly* visualized enzymatic watery saliva in plants, due to its usually fluid and dispersive nature. This project will also partially test Backus's hypothesis that enzymatic salivary secretions of GWSS aid in the cell-to-cell movement of newly inoculated *Xf* cells. Salivary enzymes may break down pit membranes, allowing the few pioneer bacterial cells inoculated during feeding to move between adjoining xylem cells. Carbohydrase enzymes with very high activity for cell wall polymer-degradation, especially  $\beta$ -1,4 glucanase (EGase, often identified as cellulase in the literature), have been found in GWSS salivary gland fractions (Labavitch 2006, unpub. data). We use immunohistological methods combined with Electrical Penetration Graph (EPG) monitoring of GWSS feeding, to determine whether *Xf* co-localizes with EGase in saliva.

## OBJECTIVES

1. Purify and characterize  $\beta$ -1,4-glucanase (EGase), a putatively cell wall-degrading salivary enzyme of GWSS, and develop antibodies for *in planta* localization of saliva.
2. Determine whether GWSS salivary proteins (injected into grape during EPG-controlled insect feeding) affect the distribution of recently inoculated *Xf*, as detected by immunocytochemistry.

## RESULTS

Objective 1 – Purify and characterize  $\beta$ -1,4-glucanase and develop antibodies.

*Study a:  $\beta$ -1,4-glucanase isolation and purification*

EGase was isolated and purified last year in the Labavitch lab, from GWSS salivary glands previously dissected in the Backus lab (Backus & Labavitch 2007).

*Study b: Determination of cell-wall degrading properties of  $\beta$ -1,4-glucanase*

The glucanase enzyme was purified based on its ability to digest carboxymethyl cellulose. However, tests showed that it could also digest xyloglucan (XyG), a major cell wall hemicellulosic polysaccharide that is present in grape tissues, including leaf petioles. At least one of the  $\beta$ -1,4-glucanase enzymes of *Xf* also digests XyG. Therefore, the ability of the tomato xyloglucanase-inhibiting protein to block the action of the GWSS glucanase was tested. Unfortunately, as was found for the *Xf* glucanase, no inhibition was detected (Labavitch, 2006).

*Study c: Development of antibody to  $\beta$ -1,4-glucanase*

Polyclonal antibodies were raised in guinea pig by Antibodies, Inc. (Davis, CA) in late November 2007, then were further purified in the Labavitch lab in December 2007. This objective was completed when antibody serum was delivered to the Backus lab, in January 2008.

Objective 2 – Determine whether GWSS salivary proteins (from EPG-controlled insect feeding) affect the presence/distribution of inoculated *Xf*.

*Studies a and b: Immunocytochemistry of probes by a) clean and b) GFP-Xf inoculative GWSS*

Our ultimate goal for this objective is to combine five challenging procedures into one large experiment with the following steps (first described in Backus & Labavitch 2007):

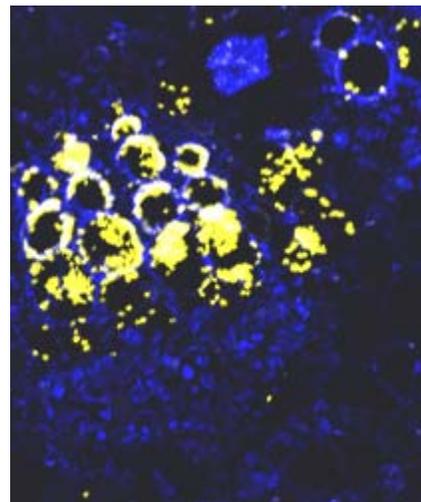
- 1) Allow one group of GWSS to acquire *Xf* expressing green fluorescent protein (hereafter, GFP-*Xf*) (*Study a*) and another (control) group to remain non-inoculative (*Study b*), then...
- 2) EPG-record a single, standardized probe consisting of pathway followed by ingestion lasting no more than three – six min, as described in Backus & Labavitch (2006), then...
- 3) Excise, histologically prepare, and section the fed-upon grape tissue, using methods that retain fluorescence of GFP, then.
- 4) Probe the sectioned tissue with primary antibody to EGase (from Objective 1) then secondary, fluorescently conjugated antibody, and finally.
- 5) Use confocal laser scanning microscopy (CLSM) to simultaneously locate and image autofluorescent salivary sheaths and cell walls, GFP-*Xf*, and fluorescently-stained EGase/saliva.

In this way, we hoped to visualize the location of both watery saliva (i.e. EGase) and sheath saliva in relation to presence, location and movement of *Xf* bacterial cells, during certain EPG waveforms.

Postdoctoral Research Associate Kim Kingston worked in the Backus lab from 1<sup>st</sup> July 2007 until 30<sup>th</sup> June 2008, under joint supervision by Backus and Labavitch. Prior to her arrival, preliminary attempts were made to achieve steps 1 – 3, above, which partially failed. We attempted to view salivary sheaths containing GFP *Xf* left by inoculative GWSS, using the fluorescence-retaining protocols developed last year (Backus & Labavitch 2007). To our dismay, sheath saliva was so strongly autofluorescent (at all excitation wavelengths) that its brightness overwhelmed and overlaid the lesser brightness of the GFP *Xf* (except in a few rare cases wherein the sheath was very diffuse, confirming presence of bacteria in the sheath; Backus 2007). Also, *Xf* were not seen outside the salivary sheath, only embedded within sheath saliva. These results confounded the rest of our tests because we could not be sure that all bacteria injected by every insect would consistently be visible.

Consequently, we spent the first six mo. of Kingston's tenure (including the first four mo. of the reporting period) successfully developing a protocol for a more reliable means of histologically detecting *Xf*, i. e. immunolocalization. We used commercially-prepared *Xf* primary antibody from rabbit (Agdia, Elkhart, IN) and secondary, anti-rabbit conjugated with Alexa Fluor 647 (Invitrogen, Carlsbad, CA) with mechanically inoculated vs. control grape petioles (**Figure 1**). We anticipate that this protocol will enable visualization of GFP *Xf* inoculated into the plant by GWSS, even if bacteria are embedded in the salivary sheath.

Also in Kingston's first six mo., we performed two major experiments to EPG-record feeding of putatively inoculative GWSS on healthy grape petioles, following the procedure outlined in step 2, above. Putatively clean GWSS were provided by David Morgan (CDFA). Recordings were performed in the dark, using a petiole whose leaf was masked by wet tissue paper and plastic wrap (according to a procedure developed with advice of Andrew McElrone, ARS Davis, CA). This was to reduce the likelihood that bacteria injected into the xylem would be rapidly pulled out of the confocal field of view by xylem tension. Following each recording, the insect head was fixed in 4% paraformaldehyde, for separate examination by confocal microscopy to verify the presence of GFP *Xf* in the cibarium and



**Figure 1.** Immunohistological confocal image of grape petiole xylem (autofluorescent cell walls, blue) mechanically inoculated with *Xf* (yellow). White coloration is overlay of yellow and blue.

precubarium. Petiole tissue was excised and fixed under dim light, then further prepared for sectioning and later confocal microscopy.

Once the EGase antibody serum arrived, we spent Kingston's last six mo. developing a successful protocol for its use in immunoprobng and -localizing EGase in GWSS salivary sheaths. The protocol visualizes the EGase primary antibody (from Objective 1) with goat-anti-guinea pig secondary antibody conjugated with Alexa Fluor 647 or 568 (Invitrogen, Carlsbad, CA) (**Figures 2a and b**).

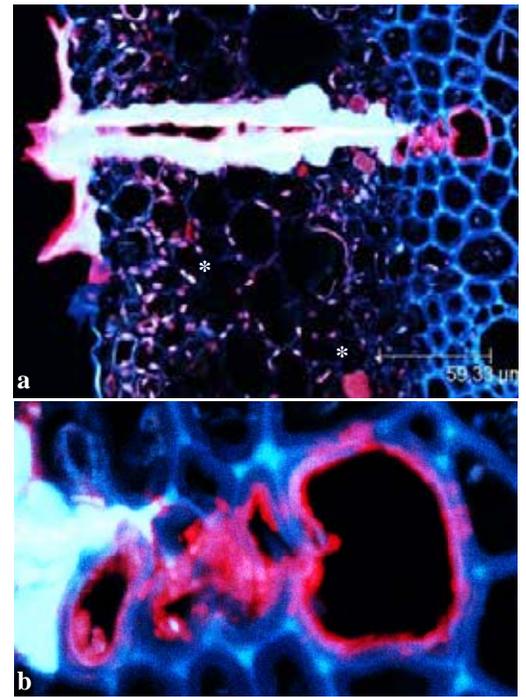
Contrary to our initial hypothesis, there was no evidence that glucanase diffused into plant tissues adjoining the salivary sheath (**Figure 2**). At no concentration of EGase antibody was a diffuse 'halo' seen only around the salivary sheath, as has been enzymatically detected surrounding aphid salivary sheaths (Ma et al. 1990). Salivary glucanase was strictly localized to the sheath, though along its entire length. The polyclonal EGase antibody also bound non-specifically to small plastids and certain vacuole contents (**Figure 2a, \*'s**). Nonetheless, these were easily distinguished from saliva by their paler coloration and their widespread distribution in all parenchymous tissues (**Figure 2a**). For most of the sheath length, EGase was co-localized with the hardening (autofluorescent) constituents of the sheath. However, the narrowest, deepest branches of the sheath (which ultimately enter the target xylem cell), were composed almost exclusively of EGase. They did not autofluoresce like the rest of the sheath. Also, EGase both bound to and evidently infiltrated the cell walls of the xylem, probably due to cell wall loosening caused by the enzyme (**Figure 2b**). This would produce a very strong seal of the stylets into the cell, as hypothesized by Backus et al. (2005). In addition, glucanase-labeled saliva was found in xylem cells distant from the sheath, indicating that it traveled from the site of injection (**Figure 3**).

Toward the end of Kingston's tenure, we performed one more EPG experiment to generate GWSS-inoculated grape petioles, this time with young, newly eclosed adults from a putatively clean GWSS colony in Fresno, CA. Thus, a total of about 160 GWSS probes were EPG-recorded on grape petiole, producing salivary sheaths for immunohistology work.

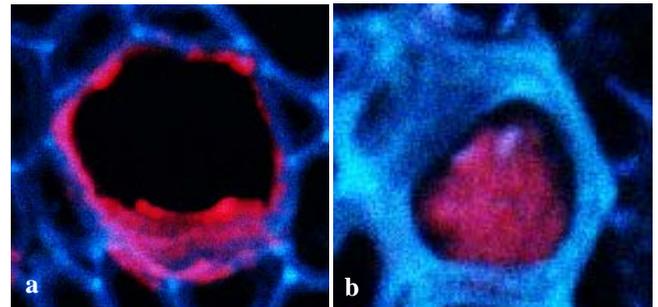
Although proposal funding has ended, work on this project will continue to completion using ARS in-house funds. Steps 4 and 5 (above) will be performed during the coming year, with the addition of immunolocalization of *Xf* from probed grape petioles. We will attempt to co-localize both *Xf* and EGase in the GWSS saliva within probed grape petioles (step 5, above).

## CONCLUSIONS

The described findings continue to support the following hypotheses: 1) cell wall-degrading salivary enzymes are injected during the earliest stages of stylet penetration, as well as further along the pathway and into a xylem ingestion cell, 2) GFP-*Xf* exit the stylets during stylet penetration, and become embedded in the salivary sheath, as well as injected directly into xylem cells, and 3) EGase-containing saliva infiltrates xylem cell walls at its site of injection and further distant; it could potentially interact with pit membranes at any site. Findings from this study will help solve the PD/GWSS problem by opening up all-new avenues for transgenic host plant resistance. Novel transgenes could be developed by engineering an inhibitor of the salivary components that aid inoculation. In addition, differences in vector efficiency among GWSS populations, or other vector species, could be related to salivary enzyme composition. Biochemical analysis of saliva in other vectors (e.g. Brazilian vectors of Citrus Variegated Chlorosis, or vectors of Oleander and Almond Leaf Scorch) could aid understanding of the epidemiology of all xylellae diseases.



**Figure 2.** a. Immunohistological confocal image of a GWSS salivary sheath in grape petiole. Single-branched sheath penetrates to a large, lignified xylem cell (autofluorescent cell walls, blue). Sheath is brightly autofluorescent in blue and green wavelengths, while the EGase fluorochrome is red. White coloration is overlay of all wavelengths. \* plastids and vacuole contents also binding polyclonal EGase. b. Higher magnification view of the terminal sheath branch and xylem cell.



**Figure 3.** a. EGase-containing sheath saliva lining a xylem cell several sections away from a salivary sheath. Note infiltration of walls. b. A large blob of EGase-containing sheath saliva inside a xylem cell, many sections away from, but in the same terminal xylem cell as, a salivary sheath.

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

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**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<sub>2</sub>O] 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<sub>2</sub>O 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 60° C for 10 min. The silica was resuspended in 100 µL of TE Buffer and incubated again for 5 min at 60° 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).

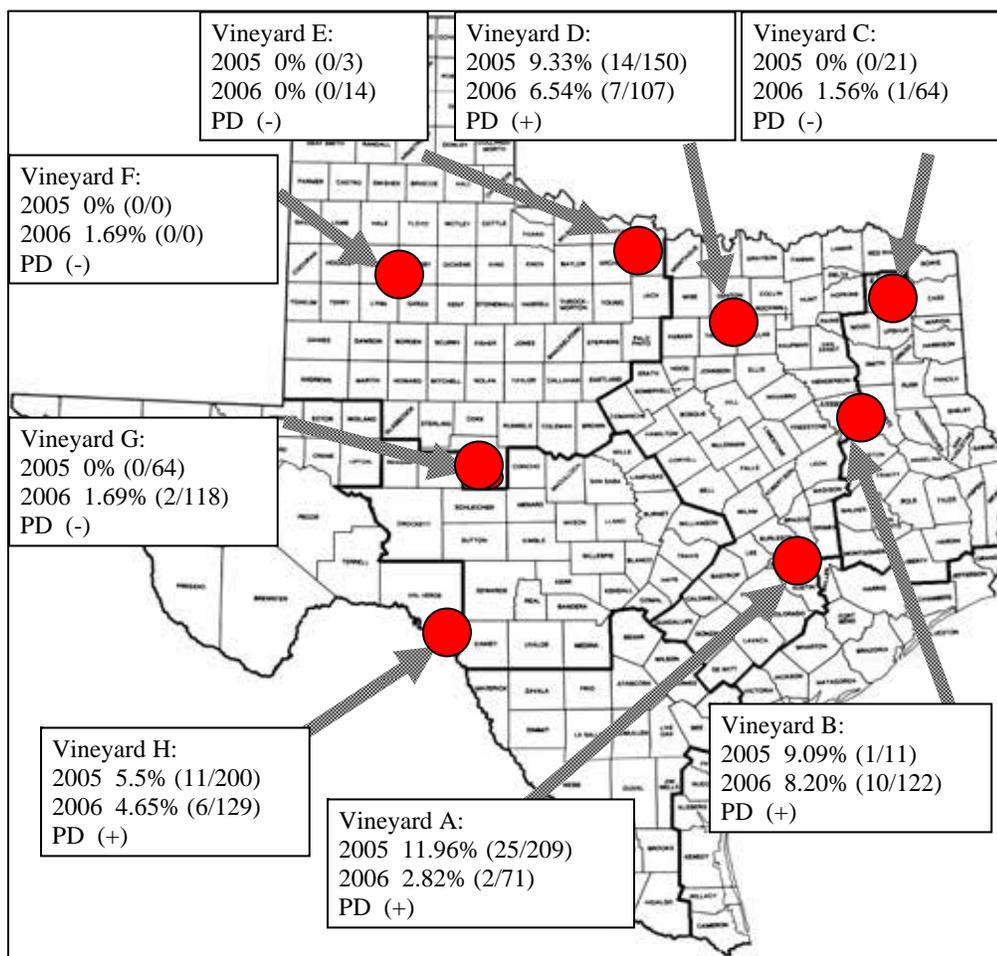
The resuspended samples were transferred to the appropriate Beckman Coulter 96-well microplates, centrifuged at 300 rpm at 2°C for 30 s, and then overlaid with one drop of mineral oil. Samples then were sequenced in a CEQ 8000 Genetic Analysis System (Beckman Coulter) using the manufacturer's protocol. 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](http://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 symptomatology 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 than 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.

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**USING AN ORANGE OIL SOLVENT TO REMOVE INSECTS FROM YELLOW STICKY TRAPS:  
IMPACTS ON THE DETECTION OF *XYLELLA FASTIDIOSA* DNA  
IN GLASSY-WINGED SHARPSHOOTER SAMPLES**

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**ABSTRACT**

*Xylella fastidiosa* (*Xf*) Wells is a plant pathogenic bacterium that causes many economically important agricultural diseases and is transmitted by the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Hemiptera: Cicadellidae). Effective detection of *Xf* in field collected GWSS in an area-wide management program can contribute to the assessment of risk associated with insect presence in vineyards. Prior to conducting molecular assays for detection of *Xf* in individual insects, GWSS must be removed from yellow sticky traps using a solvent. In this study, we determined the effect of orange oil concentration in individual GWSS on detection of *Xf* by QRT-PCR. In a ten-fold dilution series of orange oil, increased amounts of orange oil caused decreased levels of *Xf* detection in standardized positive samples. Additionally, we determined methods for lowering the concentration of orange oil found in processed field samples below the point where detection of *Xf* is negatively impacted.

**INTRODUCTION**

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Hemiptera: Cicadellidae), is the major vector of *Xylella fastidiosa* (*Xf*) Wells in the southern USA (Adlerz 1980; Blua et al., 1999). The plant pathogenic bacterium, *Xf*, has caused economic losses to a several agricultural industries in North, Central, and South America. Pierce's disease (PD) of grapevine has become a well understood *Xylella*-related disease; the vector complement is well known and the epidemiology of the disease is well documented (Hopkins et al., 2002). The introduction of GWSS into new areas is directly related to increased occurrence of PD in vineyards (Perring et al., 2001). Therefore, the management and control of PD depends heavily on the ability to closely and accurately monitor its vectors, especially GWSS.

In area-wide management studies in California and Texas, GWSS are collected using yellow sticky cards such as the Trece Inc. adhesive trap T3306 (Trécé, Inc., Adair, OK). This method works very well for monitoring population numbers and identifying species that occur in the field; however, the adhesive that coats the yellow sticky card can be problematic when applying molecular techniques (DNA and RNA studies) to these samples. Therefore, the sticky adhesive on the yellow sticky cards must be removed. This specimen extraction process involves application of a strong organic solvent to remove the adhesive. Because the downstream molecular assays involving DNA extraction and PCR both require the heating of samples, a solvent with a low flashpoint must be used. Orange oil has the lowest flashpoint of any organic solvent on the market, 118°F/48°C (Florida Chemical Co. Inc., Winter Haven, FL). In addition to its non-volatile composition, this product is inexpensive, nontoxic and effective at removing sticky adhesives. Orange oil is the most common product used in studies involving the removal of insects from yellow sticky cards and is usually applied directly to insects on the adhesive traps. Because all specimens come in direct contact with the solvent, they often absorb unknown amounts of the orange oil. As a result of this, concern was expressed as to whether or not orange oil retained in the insect bodies interfered with quantitative real-time polymerase chain reaction (QRT-PCR). It was speculated that the presence of orange oil in an GWSS specimen containing *Xf* would inhibit either the extraction of DNA, the amplification of target DNA during PCR, or the fluorescence signal emitted during QRT-PCR. In this study, a 10-fold dilution series of different volumes of orange oil mixed with positive *Xf* control specimens were analyzed by QRT-PCR to determine the amount of interference caused by the solvent. We also determined the amount of orange oil contained in a typical extracted sample and discussed the potential effect this will have on *Xf* detection in field samples.

**MATERIALS AND METHODS**

**Sample Collection.** Samples were collected using sticky adhesive-based double-sided traps (Seabright Laboratories, Emeryville, CA), each 23 x 14 cm in size, bright yellow in color (Pantone® Matching System (PMS) and coated with Stikem Special® glue, stored at -4°C, and transported to the University of Texas at Tyler in Tyler, TX. Traps were processed one at a time by individually marking GWSS that were to be recovered and then placing traps in plastic containers and soaking them in orange oil (Citrus Depot, St. Petersburg, FL) for five minutes per two-sided card. Then, using tweezers, the insects were removed individually from the traps, and placed into micro centrifuge tubes (MCTs). Each MCT was labeled according to its corresponding trap with its vineyard's location and its individual location within said vineyard.

*DNA Extraction.* Once extracted from sticky traps, GWSS bodies were separated from their heads and placed back in their original MCTs (Bextine et al. 2004). Briefly, the heads were then placed in 96 well plates, with one head per well, and covered with 200 $\mu$ L PBS buffer. The plates were placed under vacuum suction for two minutes a total of five consecutive times. The heads were removed, and 200 $\mu$ L of Lysis Buffer L6 was added to each well. The plates were centrifuged at 5,000rpm for 5 minutes, and 300 $\mu$ L of the supernatant in each well was transferred into a corresponding MCT. Afterward, 53 $\mu$ L of silica slurry was mixed into the 300 $\mu$ L solution, and the MCTs were incubated at room temperature for five minutes, and centrifuged at 2000rpm for another five minutes. Afterward, the supernatant was drawn off and discarded, and 200 $\mu$ L of wash buffer was added to each MCT. The MCTs were then centrifuged at 2,000rpm for five minutes, and the supernatant was drawn off and discarded. This washing step was repeated twice for a total of three washes. The MCTs were dried at 60°C with their caps open for ten minutes, or until the silica was dry. One hundred  $\mu$ L of TE buffer was gently mixed with the silica in each MCT, and the MCTs were incubated at 60°C for another five minutes, and centrifuged at 5,000rpm for another five minutes. Seventy  $\mu$ L of the supernatant was transferred into a sterile MCT, without picking up any silica, and the MCT's were labeled and placed in the freezer.

*QRT-PCR.* The PCR hood was left under UV light for a minimum of thirty minutes prior to use. Once UV light was turned off, the station was sterilized using 10% bleach. All samples to be run were placed in a cold block, and the master mix reagents were allowed to defrost. PCR was conducted in 10 $\mu$ L reactions, including two *Xf* positive controls and two No Template Controls (NTCs). Each sample reaction, a total of 10 $\mu$ L, included 5 $\mu$ L iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5 $\mu$ L SYBR® Green nucleic acid gel stain (Molecular Probes™, Eugene, OR), 1.7 $\mu$ L nanopure water, 0.4 $\mu$ L forward primer, 0.4 $\mu$ L reverse, and 2 $\mu$ L sample DNA. Each *Xf* positive control contained only 1 $\mu$ L DNA, and each NTC contained only 10 $\mu$ L master mix (every reagent except DNA). Each reaction was carried out in 0.1mL PCR tubes (Corbett Research, St. Neots, Cambridgeshire, UK). The prepared samples were placed into a Rotor-Gene RG-3000 QRT-PCR machine (Corbett Research, St. Neots, Cambridgeshire, UK) and run to determine if they contained *Xf* positive DNA.

*Determining Orange Oil Retained.* In order to create an applicable orange oil dilution series, the average amount of orange oil retained in each GWSS body was first determined. Ten empty MCTs were weighed and logged. The average MCT mass was found to be 965.7mg. The same ten MCTs were each filled with 100 $\mu$ L orange oil and weighed once more. The average MCT + 100 $\mu$ L orange oil mass was found to be 1044.5mg. Ten GWSS bodies were soaked in orange oil for five minutes, following the trap removal protocol. The heads were removed, placed in ten MCTs, and centrifuged at maximum rpm (14,000rpm) for one minute. The heads were then removed and the MCTs containing retained orange oil were weighed. The average mass of the MCTs + retained orange oil was 965.84mg. The average mass of an MCT containing retained orange oil minus the average mass of an empty MCT was found to be the average mass of retained orange oil in an GWSS head (965.84mg - 965.7mg = 0.14mg).

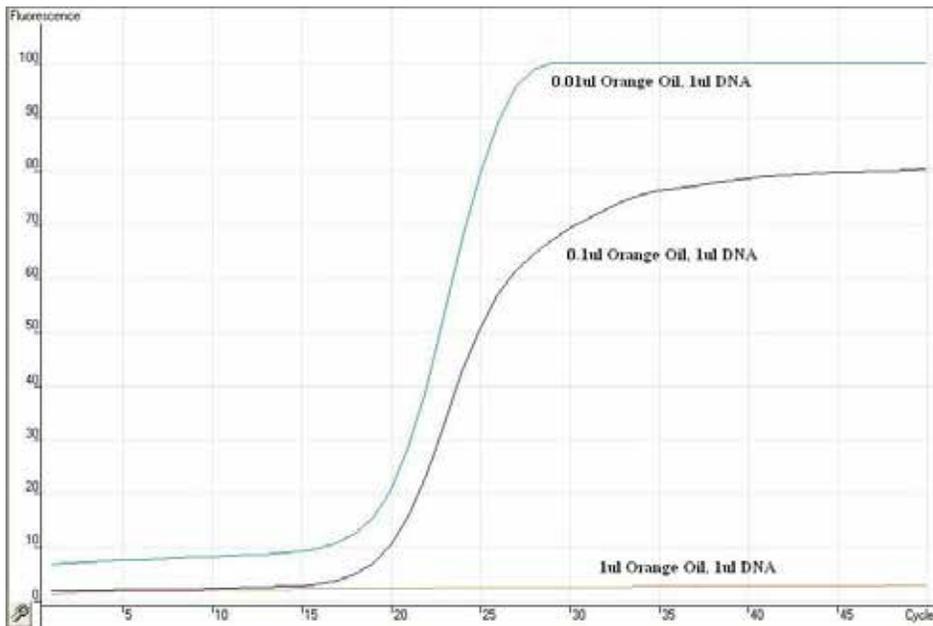
*Dilution Series.* Two orange oil dilution series were run through QRT-PCR in this test. The first was a ten-fold dilution series. Triplicates of samples containing 8 $\mu$ L master mix, 1 $\mu$ L positive *Xf* DNA, and 1 $\mu$ L of either 100%, 10%, or 1% orange oil were run through QRT-PCR. With the information gained from the ten-fold dilution series, a more precise dilution series was developed. This dilution series was a 1/1 – 1/10 dilution of orange oil. The same reaction volumes were used, and the same procedure was followed.

## RESULTS AND DISCUSSION

*Determining Orange Oil Retained.* The average mass of an MCT containing retained orange oil minus the average mass of an empty MCT was the average mass of retained orange oil in an GWSS head (965.84mg - 965.7mg = 0.14mg). The average mass of an MCT containing 100 $\mu$ L orange oil minus the average mass of an empty MCT equaled the average mass of 100 $\mu$ L of orange oil (1044.5mg - 965.7mg = 78.8mg). Since  $M1/M2 = V1/V2$ , the average mass of orange oil retained divided by the average mass of 100 $\mu$ L of orange oil was equal to the volume of orange oil retained divided by 100 $\mu$ L orange oil (0.14mg/78.8mg =  $V1/100\mu$ L). The average volume of orange oil retained by an individual GWSS head was 0.178 $\mu$ L (0.14mg/78.8mg x 100 $\mu$ L). This figure was used to determine the range of an orange oil dilution series.

*Dilution Series.* From the QRT-PCR data collected (**Figure 1**), 1 $\mu$ L of 100% as well as 1 $\mu$ L of 10% orange oil in a 10 $\mu$ L reaction completely inhibited binding of fluorescent binding proteins to *Xf* DNA, and 1% orange oil had no effect on binding.

Following the first dilution series, a more precise dilution series was developed. This dilution series was a 1/1 – 1/10 dilution of orange oil. The same reaction volumes were used, and the same procedure was followed. The result was a strong deviation from fluorescence expected under normal conditions in samples containing between 1 $\mu$ L and 0.1 $\mu$ L of orange oil. The average volume of retained orange oil per GWSS head, 0.178 $\mu$ L, is within this range, proving that the orange oil present in GWSS heads interferes with *Xf* DNA fluorescence in QRT-PCR.



**Figure 1.** Dilution Series 1, Cycling Curve Analysis. QRT-PCR with SYBR green®, 10ul reactions. 1ul oil, 1ul *X.f.* DNA (Orange). 0.1ul oil, 1ul *X.f.* DNA (Purple). 0.01ul oil, 1ul *X.f.* DNA (Blue).

## CONCLUSIONS

The presence of orange oil does inhibit the ability to detect *Xf* DNA by QRT-PCR by inhibiting the extraction of *Xylella* DNA, the amplification of target DNA during PCR, or the fluorescence signal emitted by fluorescent binding proteins during QRT-PCR. In our protocol, care was taken to avoid an overabundance of orange oil in samples. Primarily, we were extremely selective and conservative with the amount of orange oil used during insect extraction. Instead of soaking an entire trap, our procedure calls for a squeeze bottle that can directly apply small amounts of orange oil to each individual insect. Another step is the prompt transfer of each insect into a 70% ethanol wash, followed by a DI water wash, before being placed in a sterile MCT. This step ensures that each insect has been thoroughly washed of enough orange oil that contamination is no longer a concern. Seventy percent ethanol also cleanses each insect of many other contaminants as well. Concentrated orange oil can also be diluted, but loses its solvent strength the more it is diluted. We use concentrated orange oil in our trap extractions because it dissolves the sticky trap adhesive faster and more efficiently. Again, other solvents may be more efficient and less contaminating, but we must use orange oil in our extractions due to its high flashpoint. Other strong organic solvents, such as turpentine, hexanes, or ethers have flashpoints too low to be used in silica-based DNA extraction or QRT-PCR. The temperatures reached in these processes are too high for other organic solvents except orange oil.

Another factor in the inhibition of QRT-PCR performance by *Xylella* DNA may be the sticky adhesive that is dissolved in orange oil during the trap extraction process. We believe that this does not have an inhibiting effect due to the adhesive's inability to absorb into the bodies of GWSS. However, our follow up experiment will test this theory using the same methods discussed in this test.

The results of this experiment are crucial in further understanding the insect vector, GWSS. The management and control of Pierce's disease depends heavily on the ability to closely and accurately monitor its vectors. This experiment impacts any insect extraction and trap removal procedure involving organic solvents, a prominent practice in entomology across the globe.

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## COMPARATIVE GENOMICS: IDENTIFYING SIMILARITIES AND DIFFERENCES ACROSS THREE LEAFHOPPER VECTORS OF *XYLELLA FASTIDIOSA*

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### ABSTRACT

Leafhoppers are considered the second most important vector of agricultural diseases. We examined the gene expression across three leafhopper species, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*), the blue-green sharpshooter (BGSS; *Graphocephala atropunctata*), and the black-winged sharpshooter (BWSS; *Oncometopia nigrigans*), which are vectors of the plant-infecting bacterium, *Xylella fastidiosa*, which causes Pierce's disease (PD) of grapes. The use of genomic data is providing new information on the biology and relatedness of these and other leafhoppers. Using a genomics approach has also advanced the understanding of leafhopper immunity, pathology, and development. As new developments in genomics and RNAi methodologies emerge, researchers will be able to use this genetic information to design highly specific and effective management tools to reduce either leafhopper populations, and/or leafhopper-transmitted diseases. The importance of these leafhoppers as the vectors of PD, the abundance of Expressed Sequence Tags (ESTs) produced for each, and their differences in host plant preferences, provide an excellent opportunity to conduct comparative examination of these leafhoppers. Several cDNA libraries which had been made from adult GWSS, BGSS, and BWSS, plus nymphs, and tissues, provided a resource totaling almost 50,000 ESTs. When assembled, we obtained ~5,000 specific transcripts for each species for comparison. This is approximately one-third of all the predicted active genes available, as other insect genomes have demonstrated ~15,000 total genes. These were used for analyses between these species as well as for larger analysis to known genomes. Further analyses were conducted *in silico* using software programs available online Internet Resources, NCBI, EXPASY, and others to compare assembled data, predict proteins and compare them to the broader scope of insect genomes.

Many other genes of interest which have various functions in leafhopper biology and physiology have also been identified but are not reported herein. The EST sequences reported in this study have been deposited in GenBank's dbEST (see references: Hunter 2005, 2006, 2007).

### INTRODUCTION

Sharpshooter leafhoppers are vectors of a number of economically important destructive plant diseases caused by the plant infecting bacterium, *Xylella fastidiosa* (*Xf*). Understanding how these leafhoppers interact with their host plants and the pathogens they transmit is key to developing new management strategies against Pierce's disease (PD). Advances in genomic sequencing now permits researchers to examine thousands of genes which leafhoppers depend during feeding, development, and which are associated with disease acquisition and transmission. We compared the available genetic data for three leafhopper species, *Homalodisca vitripennis* (glassy-winged sharpshooter; GWSS), *Graphocephala atropunctata*, and *Oncometopia nigrigans*, (Hunter 2003, Hunter et al., 2005, 2006, 2007) which are vectors of the plant-infecting bacterium, *Xf*, which causes PD of grapes, and other 'scorch-like' diseases in other woody crops. (Hopkins and Purcell, 2002).

Sharpshooter leafhoppers, belong to the insect order Hemiptera, and feed primarily from the plant xylem, with minor amounts of feeding from the mesophyll and phloem (Backus and Hunter 1989, Hunter and Backus 1989). Xylem unlike plant phloem does not contain large amounts of sucrose and amino acids. Amino acids and soluble proteins are the primary nitrogen nutrients in xylem fluid (Andersen et al., 1989,1992). The dietary nitrogen impacts survival, growth, and reproduction of phytophagous insects (Bi et al., 2005). Consequently, the nutritionally dilute chemistry of the xylem fluid is a probable cause of the extremely high rate of feeding by leafhoppers (Brodbeck et al., 2004). The reported ability of leafhoppers to physiologically assimilate at least 99% of the amino acids, organic acids, and sugars is an evolutionary adaptation in response to their unique food source (Andersen et al., 1989; Brodbeck et al., 1999, 2004, Redak et al., 2004). This adaptation has a genetic basis and using genomics we can start to identify many of the genetic components which are key to leafhopper feeding, digestion, and growth.

Thus an important part of our project involves gaining a better understanding of the digestive physiology of leafhoppers vectors of PD. Genomics is providing the molecular tools needed to investigate the role proteins and peptides play in leafhopper nutrition. The increased comprehension of leafhopper digestive physiology also provides a more thorough understanding of the nutritional requirements, effects of host plants, and will provide the information needed to produce more effective mass rearing methods for application in the production of leafhoppers for parasitoid production.

Although the full extent to which leafhoppers, like GWSS, utilize host plant proteins is not understood, the ability to utilize xylem proteins as a nutrient source depends heavily on the presence and activity of the kinds of proteases within the digestive tract. Therefore, identifying these proteases and other enzymes will influence current tenets and advance our understanding of the underlying mechanisms of leafhopper digestive physiology. The use of expression libraries is a timely approach to understanding the genetic basis of proteolytic activity as it relates to insect development (Hunter et al., 2003; Sabater-Munoz et al., 2006), feeding and digestion (Colebatch et al., 2002; Coudron et al., 2007).

## OBJECTIVES

Apply comparative genomics to advance the understanding of leafhopper biology, digestion, and development. These data support development and application of emerging management strategies which rely on an understanding of leafhopper genetics.

## RESULTS

The datasets were produced in the Hunter lab (2005-2007), with sequencing performed at the Genomic lab, ARS, U.S. Horticultural Research Lab, Ft. Pierce, FL.

**Sequence Analysis:** Base calling was performed using TraceTuner™ (Paracel, Pasadena, CA) and low-quality bases (quality score <20) were stripped from both ends of each Expressed Sequence Tag (EST). Quality trimming, vector trimming, and sequence fragment alignments were executed using Sequencher™ software (Gene Codes, Ann Arbor, MI). Sequencher contig assembly parameters were set using a minimum overlap of 50 base pairs (bp) and 90% identity. Contigs joined by vector sequence were flagged for possible misassembly and manually edited. Putative sequence identity was determined based on BLAST similarity searches using the NCBI BLAST server ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) with comparisons made to both non-redundant nucleic acid and protein databases using BLASTN and BLASTX, respectively (Altschul et al., 1997). Matches with an E-value  $\leq 10$  were considered significant and were classified according to the Gene Ontology (GO) classification system (Schäffer et al., 2001). A partial list of ~29 transcripts (**Table 1**) show homologous matches between leafhoppers, and the E-values showing relative homology. As the value approaches zero, the more significant the homology match (yellow), as sequences diverge, having less homology the values become farther away from zero, approaching a positive number (*note*: all values in Table 1 under E-value are negative or zero).

**Digestive Enzymes:** Aminopeptidases, several cathepsin L-like cysteine proteases, and other proteases have been identified in these leafhoppers which are also in other piercing-sucking feeding insects (Foissac et al., 2002, Wright et al., 2006, Zhu et al., 2003). In aphids, a cathepsin B protease has been shown to be constitutively expressed in all aphid individuals, suggesting gene duplication and evolution of a novel biological function of cathepsin B in the aphid lineage (Houseman and Downe 1983). Cathepsin B proteases were also identified in these leafhoppers and may show similar duplication.

Cleavage of food proteins into peptides and amino acids is an important process for which an array of proteases of different substrate specificity and enzymatic activities are produced in the alimentary tract and are involved in protein digestion (Terra et al., 1996, Sajid and McKerrow 2002). Gene duplications relevant to biological requirements such as those which encode digestive proteases, have been documented in: lepidopteran insects (Chougule et al. 2005), coleopteran insects (Zhu-Salzman et al. 2003; Brown et al. 2004), parasitic helminths (Dvora ěk et al. 2005), and will most likely be found to have occurred within leafhoppers. The number of genes associated with leafhopper biology continues to expand as more genetic information is produced and compared from different species of leafhoppers (**Figure 1**). Annotation of these data advances current understanding of leafhopper biological pathways while providing clues to the genetic basis of such processes in insect-pathogen, and insect-plant interactions (**Figures 2 and 3**). The availability of genomic data for these leafhoppers continues to increase, thus uses of the current data provides a solid foundation for future studies in leafhopper functional genomics.

## CONCLUSIONS

The information gained from this study provides the first investigation using comparative genomics of the transcriptomes from three leafhopper vectors of PD of grapes: *H. vitripennis*, *G. atropunctata*, and *O. nigricans*. Amino acid sequence comparisons BLASTX, BLASTP with other known proteins relies on conserved motifs of specific domain(s), NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/blast>). *In silico* analysis based on protein domains is a widely accepted method which continues to increase in quality and demonstrates the application of Bioinformatics to address many biological questions. Many of the discoveries made in other insects, such as *Drosophila*, Honey Bee, or Lepidopteran species, can be applied within the Hemiptera when the same genetic transcripts can be identified. For example, we increased our

understanding of the roles and pathways of heat shock proteins in leafhoppers by examining the data completed in Locusts, Flies, and Nematodes. The same is true for digestive enzymes.

The increasing application of transcriptional data is leading the way in the development of new strategies to reduce plant diseases and their insect vectors. Application of RNAi against a wide range of insect species from spruce budworm to whiteflies are viewed as the future in insect pest control, and many new methods which incorporate the use of native endophytic bacteria and/or viruses as the mechanism for delivery or expression of dsRNA within plants are being widely evaluated. The main advantages of applying genomic data in this manner to solve agricultural problems is that the plants are not 'transformed', thus the quality of the crop is not altered, saving time, money, and reducing the effort needed to find solutions to many emerging devastating agricultural problems. Collectively, these genetic sequences provide the foundation needed for further functional genomic studies which will enable the development of more biorational management strategies to reduce losses from the diseases spread by these and other leafhopper pests.

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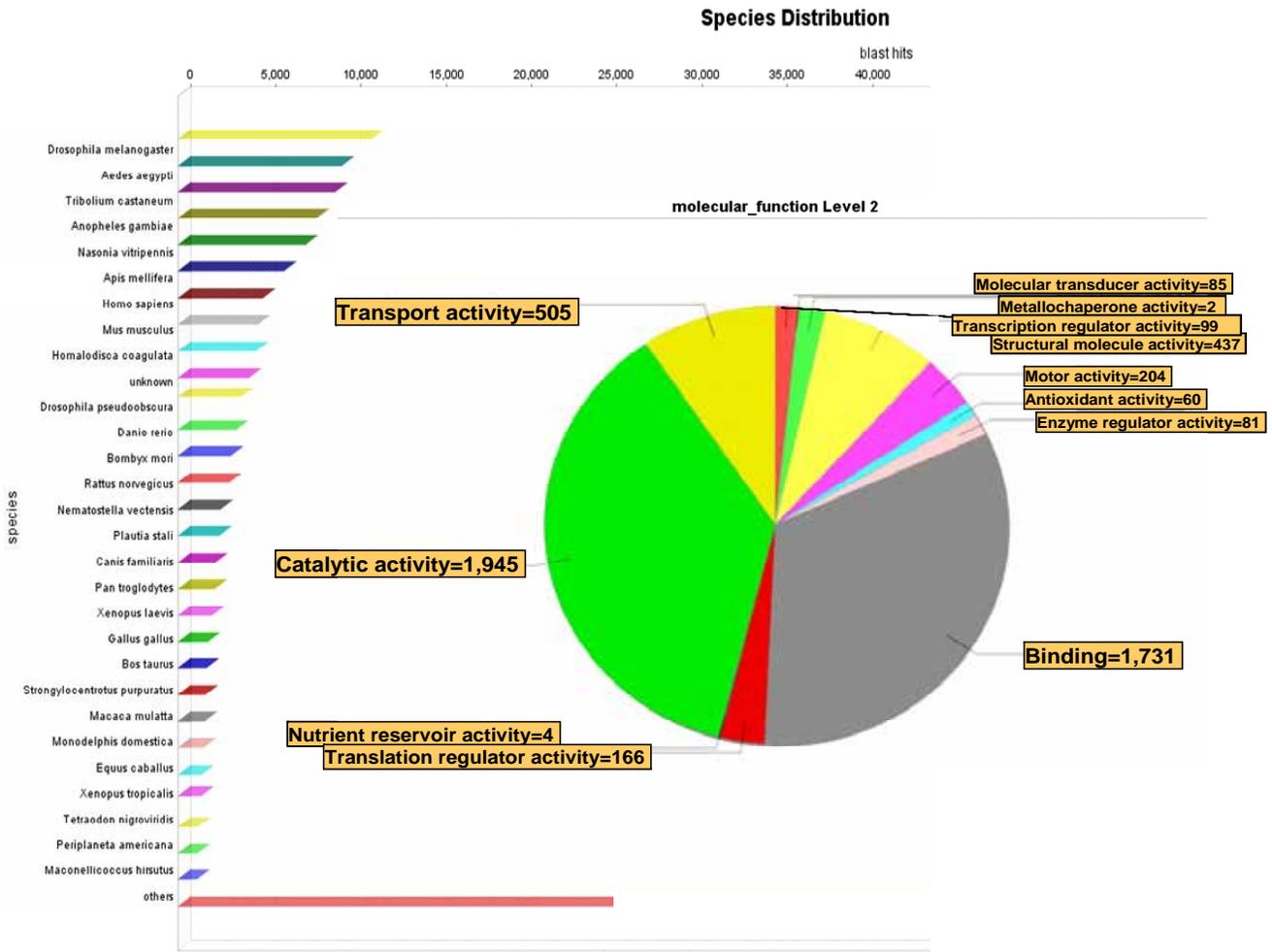
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**Table 1. Partial Comparison of cDNA's in three leafhopper species, *Homalodisca vitripennis*, *Graphocephala atropunctata*, and *Oncometopia nigricans*.** Analysis using BlastX, Values approaching zero are more significant in sequence identities (**Yellow**). Genes which have more variability (**Blue**). Sequence homology was greater between *Homalodisca* and *Oncometopia* than to *Graphocephala*, which supports current taxonomy separating these leafhoppers. Only a partial list is shown for sequences within Molecular Function.

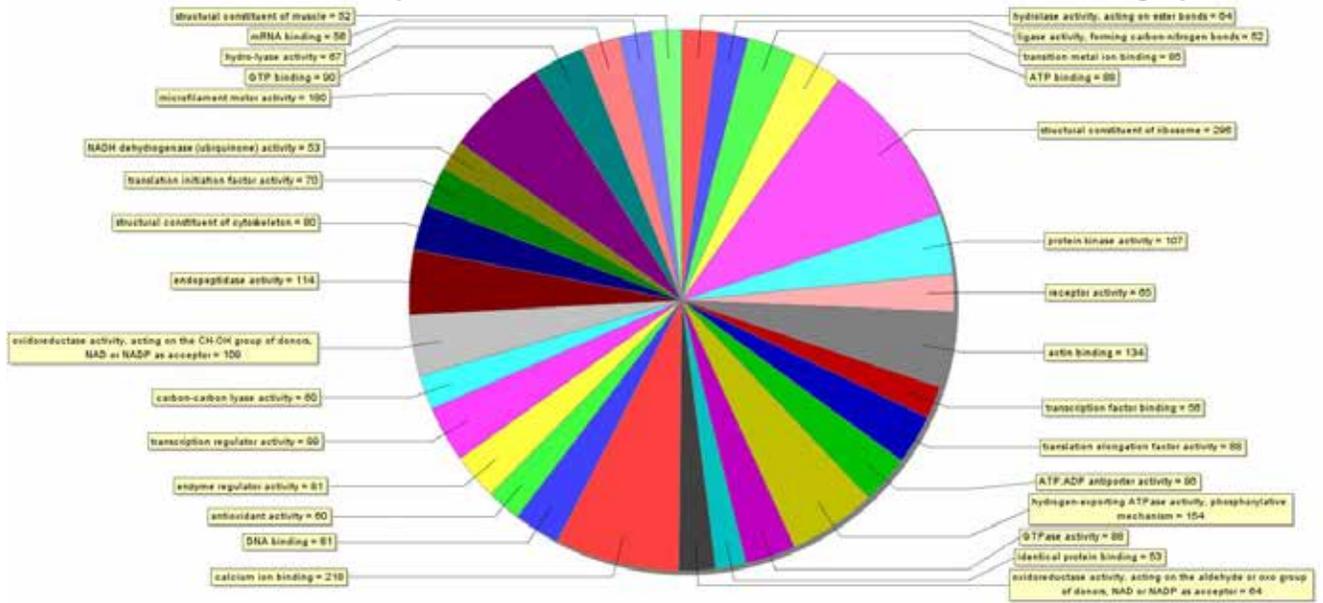


Accession	EC #	BLAST DeKegg Ids	GO TYPE	GO1	GO2	GO3	HC_ID	E value (HC)	GA_ID	E value (GA)	ON_ID	-value (ON)	
Q7QFH6		AgCP1442	Molecular Function	motor activity	structural molec	structural	WHMg 2758	0	WHGA067-6	5.4086E-102	WHON042_E12	1.1E-134	
Q86GF8		Hypothetic Citrate cycle (TCA cycl	Molecular Function	aconitate hydratase	mitochondrion	tricarboxyl	WHMg 2485	0	WHGA2364	7.01792E-63	WHON0097	0	
Q6PPH6		Putative cytoplasmic actin A3a1	Molecular Function	motor activity	structural molec	structural	WHHC51631	0	WHGA2663	4.56082E-67	WHON0017	0	
Q6RFY9	[3.6.5.3]	Putative elongation factor 1-alpha	Molecular Function	translation elongatio	GTP binding	cytoplasm	WHHC51398	0	WHGA0038	0	WHON0147	0	
Q6PPH5		Putative muscle actin	Molecular Function	motor activity	structural molec	structural	WHHC51395	0	WHGA2643	1.92228E-73	WHON0021	0	
Q7PMM3	1.6.5.3	ENSANGP0000000024398	Molecular Function	iron ion binding	electron transp	mitochondrion	WHHC2058	0	WHGA1900	8.2498E-107	WHON1268	0	
Q7PPE7	2.7.1.40	ENSANGP0000000021580	Molecular Function	magnesium ion bind	pyruvate kinase	glycolysis	WHHC0110	0	WHGA2158	1.54504E-62	WHON0828	2.1E-138	
Q6PPH2		Putative activated protein kinase 1	Molecular Function	receptor activity	kinase activity		WHHC2042	0	WHGA006-44	1.6439E-120	WHON1478	1.98E-48	
Q6PPH6		Putative cytoplasmic actin A3a1	Molecular Function	motor activity	structural molec	structural	WHHC00652	0	WHGA2663	4.56082E-67	WHON0017	0	
Q6PPH6	1.14.19.1	Putative delta-9 desaturase 1	Molecular Function	stearoyl-CoA 9-desat	ion binding	endoplasm	WHHC0256	0	WHGA0206	0	WHON0283	0	
Q6RFY9	3.6.5.3	Putative elongation factor 1-alpha	Molecular Function	translation elongatio	GTP binding	cytoplasm	WHHC0034	0	WHGA0038	0	WHON0147	0	
Q6PPH0	4.1.2.13	Putative fructose-1,6-bisphosphate	Molecular Function	fructose-bisphospha	glycolysis	lyase activ	WHHC0177	0	WHGA1213	0	WHON0128	0	
Q6PPH5		Putative muscle actin	Molecular Function	motor activity	structural molec	structural	WHHC0074	0	WHGA2643	1.92228E-73	WHON0021	0	
Q6PPH1		Putative rhodopsin	Molecular Function	rhodopsin-like recep	receptor activity	G-protein	WHHC0120	0	WHGA051-49	3.1982E-122	WHON0281	0	
Q6PPH3		Glycerol 3-phosphate	Molecular Function	glycerol 3-phosphate	metabol	glycolysis	WHMg 1552	4.3383E-168	WHGA0261	1.9645E-169	WHON0243	4.9E-173	
Q6PPH2		Putative activated protein kinase	Molecular Function	receptor activity	kinase activity		WHHC2008	4.6847E-166	WHGA2745	9.75547E-72	WHON1478	1.98E-48	
Q6PPH0		Putative fructose-1,6-bisphosphate	Molecular Function	fructose-bisphospha	glycolysis	lyase activ	WHMg 2297	2.3954E-147	WHGA1213	0	WHON013_H01	2.59E-19	
Q17083		Vitellogenin precursor	Molecular Function	lipid transporter	actin	lipid transport	nutrient re	WHHC0041	3.3648E-143	WHGA019-70	2.47424E-39	WHON0059	1.1E-105
Q9VFF0		CG3731-PA	Molecular Function	metalloendopeptidaz	proteolysis		WHHC52247	5.2528E-142	WHGA085-15	4.12314E-65	WHON036_H10	5.27E-30	
Q7PXA8		AgCP12715	Molecular Function	catalytic activity	GTP binding	tricarboxyl	WHHC1365	0.5226E-136	WHGA2584	2.53721E-45	WHON007_F09	2.43E-73	
Q7PFS3		ENSANGP0000000024398	Molecular Function	electron transporter	iron ion binding	tricarboxyl	WHHC2462	1.1502E-129	WHGA1364	1.55212E-69	WHON030_F08	2.06E-64	
Q7PPE7	[2.7.1.40]	ENSANGP0000000021580	Molecular Function	magnesium ion bind	pyruvate kinase	glycolysis	WHSG044_B07	7.9038E-108	WHGA2158	1.54504E-62	WHON0828	2.1E-138	
Q7PPE7		ENSANGP0000000021580	Molecular Function	magnesium ion bind	pyruvate kinase	glycolysis	WHMg 2868	2.4039E-107	WHGA2158	1.54504E-62	WHON0828	2.1E-138	
Q9VFF0		CG3731-PA	Molecular Function	metalloendopeptidaz	proteolysis		WHMg045_G12	6.9007E-105	WHGA085-15	4.12314E-65	WHON036_H10	5.27E-30	
Q9V4D3		CG1970-PA	Molecular Function	electron transporter	mitochondrion	electron tr	WHSG031_H08	6.3715E-104	WHGA2174	1.50812E-85	WHON032_H08	4.29E-96	
Q17083		Vitellogenin precursor	Molecular Function	lipid transporter	actin	lipid transport	nutrient re	WHHC0115	4.2689E-103	WHGA1463	4.34174E-57	WHON0059	1.1E-105
Q6PPH2		Putative ferritin GF2	Molecular Function	binding	iron ion transport	iron ion ha	Contg 1413	3.50513E-95	WHGA0709	7.98921E-91	WHON0085	2.56E-92	
Q7QBS4		AgCP2476	Molecular Function	male enzyme activ	malate metabol	oxidoreduc	WHSG041_F10	3.9499E-95	WHGA1635	5.71834E-84	WHON034_G07	5.86E-93	
Q6PPH2		Putative ferritin GF2	Molecular Function	binding	iron ion transport	iron ion ha	WHMg 1739	4.47344E-95	WHGA0709	7.98921E-91	WHON0085	2.56E-92	



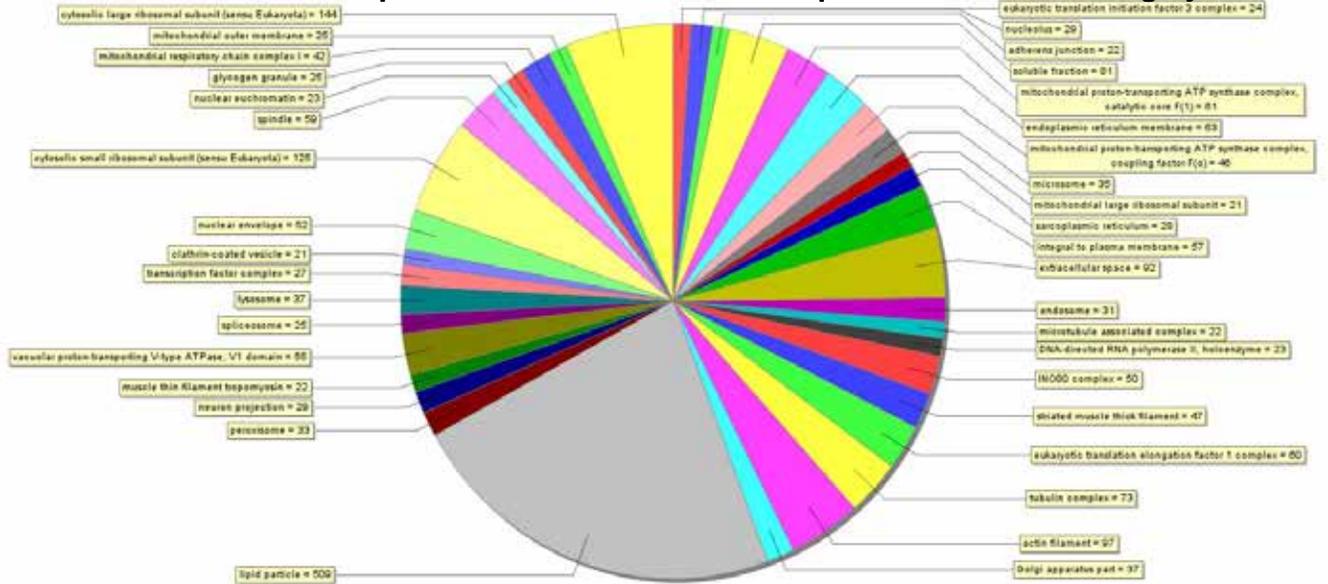
**Figure 1. Composite figure** showing distribution of *Homalodisca vitripennis* transcripts across other species (along left), with the top 6 species homologies being in these insects whose genomes have been completed: *Drosophila melanogaster*, *Aedes aegyptii*, *Tribolium castaneum*, *Anopheles gambiae*, *Nasonia vitripennis*, and *Apis mellifera*. **Molecular functions** of transcripts gave the greatest number within: Catalytic activity=1,945; Binding=1,731; and then transporter activity=505. Broad Categories. Represents EST's from three cDNA libraries, Adults, 5<sup>th</sup> instar, and Midgut. *H. vitripennis*, (Blast2GO analysis).

### Sequence Distribution: Molecular Function- cutoff 50.0/category



**Figure 2. Sequence Distribution: Molecular Functions.** Categories had to have at least 50 members. Represents EST's from three cDNA libraries, Adults, 5<sup>th</sup> instar, and Midgut. *Homalodisca vitripennis*, (Blast2GO analysis). Highest Categories in descending order: Ribosome structure= 296, Calcium ion binding=218, ATPase activity=154, Actin binding= 134, Microfilament motor activity=180, Endopeptidase activity=114, Oxidoreductase activity= 109, Protein Kinase activity= 107.

### Sequence distribution: Cellular Component- cutoff=20.0/category



**Figure 3. Sequence Distribution: Cellular Component.** Categories had to have at least 20 members. Represents EST's from three cDNA libraries, Adults, 5<sup>th</sup> instar, and Midgut. *Homalodisca vitripennis*, (Blast2GO analysis).

## ASSESSING THE POST-WINTER THREAT OF GLASSY-WINGED SHARPSHOOTER POPULATIONS

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**Reporting Period:** Results reported here are from work conducted October 2007 to September 2008.

### ABSTRACT

Glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, appears to be limited to discrete regions within the San Joaquin Valley where winter temperatures are mild and the temperature rarely drops below freezing. Prior research indicates that GWSS adults cannot feed at maximum daily temperatures below 50°F (= 10°C), thereby reducing its ability to survive cold winters. We verified the impact of cool temperatures on GWSS adults by exposing them to a regime of seasonal temperatures (within temperature cabinets) that reflect a variety of areas within the state. As expected, mortality rates varied greatly among sites tested, and it appears that mortality is related to both length of exposure as well as intensity of exposure (i.e., amount of cold endured). Using temperature records to calculate numbers of cooling degree days, we constructed ten GIS maps to delineate areas where post-winter GWSS mortality should be substantial, thereby providing a tool to estimate the springtime GWSS threat to different regions. However, estimates of post-winter GWSS mortalities were usually smaller (< 90%) than expected across much of the agricultural production areas of the Central Valley.

### INTRODUCTION

The initial arrival of glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, into California's Orange and Ventura counties was predicted to dramatically change Pierce's disease (PD) epidemiology within infested areas (Varela et al. 2001). The insect soon spread into other southern California localities. PD devastated the wine grape industry in the Temecula Valley resulting in significant losses. First detected in Kern County in 1998, GWSS is now present in the San Joaquin Valley. However, the rapid population expansion first observed in southern California appears to be limited to discrete regions within the San Joaquin Valley coincident with citrus production areas where overwintering populations are highest and winter temperatures are relatively mild compared to locations elsewhere in the San Joaquin Valley. Additionally, persistent, localized GWSS populations are present within the urban areas of Fresno, Sacramento, and San Jose Counties where a range of perennial plant host types and slightly elevated daytime high and evening low temperatures might favor the survival and persistence of established populations.

Hoddle (2004) used the climate modeling program "CLIMEX" to estimate the potential worldwide distribution of GWSS. His reported estimates for California (when all localities received supplemental irrigation water) suggested that GWSS could establish reproducing populations along much of the California coast from San Diego north to the Eureka vicinity and within the Central Valley from Bakersfield north to the Redding vicinity. He did propose cold stress as a potential limitation to the establishment of GWSS in states north of California (i.e., Oregon, Washington). However, other observations and studies suggest that low winter temperatures may be the "bottleneck" that limits GWSS survival and distribution in the higher altitudes and northern regions of California (Pollard and Kaloostian 1961, Russell Groves et al., unpublished data from 2003).

CDFR-funded research showed that GWSS adults do not feed near or below 50°F (10.0°C), and that individuals will die if held below 50°F for long periods (e.g., 15 or more days) even in the presence of food and water (Johnson et al. 2006). Also of significant importance is that the overwintering adult cohort is responsible for producing the offspring in the spring, which may start as early as late-February in some southern California areas (Krugner 2007). Given this, if the daily maximum temperature infrequently surpasses the thermal activation threshold (50°F) necessary for GWSS ingestion, then GWSS survivorship may be curtailed by extended periods of cool temperatures in specific microclimatic regions of California. We experimentally showed this phenomenon using programmable, temperature cabinets to simulate fluctuating diurnal temperature regimes based on January temperatures in the locations of Riverside (Riverside County.), Oakville (Napa County), and Buntingville (Lassen County), CA. In our study, the Riverside temperature always exceeded 50°F (= 10°C),

and about 20% of the test insects remained alive after 115 days; for Oakville it daily exceeded 50°F for 18 hours and only 10% of GWSS survived after 115 days; and for Buntingville the temperature never reached 50°F, and the entire test group died within 20 days (Youngsoo Son et al., unpublished data). We have applied the concept of cooling degree-days (CDD) to estimate the impact of cool temperatures on GWSS survival. The equation for  $CDD_{GWSS}$  may be expressed as:

$$\text{Daily } CDD_{GWSS} = \left[ \begin{array}{l} |T_m - 50|, \text{ if } T_m < 50 \text{ } ^\circ\text{F} \\ 0, \text{ otherwise} \end{array} \right]$$

where  $T_m$  = daily mean temperature in a given locality,  $|T_m - 50|$  = absolute value of difference between  $T_m$  and the feeding threshold of 50°F when the mean daily temperature is lower than 50°F. Daily  $CDD_{GWSS}$  equals zero if the daily mean temperature ( $T_m$ ) is higher than 50°F. By summing the  $CDD_{GWSS}$  for each day over an extended period, one can estimate the cumulative  $CDD_{GWSS}$  over the specified time period for that locality. Using unpublished field data collected by Don Luvisi, Farm Advisor *Emeritus*, in 2001-2002, we plotted the relationship between cumulative  $CDD_{GWSS}$  and GWSS survival at various sites in the vicinity of Bakersfield (Kern County). Based on a curvilinear regression, GWSS survival dropped to 0% when about 321  $CDD_{GWSS}$  in °F (or 178 in °C) were accumulated.

Because most of our previous CDFA-funded work on the impact of cool temperatures on GWSS feeding and survival was conducted using constant temperatures, it was necessary to validate our findings under actual fluctuating temperatures in the field. Prior efforts to field-validate the impact of cool winter temperatures on caged GWSS adults in the crop production areas of the San Joaquin Valley (e.g., east and west of Bakersfield, central Fresno and Merced Counties) and farther north (Napa and Sonoma Counties) were prevented due to concerns over potential escapes of GWSS individuals. Fortunately, we were permitted to establish one field test comparing GWSS adults caged in the urban area of Bakersfield versus caged GWSS in Riverside (UCR Citrus Experiment Station). The GWSS individuals died in a shorter amount of time at the cooler Bakersfield site than the Riverside site. However, only one field test of our hypothesis using fluctuating temperatures is inadequate. Therefore, we proposed to test the impacts of fluctuating temperatures on GWSS survival using programmable temperature cabinets as we have done for the study mentioned above. These additional studies would provide insights into the benefits of using cumulative  $CDD_{GWSS}$  to estimate GWSS survival. We also planned to analyze historical temperature data for various locations within the agricultural production areas of California to determine if winter conditions (e.g. November to March) would permit significant GWSS survival based on  $CDD_{GWSS}$  accumulation. The eventual product that we aim to produce from these efforts will be the production of GIS maps that estimate  $CDD_{GWSS}$  accumulation over the winter months to provide estimates of the ability of local GWSS populations to pose a substantial threat to local agriculture in the following growing season (i.e., a risk assessment). As resources for GWSS management dwindle, government agencies will be forced to make decisions on which regions should receive area-wide treatment to suppress GWSS populations. Our studies suggest that the presence of the GWSS threat may vary with the severity of local winter temperatures. An annual estimation of overwintering GWSS survival across agricultural regions will provide insights into where resources for GWSS suppression should be most effectively allocated.

## OBJECTIVES

1. Verify impacts of winter temperatures on GWSS survival from selected California sites;
2. Quantify and compare variation in “cooling degree day” accumulation within and among selected California sites using historical temperature data; and
3. Construct Geographical Information Systems (GIS) maps that estimate GWSS survival during the winter period.

## RESULTS AND DISCUSSION

### Objective 1

Verify impacts of winter temperatures on GWSS survival from selected California sites

Dr. Hannah Nadel conducted experimental studies in temperature-controlled growth chambers at the University of California at Riverside. Laboratory studies were conducted because using live GWSS in field-cage studies was prohibited outside of the GWSS-infested areas of California. Cabinets were programmed to run various fluctuating, diurnal temperature patterns that were representative of historical patterns from selected sites within California’s agricultural regions. For nine CIMIS sites (i.e. Riverside, Santa Ynez, Porterville, Merced, Davis, Oakville, McArthur, Gerber, and Arvin), mean daily maximum and minimum temperatures were calculated for the months of November, December, January, February, and March. GWSS adults were caged under a given temperature regime (e.g., McArthur) for a five month period. In chronological order (November, December, January, February, and March), the temperature cabinets were programmed to simulate the average maximum and minimum temperature patterns for the individual months (i.e., 30 days for November, 31 days for December, 31 days for January, etc.). To avoid mortality due to freezing, the minimum temperature was set at 3°C.

Adult GWSS were collected by beat-netting from lemon trees at the UCR Agricultural Operations citrus orchard in Riverside, CA, between late October and early December 2007. They were held on potted sweet orange and prostrate acacia in mesh

and vinyl cages in a greenhouse at  $25 \pm 4^\circ\text{C}$  with natural light (supplemented with sodium vapor lamps L:D 12:12) for 4 – 7 days before use.

Two plant species were selected as winter hosts for the study, ‘Washington Navel’ orange (*Citrus sinensis* [L.] Osbeck) grafted on trifoliolate orange (*Poncirus trifoliata* [L.] Rafinesque) rootstock, and prostrate acacia (*Acacia redolens* Maslin cv ‘prostrata’). Grapevines were not used as originally planned because of difficulty locating nursery stock not treated with insecticides. Prostrate acacia is a leguminous evergreen shrub that is an overwintering host for GWSS (H. Nadel, personal observation). One orange (75 cm tall) (TreeSource Citrus Nursery, Exeter, CA) and one acacia (Parkview Nursery, Riverside, CA) were potted together in a 180 cm<sup>2</sup> (7-inch) pot and all plants were acclimated at least 1 month in a greenhouse before the study started. A 3.0 mm layer of white sand was placed over the potting medium to facilitate observation of insects on the soil. A 10-day study revealed that the nursery plants were apparently free of toxic residues.

Exposure of GWSS to simulated November temperatures began on the following dates: Riverside 11/9/07; Arvin 11/14/07; McArthur, Oakville and Merced 11/16/07; Porterville, Gerber and Davis 11/30/07; and Santa Ynez 12/6/07. Five male and five female GWSS were transferred in vials from holding cages to each experimental cage. Seven replicate cages were placed individually in water saucers in each temperature cabinet and the plants and insects allowed to acclimate at 18°C for 24 hours before winter temperature simulations began. Cabinets were lighted from 6:00 AM to 6:00 PM by four 32 W fluorescent tubes and two 15 W incandescent bulbs.

GWSS mortality was recorded weekly. Cages were removed from temperature cabinets only long enough to examine and remove dead insects, and were quickly returned (2-5 min). Insects that appeared to be dead were removed from cages, placed on paper under room temperature (20-21°C), and covered with a clear vial. Those that did not revive within two hours were recorded as dead; revived GWSS were returned to their respective cages. Examination of cages was done during the warmest hours of the simulated day, when the insects were likely to show movement. Dead insects were counted and sexed. The potting medium was kept moist with weekly or biweekly watering, as needed.

Numbers of live and dead individuals were counted weekly until all insects died or the 5-month study period ended. The cumulative CDD<sub>GWSS</sub> were calculated for each location regime (e.g., Riverside, McArthur) based on temperatures recorded with HOBO recorders within the temperature cabinets (**Table 1**) and percent survival will be compared among regimes using survival analysis. The numbers of cumulative CDD<sub>GWSS</sub> required to kill all GWSS individuals per cage will be compared across location regimes to determine if the value to kill all test insects remains fairly constant across different diurnal temperature patterns. Mortality in all environments was  $\geq 97\%$  at the end of the study (**Figure 1**). As expected, all GWSS died in the McArthur environment by early December, after exposure to temperatures below feeding threshold. All insects died in the Davis and Oakville environments before accumulated CDD reached the average predicted value for 100% mortality. Although the Riverside environment accumulated no CDD, ~ 98% of the insects died by the end of the study. A modification of the planned analysis will therefore be necessary, possibly including a senescence function. It was apparent that there was a relationship between the rate of CDD accumulation and how quickly the insects died.

### **Objective 2**

Quantify and compare variation in “cooling degree day” accumulation within and among selected California sites using historical temperature data

Daily maximum and minimum temperature data were downloaded for 10 winter periods (November through March 1997 – 2007) from 15 CIMIS stations in several climatic regions of California, including areas expected to be suitable and unsuitable for GWSS winter survival. Two CIMIS stations, Merced and Porterville, were operative less than 10 years prior to 2007, and had only eight and seven years of data, respectively. CDD were calculated for each date and summed for each winter month, then averaged (**Figure 2**). Most of the sites accumulated less than 300 CDDs (based on °C). Five sites accumulated less than 200 cumulative CDDs.

### **Objective 3**

Construct Geographical Information Systems (GIS) maps that estimate GWSS survival during the winter period

We produced 10 GIS maps that show estimates of post-winter mortality of GWSS populations (estimated as % mortality) in regions across California following the periods of November through March for each year from 1998-1999 to 2007-2008. These maps were based on temperature data collected during the target months of November through March by CIMIS and the Western Regional Climate Center (WRCC). We estimated the cumulative CDD<sub>GWSS</sub> based on mean daily temperatures for about 340 temperature monitoring sites. One hundred percent mortality was achieved at 321 cumulative CDD<sub>GWSS</sub> (based on °F). Spatial statistics techniques using ESRI ArcGIS® Geostatistical Analyst were used to create interpolated surface maps using an Inverse Distance Weighted Analysis with a standard search of 206 points using 15 neighbors (at least 10 were found for each search). Two examples of the maps are provided showing dramatic differences in estimated GWSS mortality in the Central Valley in 1998-1999 (**Figure 3A**) versus 2007-2008 (**Figure 3B**) with much less mortality during the latter period.

Contrary to our initial assumptions, estimated post-winter mortality was not as high as we expected (i.e., 100%) in most of the agricultural areas of the state and was quite variable throughout the ten years examined. Within the Central Valley, estimated GWSS mortality resulting from cool temperatures (that inhibit normal feeding) usually varied from 80 to 95%. Based on these results, we now realize that there is a need to be able to estimate the size of GWSS populations at the end of the winter months as well as the potential for increase in various areas based on climatic conditions. Small post-winter populations in cool areas (Santa Ynez) may not pose a threat to agriculture compared to large post-winter populations in warmer areas (e.g., Merced). Also of importance is the time when GWSS females initiate egg laying in the spring. Egg laying may be as soon as late February in Riverside compared to later dates farther north in cooler areas.

## CONCLUSIONS

This project has generated significant new information regarding the impact of California winter temperatures on GWSS survival and also provides a practical tool to use in the decision making process for GWSS management. However, estimated post-winter GWSS mortality due to cold inhibition of feeding was smaller (< 90%) than expected across much of the agricultural production areas of the Central Valley in most of the winters for which maps were produced. In much of the Central Valley, mortality estimates ranged from 80 to 90%, which may be insufficient to prevent the initiation of threatening spring populations of the GWSS. However, occasionally estimates were as high as 90 to 99%. Another important factor is geographical location because GWSS populations in southern latitudes are typically able to initiate egg-laying earlier than populations farther north. More northern populations will suffer greater temperature-related mortality before they can initiate egg-laying activities.

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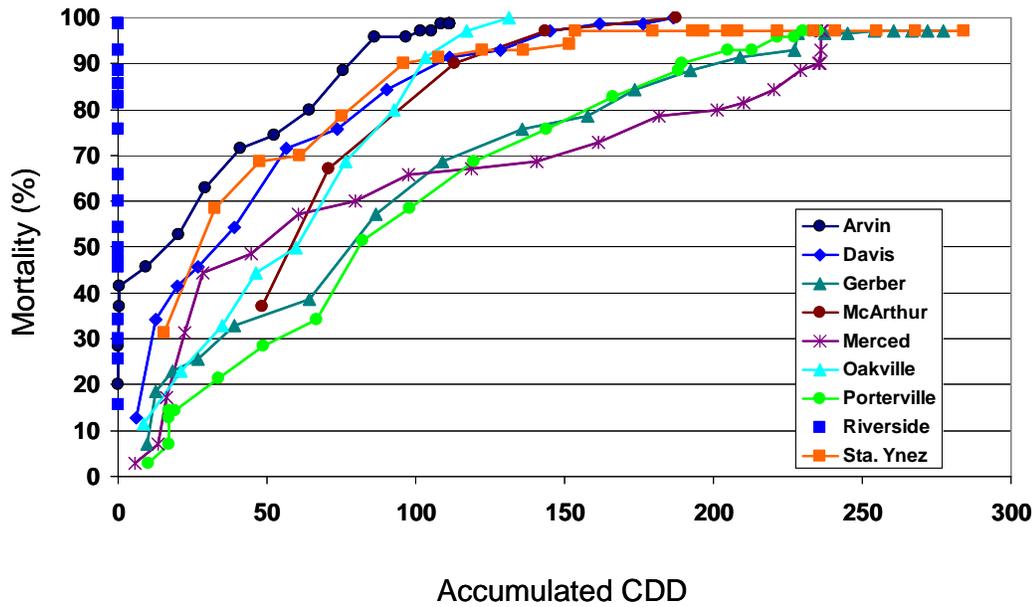
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## FUNDING AGENCIES

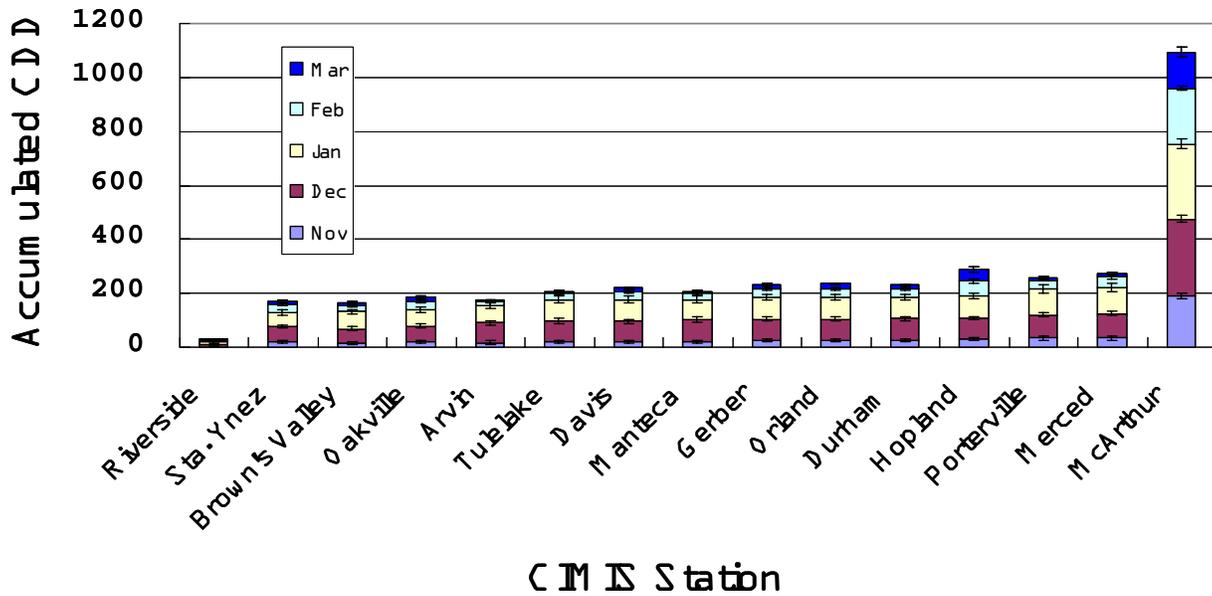
Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and the USDA Agricultural Research Service.

**Table 1.** GWSS adult mortality and total CDD (based on a feeding threshold of 10°C) accrued to reach > 95% mortality.

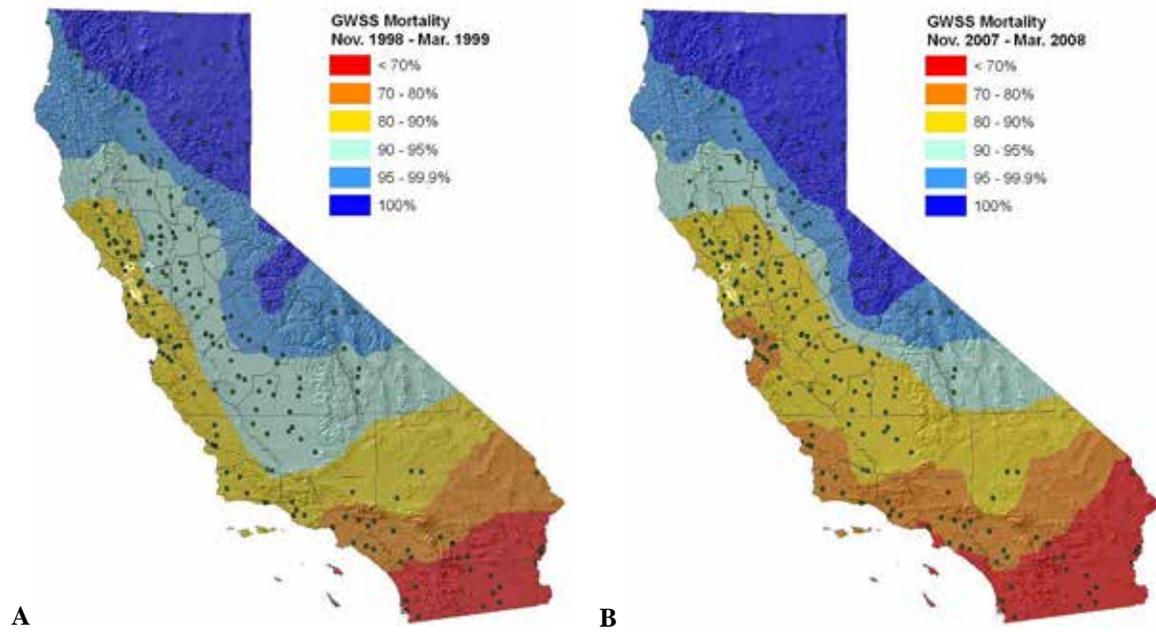
CA Site	Accumulated CDD	Mortality (%)	Weeks elapsed
Riverside	0	98.6	17
Arvin	86	95.7	12
Oakville	117	97.1	9
McArthur	144	97.1	4
Davis	145	97.1	11
Santa Ynez	154	97.1	11
Porterville	221	95.7	18
Merced	236	97.1	21
Gerber	237	96.7	16



**Figure 1.** Relationship between accumulated CDD (Based on °C) and GWSS mortality under simulated winter conditions for nine California sites. Points represent mean weekly mortality data.



**Figure 2.** Mean ( $\pm$  SEM) accumulated CDD (in °C) for each winter month at CIMIS sites in California over a recent 10-year period (November 1997 through March 2008). The selected CIMIS stations represent climates in the San Joaquin Valley (Arvin, Porterville, Merced, Manteca), Sacramento Valley (Davis, Brown’s Valley, Durham, Orland, Gerber), south coast (Riverside), central coast (Sta. Ynez), north coast (Oakville, Hopland), Cascades Range (McArthur), and Klamath Basin (Tulelake Fire Station).



**Figure 3.** Estimated percentage of cold-induced mortality of adult GWSS populations throughout California regions experiencing different levels of cumulative cooling day degrees (CCD) from A) November 1998 thru March 1999 and B) November 2007 thru March 2008. Dark blue represents 100% GWSS mortality (> 321 cumulative CDD based on °F or > 178 cumulative CDD based on °C) and red represents 0 to 69% GWSS mortality. Green circles indicate CIMIS weather stations.

# REPRODUCTIVE BEHAVIOR OF A KEY VECTOR OF *XYLELLA FASTIDIOSA* IN TEXAS

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**Reporting Period:** The results reported here are from work conducted July 2005 through October 2008. Greenhouse data are from studies carried out in 2002-2003.

## ABSTRACT

The glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) is a xylem specialist in the Hemiptera Auchenorrhyncha which has the potential to transmit *Xylella fastidiosa*, the causal agent in Pierce's disease of grapevine. It is the most common leafhopper associated with vineyards in Texas. Using wild GWSS adults collected in Central Texas parks and vineyards, egg loads and biometrics were obtained and used to determine the seasonality of reproduction in females of this insect species. Captive adults reared in the greenhouse during the summer and fall months were studied throughout their lifespan. During this study, caged females produced large numbers of eggs in the same seasons as wild females do. Fall females delayed oviposition by about 75 days, with nymphal emergence being postponed then until late winter. More suitable rearing conditions are needed to reduce the duration of the pre-oviposition period in fall emerged females.

## INTRODUCTION

Between 1995 and 2004, (fresh) grape production in the United States averaged 6.5 million tons annually (NASS, Crops Branch, 2005). With citrus and commercial apples, grapes are the most important fruit crop in the country. In recent years, grapes have been a very popular fruit crop in Texas, the fifth largest wine producing state in the nation. There are currently over 220 family-owned vineyards and about 3,700 acres under production; this industry contributes over \$1 billion a year to the state's economy (Texas Wine and Grape Growers Association). The leading grape varieties are the French/European hybrids of *Vitis vinifera* traditionally associated with the highest quality wines. These varieties are susceptible to Pierce's disease (PD), an incurable and fatal bacterial infection of disseminated by xylem specialist insects such as the glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS; Hemiptera: Cicadellidae). These insect pests are often designated as vectors. Certain grape varieties such as Blanc du Bois, Le Noir, Champlein or the muscadines may be better adapted for Texas and are considered fairly immune to this disease, whereas the most popular *V. vinifera* are susceptible to diverse degrees. PD of grapevine is the most important limiting factor to grape production in Texas (Texas Pierce's Disease Task Force 2004). A research program was initiated in 2002 with funding from the U.S. Department of Agriculture. Within this program, researchers are provided an opportunity to study the vectors in their natural habitat and their interaction with cultivated vines and other vegetation.

## OBJECTIVES

1. Wild GWSS populations have been monitored in Central and North Central Texas since 2003-2004. A number of adult females were sorted out to assess biometrics and evaluate their reproductive status through time based on egg load.
2. Using GWSS reared in captivity, total fecundity, daily oviposition pattern and longevity of GWSS females produced in summer and fall months were determined. Embryonic and nymphal survival and sex ratio of the first generation were measured in both seasons.

## RESULTS

GWSS populations are monitored using sticky traps in Texas vineyards and *in situ* in urban areas of Central and North Central Texas. Wild adult females used in this study were from a subsample of insects harvested from July 2005 to October 2008 and stored at 10°C until processed. Dissections of the ovaries were carried out under stereomicroscope and egg loads were assessed individually. Only mature eggs were counted. Over 3,000 adult GWSS females were sorted out, with numbers varying from 44 in the combined months of January 2006-2008 to 700 in the months of June 2006-2008. These data reflected well the seasonal abundance of GWSS in our area (Lauzière et al. 2008). Females exhibiting active egg production (vitellogenesis) were harvested from February to September. Eggs loads varied significantly between months ( $F = 60.0$ ;  $df = 11, 3152$ ;  $P < 0.0001$ ). Highest egg loads were observed in March ( $13.8 \pm 7.2$  eggs/female;  $n = 155$ ). The length of the left hind tibia was measured individually and used as an indicator of adult size. Tibia are relatively well conserved in trap collected adults and therefore a suitable choice for this study. The size of the tibia in wild females varied significantly between months ( $F = 36.8$ ;  $df = 11, 3069$ ;  $P < 0.0001$ ). The largest females were caught in May, June and July ( $4.1 \pm 0.2$  mm;  $n = 1605$ ), the smallest ones in December ( $3.9 \pm 0.2$  mm;  $n = 44$ ).

GWSS adults were reared to adulthood under greenhouse conditions using black-eyed peas and hibiscus as hosts. Newly emerged females ( $n = 30$ ) were selected and individually introduced into cages, each with five adult males. Adults and plants (leaves) were monitored once daily. The duration of the pre-oviposition period (time elapsed between adult emergence and first egg laid) was determined. Oviposition was recorded as the number of eggs per day. Host plants were renewed every

two weeks and observations continued until females died. After their death, tibiae were individually measured under stereomicroscope. Females reared during the summer months initiated oviposition within 13 days of the last molt to adulthood and deposited on average 187 eggs (range: 36-457) in approximately one month. The duration of the pre-oviposition period in females reared during the fall months was 5.6-fold longer than in summer individuals ( $F = 48.12$ ;  $df = 1, 48$ ;  $P < 0.0001$ ). Both the oviposition ( $F = 2.30$ ;  $df = 1, 48$ ;  $P < 0.0001$ ) and post-oviposition ( $F = 1.21$ ;  $df = 1, 48$ ;  $P < 0.0001$ ) periods were not affected by the season. The mean number of eggs laid by females in confinement did not vary significantly with seasons ( $F = 2.2$ ;  $df = 1, 49$ ;  $P = 0.2$ ). In both seasons, a high proportion of females ( $\geq 80\%$ ) deposited at least one egg during their lifespan and season did not affect the egg laying ability of captive females ( $\chi^2 = 1.2$ ;  $df = 1$ ;  $P = 0.3$ ). Three of the summer females and six of the fall females never laid eggs in captivity. The mean size of an egg mass was 5.4 and 5.7 eggs in the summer and fall, respectively. The largest egg mass contained 39 eggs in the summer and 37 eggs in the fall group. A mixed model analysis showed that in greenhouse-reared individuals, both the size of the female ( $F = 0.1$ ;  $df = 1, 54$ ;  $P = 0.8$ ) and the season ( $F = 0.2$ ;  $df = 1, 54$ ;  $P = 0.7$ ) did not affect total fecundity. However, the total number of eggs laid by GWSS females was significantly and positively affected by their longevity ( $F = 5.5$ ;  $df = 1, 54$ ;  $P = 0.02$ ). Unlike many insect species where longevity is positively determined by size, in captive *H. vitripennis*, we observed that longevity was strongly dependent upon the season in which the adults emerged ( $F = 124.0$ ;  $df = 1, 176$ ;  $P < 0.001$ ). Mated summer adult females lived on average  $49.3 \pm 2.9$  days, as compared to  $104.0 \pm 7.9$  days in the fall counterparts. The viability of *H. vitripennis* eggs exceeded 77% and there was no significant difference observed between seasons ( $F = 0.8$ ;  $df = 1, 49$ ;  $P = 0.4$ ). However, nymphal survival to adulthood was season dependent ( $F = 5.5$ ;  $df = 1, 49$ ;  $P < 0.001$ ). The ultimate percentage of adult emergence (from egg to adult) was also affected by the season with significantly more adults obtained from eggs laid by the fall emerged females. Of all the F1 adults emerged, the sex ratio was near 50:50 in both seasons ( $F = 0.3$ ;  $df = 1, 49$ ;  $P = 0.6$ ).

## CONCLUSIONS

In captivity, the onset of the oviposition period in summer females occurred two weeks after emergence, whereas in fall emerged females, oviposition was delayed for about 75 days, which in turn postponed nymphal emergence to mid February-early March of the following year. Under the greenhouse conditions tested, i.e., warm temperatures and supplemented artificial lighting maintained throughout the winter, adult fall females behaved as they would have under field conditions (mortality due to harsh weather and predators excluded) where no reproduction was observed between November and January. Additional research is underway to better understand reproductive behaviors in *H. vitripennis*, stimulate mating and reproduction in captivity off season so that a reliable rearing procedure that would allow fall-winter production of GWSS immatures needed for other research activities can be developed.

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## INVESTIGATION INTO THE INCIDENCE AND DISTRIBUTION OF PIERCE'S DISEASE AND ITS VECTORS IN A PREVIOUSLY CONSIDERED "VERY LOW RISK" TEXAS WINEGRAPE GROWING REGION.

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### ABSTRACT

An ecological model taking into account climactic, environmental and ecological factors was presented as a poster at the 2007 CDFA Pierce's Disease Symposium. (Lewis, 2007) The resulting risk map showed that conditions in the High Plains growing region of Texas were similar enough to other regions where Pierce's disease (PD) had been previously diagnosed to suggest this area could be vulnerable to the disease as well. In response, considerations for a new risk map for the state of Texas were proposed and sampling of suspect vineyards was carried out. Results of this sampling were used as a basis for the proposal of a program to examine the distribution of disease in this growing region. Additionally, possible agents of transmission were explored starting with an evaluation of the local insect population for putative vectors. The disease was found to be widely distributed in this growing region and a number of xylem specialists previously unrecognized to reside in this area were identified. Further analysis showed a percentage of these putative vectors to be positive for multiple strains of *Xylella fastidiosa*.

### INTRODUCTION

There have been periodic observations and diagnostic results to indicate Pierce's disease (PD) has been an ongoing problem in the far west Texas grape growing region for decades. The vineyard originally diagnosed with PD in west Texas is situated at an elevation of 5410 ft. above sea level and experiences average winter low temperatures of 32° F with frequent drops well below freezing. When in 2007, testing for possible causes to explain symptoms observed in vineyards in far west Texas returned negative results, PD was considered as a possible cause. Sampling of all west Texas vineyards in commercial production indicated that PD had at that time become geographically widespread in this growing region. In the process of compiling statewide ecological and environmental information for addition to a GIS for vineyard information for the state of Texas, similarity of environmental and ecological conditions between these vineyards and those in what was then described as an "extremely low risk" wine grape growing region in the Texas High Plains became evident (Perry and Bowen 1974). Based on this realization, a new risk model for the state of Texas was initiated taking these factors into account.

During the compilation of information for this GIS, current and historical information regarding vineyard establishment and decline, disease symptoms, and cultural practices was obtained. During the course of this data collection, it was ascertained that for several years, growers in this area had reported symptoms of "unusual" leaf discoloration, decline in yield and fruit quality, higher than expected loss of vines to winter injury, and unexplained difficulty in successfully establishing previously successful varieties such as 'Chardonnay' (Lewis unpublished data, Burns personal communication September 2007). While this region has historically been considered to be in a zone of "very low risk" for PD, the combination of these factors lead researchers and extension personnel to consider the possibility that PD might be implicated in some of these previously unexplained grower reports.

While the premise for this study was realized well past the optimal season for the collection of plant samples for detection of *Xylella fastidiosa* (*Xf*), the decision was made to begin a preliminary sampling of some of the vines in this area to be used both to potentially justify further investigation into PD in this growing region and to serve as a pilot study for a potential research program, should these results be positive. While certainly not conclusive, based in part on the deteriorated quality of the plant material at the time of sampling; this investigation returned positive results for *Xf* by QrtPCR from 31 of 39 samples in 12 of 12 vineyards with 3 negative and 5 inconclusive results.

Due to what was historically considered a very low potential for PD in this area, the High Plains was not considered a high priority area for the evaluation of potential vectors for *Xf*. Trapping in this area was limited to 12 traps in three vineyards, returning only 10 individual insects representing eight species of potential vectors over a three year period. The near absence of potential vectors in this area, presented as a contradiction in the understanding of how *Xf* infection may have become so apparently widespread across this region. Non-traditional possibilities that might account for disease spread in this area needed to be considered, however a more thorough investigation into potential vector populations needed first to be completed. It was in order to narrow the gap in knowledge and understanding of both the incidence of *Xf* and of the potential for the range of known vectors to extend into this region that this project was designed and initiated. Over time, a

comprehensive understanding of both the vector ecology and bacterial pathogenicity in this area may increase the understanding of the epidemiological effects of PD in temperate regions world wide.

## OBJECTIVES

1. Assess the distribution of *Xf* infection and PD in the Texas High Plains Growing Region.
2. Assess the presence and distribution of putative vectors of *Xf* in the Texas High Plains Growing Region.
3. Evaluate putative vectors for association with *Xf*.

## RESULTS

### Objective 1

*Assess the distribution of PD in the Texas High Plains Growing Region.*

Twenty-six vineyards in the High Plains were selected for potential PD infections based on a number of criteria. Eleven vineyards had previously tested positive for *Xf* in a 2007 pilot survey with QRT-PCR. A representative sample was selected to include the range of growing conditions in the High Plains grape growing region. In addition to meeting ecological and geographic criteria, several of the selected vineyards had histories of unexplained symptoms and mortality. Each vineyard was sampled in May, July, and October. The sampling procedure consisted of collecting petioles with blades attached from vines exhibiting suspicious symptoms. A minimum of 3-4 petioles were removed, starting with the basal position and moving acropetally to the end of a cane. When no symptoms were present, a random sample of vines were included to represent the entire vineyard, bringing the total number of vines to 10/vineyard. The presence of foliar scorching, green islands, matchsticks and other appropriate observations were made for the sampled vines.

Samples were placed in an ice chest with blue ice and returned to the laboratory for processing with ELISA, QRT-PCR, and direct isolation of the pathogen on culture media. ELISA analyses were done according to manufacturer's instructions (Agdia, Elkhart, IN 46514). QRT-PCR procedures have been established and described in previous studies (Schaad et al. 2002). Culturing of tissue samples for pathogen isolations was done on PW media according to previously accepted practices.

**Table 1.** Summary of survey and diagnostic results for PD on vines testing positive in Texas High Plains vineyards in samples collected in June, 2008.

Vineyard No.	No. Samples Processed	No. ELISA Positive	No. QRT-PCR Positive	No. Isolations
1	20	3	2	0
2	19	1	1	0
3	13	1	1	0
4	19	1	2	0
5	18	1	0	0
6	22	3	1	0
7	21	2	2	0
8	16	0	1	0
9	22	4	4	0
10	19	1	0	0
11	16	0	2	0
12	16	0	1	0
13	16	0	1	0

Thirteen of the 26 surveyed vineyards tested positive for PD (**Table 1**). Seven of those 13 positive vineyards were among those testing positive in the original 2007 pilot survey. Although QRT-PCR proved to be more sensitive to detection of the pathogen in terms of numbers of vineyards, in a few cases ELISA positives were obtained with the sample testing negative by QRT-PCR. In no case was the pathogen isolated from the samples, regardless of the results of the indirect tests. Due to the implications of expanding the recorded range of PD into a previously unaffected, major Texas grape growing region every precaution has been taken to insure the reliability of the diagnostic results. The results of the ELISA and QRT-PCR are considered to be sufficiently reliable to conclude that *Xf* infections of grape are widespread on the Texas High Plains. However, the diagnostic step of isolating the pathogen and completing strain analyses will continue to be pursued in order to further define the status of PD on the High Plains.

The results from the 2008 sampling extend the range of PD in Texas well into the High Plains growing region. The apparent widespread distribution of the disease within this region would suggest that it has been present in this area for several years and that a competent vector of the bacteria is active in the environment. The High Plains of Texas is the most productive wine-grape region in the state, with approximately 1500 acres in commercial production with production expanding yearly.

Environmental conditions are very different from most other grape growing regions of the state where PD has become a limiting factor. It is possible that PD has been responsible for many of the chronic problems for vine production in the past on the High Plains. The environmental conditions on the High Plains may be less conducive to epidemic development of PD, such as that seen in the more southern Texas growing regions. If so, the economic impact and prospects for control may be vastly better than in those areas where the disease has been historically devastating.

## Objective 2

*Assess the presence and distribution of putative vectors of Xf in the Texas High Plains Growing Region.*

A number of trapping sites within the High Plains growing region were selected in an effort to identify the presence and distribution of putative vectors for Xf in grapevines. In order to identify occupant vector species in the High Plains, a trapping program which included both active and passive trapping methodologies was designed and implemented during the 2008 growing season. Results available for this report come from the passive portion of this program.

Nineteen sites were selected for the placement of yellow sticky traps. These traps were scheduled to be collected on a 14 day rotation, which was at times amended due to adverse weather or vineyard conditions which made trap collection on the planned day impossible. Other factors forced researchers to change the collection schedule for three trapping areas to once monthly.

Traps placed within vineyards were placed at a density of 10 traps per acre. A trapping protocol developed to both address the unique environmental conditions and lack of knowledge of target species was utilized (unpublished). Trap height varied with trellis height but was standardized primarily to be wholly or partially within the canopy level for that location.

Additionally, in some locations traps were standardized to heights both “short” approximately two feet from the ground and “high” approximately four feet from the ground. Finally, in some “non-vineyard” locations, traps were set using local vegetation as the indicator for trap height. This technique helped to increase trapping efficiency for a variety of insect species, giving a more comprehensive representation of the resident population of putative vectors.

Trapping locations within vineyards were selected based on the following criteria:

1. The 11 vineyards that tested positive for presence of Xf in the 2007 pilot study were given priority.
2. Vineyards not included in any pre-existing trapping programs were prioritized over those that were.
3. Vineyards were grouped into five geographic areas in order to properly assess the entire growing region. A minimum of one vineyard per geographic region was selected.
4. Vineyards were further selected based on surrounding vegetation in order to include as many ecological/environmental types as possible.

Trapping sites outside vineyards were selected in order to adequately represent as many of the environmental and ecologically distinct areas within the region as possible. In addition, both areas directly adjacent to and several miles from the nearest vineyard were selected.

This report contains the first nine trapping periods in the 2008 cycle beginning in May and ending in September. As previously stated, some trapping periods were either shortened or lengthened as a result of weather conditions, or other vineyard considerations including pesticide applications and harvesting (**Tables 2 and 3**).

This program resulted in the capture of approximately seven distinct species of xylem feeders, six which are known vectors of Xf in grape. Of these, the range of at least two were not known to extend into this area and at least one species is currently classified as “undescribed.” Previous to this study, 10 individual insects representing seven species of xylem feeders had been observed by vineyard trapping efforts in this area over a three year period (Lauzière et al. unpublished).

**Table 2.** Putative vector's captured during the months of May through August of 2008 on traps set inside vineyards. In addition to total # of xylem specialists recovered, data is reported for the three most commonly occurring species in this study.

Trapping Site	Total # Xylem Specialists	<i>H. vitripennis</i>	<i>G. hieroglyphica</i>	<i>Cuerna sp.</i>
1	15	0	8	3
2	8	0	5	2
3	2	2	0	0
4	2	0	2	0
5	0	0	0	0
6	7	0	0	7
7	7	0	0	7
8	17	0	0	17
9	8	0	1	7
10	1	0	1	0
11	6	0	0	4
12	4	0	0	4
13	10	0	0	6
14	13	0	0	12

**Table 3.** Putative vector's captured during the months of May through August of 2008 on traps set outside vineyards. In addition to total # of xylem specialists recovered, data is reported for the three most commonly occurring species in this study.

Trapping Site	Total # Xylem Specialists	<i>H. vitripennis</i>	<i>G. hieroglyphica</i>	<i>Cuerna sp.</i>
15	59	0	53	1
16	2	0	0	2
17	1	0	1	0
18	199	0	198	0
19	1	0	1	0
20	32	1	2	26
21	2	1	0	1

### Objective 3

#### *Evaluate putative vectors for the presence of Xf.*

A number of insects from this region representing eight species were examined by PCR using an established protocol (Schaad et al. 2002), for the presence of *Xf*. Of those tested, approximately 30% tested positive for *Xf*. The insects that were selected for testing were chosen based on overall condition and preservation of the available specimens and represent a random sample of insects with regard to where within the research area they were captured. Specimens from this area are in the process of being analyzed in order to determine which specific strain of the bacteria is present in these insects. As of the time of this report, this data is still pending. The presence of *Xf*, in xylem specialists captured on the High Plains would suggest that it is likely that vectors are active in this region. It appears likely that at least some portion of the widespread presence of *Xf* and PD in this growing region is the result of vine to vine transmission by insect vectors.

### **CONCLUSIONS**

This study was designed as a first step to examining the occurrence of *Xf* infection in the High Plains growing region as well as the potential for increased incidence of infection over time.

Findings from Objective 1 would suggest that *Xf* infection occurs widely in this area and that PD should be considered in previously unexplained grower reports of vine decline and reductions in fruit quality and yields as well as other symptoms such as higher than expected percentages of vine loss due to winter injury. During this study, symptoms indicative of PD

including leaf blade scorching, petiole retention and uneven periderm formation were observed in vineyards across the region.

The presence of *Xf* in samples taken in early June, supports the hypothesis that the bacteria is able to over-winter in vines in this area both serving as a source for inoculum for subsequent spread within and among vineyards, and potentially impacting vine health from season to season. The widespread distribution of infection appears to imply that infection may have been actively spreading in this area for some time. Coupling the current distribution of the disease with the historical accounts reported by growers further supports the conclusion that *Xf* has been present and active in this growing region for a number of years. Lack of suitable historical records and the relatively new nature of large scale grape production in this area will make it extremely difficult to ever make an accurate estimate of how long PD may have been impacting vineyards in this region.

The data collected in order to satisfy Objectives 2. and 3. support the conclusion that the potential exists for continued spread of *Xf* in the High Plains region of Texas. While the first season of trapping recovered low numbers of potential and known vectors, the diversity of these putative vectors was moderately high and species known to be very competent vectors of the bacteria were among those recovered. Of the species captured, 30% of these species were found to be carrying *Xf*. Because the 2008 trapping program results contained in this report represents only a single “short” (May-Aug) season of collection and the trapping protocol was in the process of being refined, this data is far from comprehensive. Only with several seasons of collection and an increase in trapping intensity can an in depth understanding of the vector ecology of this area begin to become clear. A solid understanding of how vectors behave in the environment over time is necessary in order to develop targeted strategies for management of this disease.

The newly recognized range of PD in this growing region brings attention to the danger of making assumptions about the susceptibility of vineyards to disease based on geographical location or historic climactic patterns. While it is impossible to be certain, it is logical to conclude that a lack of recognition of disease and the subsequent complacency with regard to preventive strategies to control the spread of the bacteria may be at least partially responsible for the widespread presence of the bacteria in this region today. Based on the preliminary findings of this study, it seems prudent to re-evaluate the potential for vineyards across much of temperate North America to be affected by PD, and to reconsider the potential for further disease spread into regions currently considered to be at very low risk.

Continuation of this study will further define the vector population in this area as well as serve to provide an initial understanding of the local ecology of the guild of xylem specialists in this and other temperate regions. More in depth knowledge of possible migratory, reproductive, and over-wintering behaviors as well as feeding strategies of the individual species in this group will aid in both an understanding of the current and historical epidemiology of the disease as well as the development of control strategies specifically targeted at this region. Preliminary data suggests that this guild may have a very different species composition and exhibit behaviors that differ from their functional equivalents in warmer and/or higher precipitation areas. Further, continued investigation into the long term health and productivity of individual vines and vineyards will aid in the understanding of the epidemiology both at the vineyard and the landscape level of this disease in more temperate regions. This is an area of knowledge for which there is currently very little data and could prove invaluable in the future protection of vineyards in these lower temperature regions.

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# COLD STORAGE OF *GONATOCERUS ASHMEADI* GIRAULT: EXTENDED EMERGENCE, AND PARENTAL AND PROGENY FITNESS

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**Reporting Period:** The results reported here are from work conducted January 2008 to September 2008.

**ABSTRACT**

The emergence pattern was changed after immature *G. ashmeadi* were stored within their *Homalodisca vitripennis* egg hosts under a fluctuating temperature and a short-day photoperiod for 30 d. The fitness of parasitoids collected from three emergence peaks coming 1-5, 12-16, and 23-26d after the onset of the first emergence was investigated by examining developmental and reproductive parameters. Likewise, these parameters were also determined for the F<sub>1</sub> and F<sub>2</sub> progeny. The development to adulthood of the parental parasitoids collected from second and third emergence peaks was delayed by approximately 106% and 279% while the parasitoids collected from first emergence period had no developmental delay. Compared to the control group, the parasitism of the egg hosts by parasitoids collected from the first, second, and third emergence periods was decreased by 43, 68 and 80%; the fecundity by 53, 84 and 89%; and the longevity by 27, 72 and 67%, respectively. The F<sub>1</sub> parasitoids derived from parents collected from second and third emergence periods also had a lower incidence of parasitism. The fecundity and longevity of F<sub>1</sub> and F<sub>2</sub> parasitoids derived from parents collected from second and third emergence periods were significantly reduced. However, development and emergence of the F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> parasitoids were not influenced by any of the periods from which their ancestors emerged.

**INTRODUCTION**

Dormancy is one of the major strategies employed by insects and mites to survive harsh environmental conditions (Leopold, 1998). It is an adaptive response of arthropods to adverse environmental conditions by often entering a state of diapause or quiescence. Diapause is an indirect response to unfavorable conditions. It is mediated via the endocrine system, resulting in developmental arrest and adaptive physiological changes (Blum, 1985). Development usually resumes upon exposure to the appropriate environmental signals. Quiescence is a direct response to harsh conditions and results in suppression or arrest of development. Once adverse conditions cease, the organism can quickly recover and resume immediate development (Tauber et al., 1986). Either diapause or quiescence can be used in devising cold storage methods to facilitate mass-rearing of beneficial insects in classic biological control programs.

The egg parasitoid, *Gonatocerus ashmeadi* Girault, is one of the most common natural enemies of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar), in California. Augmentative release of this parasitoid is a feasible approach for control of the GWSS in affected areas where other methods are not accepted or appropriate. Mass-rearing parasitoids for control of the GWSS becomes problematic when there is a shortage of host eggs because there is currently no artificial diet to rear these wasps. Shortage of GWSS eggs can occur because there is a reproductive diapause in this insect that occurs during the winter months. Oftentimes there are insufficient numbers of parasitoids in the colder areas of California to produce an impact on sharpshooter populations (Morse et al, 2005), so it is important to develop effective methods to store a large number of hosts and parasitoids to meet these fluctuating demands in the field.

The effects of cold storage on parasitized and non-parasitized eggs of the glassy-winged sharpshooter have been recently studied to aid the mass-rearing of *G. ashmeadi* (Chen and Leopold, 2007, Chen et al, 2008a). Chen et al (2008b) also developed a method to store adult *G. ashmeadi* and examined the subsequent storage effects on maternal and progeny fitness. Storage at 4.5-7.5°C for 30 d induces quiescence in parasitoids stored within *H. vitripennis* eggs deposited beneath the surface of euonymus (*Euonymus japonica* Thumb.) leaves (Chen et al, 2008a). After cold storage, emerging adults have three emergence peaks after the initial onset (Chen et al. 2008a) whereas parasitoids reared continuously at 16-32°C have only one peak (Chen et al. 2006). In this study, we examined the biological and reproductive fitness of the parental generation of this parasitoid collected separately from the three emergence peaks and also of the F<sub>1</sub> and F<sub>2</sub> progeny to determine whether the gated emergence response elicited by extended storage had an effect on the fitness of maternal and progeny *G. ashmeadi*.

**OBJECTIVES**

1. Determine whether the extended emergence pattern affects reproduction and/or development of the post-storage parental generation.
2. Determine whether the extended emergence pattern affects fecundity and longevity of parental, F<sub>1</sub> and F<sub>2</sub> generations and progeny development.
3. Determine the post storage incidence of parasitism, emergence pattern and sex ratio of the parents and their progeny.

## RESULTS AND CONCLUSIONS

### **Objective 1. Adult emergence pattern and developmental time of post-storage parasitoids.**

After the parasitoids were stored within their host at 4.5, 6.0 and 7.5 °C, (each temperature changing at eight h intervals over a 24 h period for 30 d) the emerging adult parasitoids displayed an emergence pattern consisting of three peaks while the control group only had one emergence peak (**Figure 1**). To determine possible effects on development after cold storage, parasitoids were collected during each period of emergence (i.e., 1-5 d; 12-16 d; and 23-26d after the initial onset of emergence). Development time in this study was measured from the removal from cold storage to the medium time of adult emergence. Wasps emerging from the control group were also collected during their emergence peak. **Figure 2** shows that development time of stored parasitoids varied significantly with the emergence periods ( $F = 835.72$ ,  $df = 3,15$ ,  $P < 0.0001$ ). After storage for 30 d, more than 60% of the wasps emerged during first period and the development time was similar to the control group (**Figure 1**). These results indicate that a large of number of immature *G. ashmeadi* could quickly recover from the cold-induced quiescence and resume normal development. However, when compared to the control, the development time of wasps collected from second and third emergence periods was delayed approximately one-(106%) and three-fold (279%), respectively. Approximately 26% and 4% of parasitoids collected from the second and third emergence periods (Chen et al, 2008a) did not quickly resume development after removal from cold storage. It is uncertain what causes this significant delay in development time. These results are being studied further.

### **Objective 2. Parasitism, fecundity and longevity of parents and progeny.**

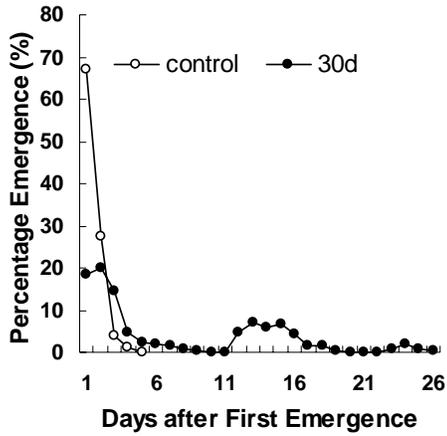
A repeated measure ANOVA showed that the incidence of parasitism by the parasitoids varied significantly with generation ( $F = 6.08$ ,  $df = 2,56$ ,  $P = 0.004$ ) and emergence period ( $F = 13.73$ ,  $df = 3,28$ ,  $P < 0.0001$ ) and that there was significant interaction between generation and the emergence period of the parasitoids ( $F = 2.37$ ,  $df = 6,56$ ,  $P = 0.042$ ). Parental ( $F = 9.77$ ,  $df = 3,29$ ,  $P < 0.0001$ ) and the  $F_1$  ( $F = 2.78$ ,  $df = 3,28$ ,  $P = 0.034$ ) generations showed a significant decrease in parasitism of host eggs across the emergence periods. When compared to the control group, the parasitism by the wasps collected from the first, second, and third emergence periods declined by 43, 68 and 80%, respectively. There was no difference in the rate of parasitism by the  $F_2$  generation whose grandparents were collected from different emergence periods ( $F = 1.49$ ,  $df = 3,36$ ,  $P = 0.235$ ) (**Figure 3A**).

Lifetime fecundity varied significantly with generation ( $F = 24.02$ ,  $df = 2,56$ ,  $P < 0.0001$ ) and the emergence period ( $F = 37.55$ ,  $df = 3,28$ ,  $P < 0.0001$ ). There was a significant interaction between generation and the emergence period of parasitoids ( $F = 6.07$ ,  $df = 6,56$ ,  $P < 0.0001$ ). The fecundity of parental parasitoids was significantly influenced by the period that the wasps emerged. Compared to the control group, the fecundity of parasitoids collected from the first, second, and third emergence periods decreased by 53, 84 and 89%, respectively. There was also a significant decrease in fecundity of  $F_1$  and  $F_2$  parasitoids derived from the parents that were collected during the second and third emergence periods (**Figure 3B**). Longevity of the parasitoid varied significantly with generation ( $F = 9.81$ ,  $df = 2,56$ ,  $P = 0.0002$ ) and the emergence period ( $F = 54.64$ ,  $df = 3,28$ ,  $P < 0.0001$ ). There was no significant interaction between generation and the emergence period of parasitoids ( $F = 1.36$ ,  $df = 6,56$ ,  $P = 0.247$ ). The longevity of parental parasitoids was significantly influenced by the emergence period. Compared to the control group, the longevity of parasitoids collected from the first, second, and third emergence periods decreased by 27, 72 and 67%, respectively. The longevity of  $F_1$  and  $F_2$  parasitoids derived from the parents that were collected during the first emergence period was similar to that of the control group. However, there was a significant decrease in longevity of  $F_1$  and  $F_2$  parasitoids derived from the parents there collected during the second and third emergence periods (**Figure 3C**).

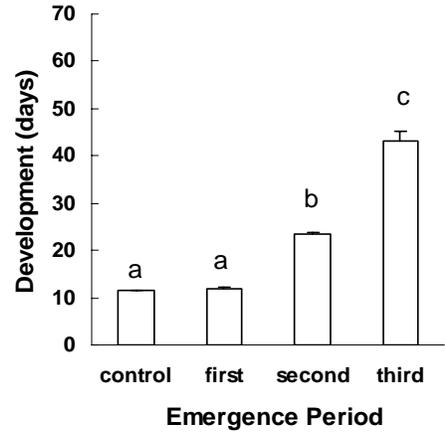
### **Objective 3. Development, emergence and sex ratio of $F_1$ , $F_2$ and $F_3$ generations.**

A repeated measures ANOVA showed that emergence of parasitoids was not significantly influenced by generation ( $F = 2.45$ ,  $df = 2,50$ ,  $P = 0.097$ ) and the period ( $F = 0.47$ ,  $df = 3,25$ ,  $P = 0.703$ ) that the parasitoids emerged. There was no significant interaction between generation and the emergence period of parasitoids ( $F = 0.74$ ,  $df = 6,50$ ,  $P = 0.620$ ) (**Figure 4A**). The development time of parasitoids emerging from non-stored, recently collected *H. vitripennis* eggs (< 24 h old) was not significantly influenced by generation ( $F = 2.81$ ,  $df = 2,36$ ,  $P = 0.074$ ) or the peak ( $F = 2.37$ ,  $df = 3,18$ ,  $P = 0.079$ ) from which the parasitoids emerged.

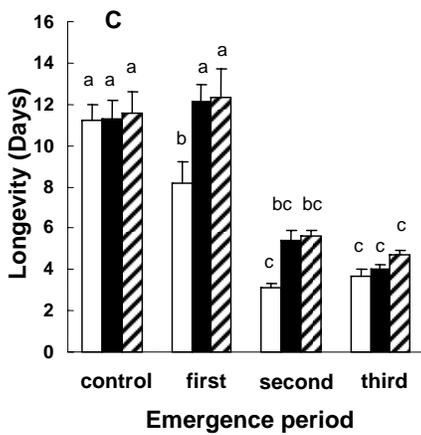
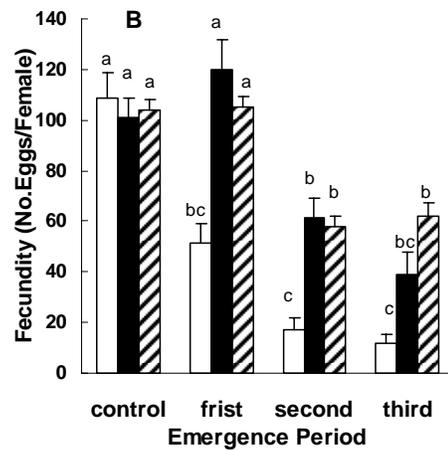
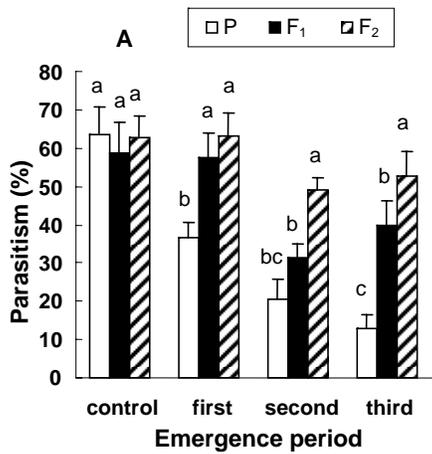
There was no significant interaction between generation and the emergence period of parasitoids ( $F = 0.74$ ,  $df = 6,36$ ,  $P = 0.082$ ) (**Figure 4B**). Data on sex ratio of  $F_1$ ,  $F_2$  and  $F_3$  generations are not shown here because of a lack of a sufficient number of replicates. The complementary experiments are in the process of being conducted.



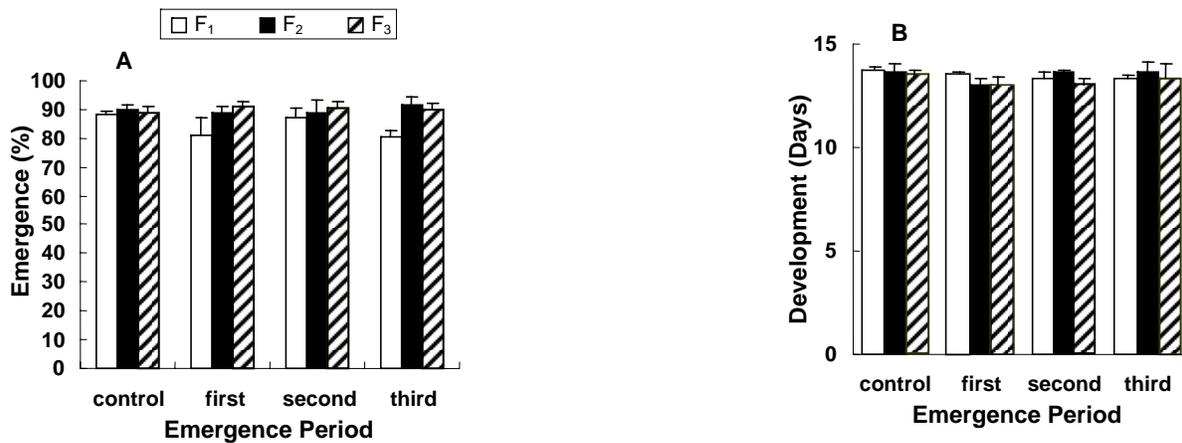
**Figure 1.** Adult emergence pattern of *G. ashmeadi* after being held in cold storage under the 4.5-7.5 °C daily fluctuating temperature for 30 d.



**Figure 2.** Development time of *G. ashmeadi* collected from various emergence periods after being held in cold storage under the 4.5-7.5 °C daily fluctuating temperature for 30 d. Columns denoted by differing letters are significantly different.



**Figures 3 A-C.** Parasitism (A), fecundity (B), and longevity (C) of the parental, F<sub>1</sub> and F<sub>2</sub> generations of *G. ashmeadi* collected from various emergence periods after cold storage under the 4.5-7.5 °C daily fluctuating temperature for 30 d.



**Figures 4A & B.** Developmental time (A), emergence (B) of the F<sub>1</sub>, F<sub>2</sub> and F<sub>3</sub> generations of *G. ashmeadi* collected from various emergence peaks after cold storage of their ancestors that were stored under the 4.5- 7.5°C daily fluctuating temperature for 30 d.

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## FAUNISTIC ANALYSIS OF SHARPSHOOTERS IN PLUM ORCHARDS OF RIO GRANDE DO SUL STATE, BRAZIL

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### ABSTRACT

Plum leaf scald (PLS) is a severe disease caused by *Xylella fastidiosa* (*Xf*), which drastically affects cultivation of plum in Brazil, but little is known about its epidemiology. We carried out faunistic analyses of sharpshooter leafhoppers in two plum orchards of Rio Grande do Sul (RS) State, in order to identify potential vectors in this crop, based on their ecological characteristics. Sharpshooters were sampled fortnightly by yellow sticky cards placed on the plum tree canopy (1.7 m above ground) and just above the ground vegetation (0.5 m), in 10 sampling points per orchard. A total of 23 and 18 species were trapped in orchards located in the municipalities of Farroupilha and Bento Gonçalves, respectively, in 50 sampling dates from Sept/2006 to Sept/2008. Seven sharpshooters were classified as predominant (dominant, very abundant, very frequent and constant) in the plum orchards: *Bucephalagonia xanthophis* (Berg), *Dilobopterus dispar* (Germar), *Erythrogonia dorsalis* (Signoret), *Macugonalia cavifrons* (Stal), *Molomea lineiceps* Young, *Sibovia sagata* (Signoret) and *Spinagonalia rubrovittata* Cavichioli. Among them, *D. dispar*, *M. cavifrons* and *M. lineiceps* are likely associated with disease spread because of their activity on the plum canopy. Considering the diversity of sharpshooter species in plum orchards, additional data on spatial patterns of disease, alternative hosts of *Xf*, vector infectivity and transmission efficiency are needed to determine key vectors for PLS spread.

### INTRODUCTION

A major factor limiting cultivation of plum in Brazil is the widespread occurrence of Plum leaf scald (PLS), a bacterial disease caused by *Xylella fastidiosa* (*Xf*) (Raju et al. 1982). First detected in plum in Pelotas, Rio Grande do Sul (French & Kitajima, 1978), PLS is now endemic in most plum production areas not only in RS, but also in the states of Santa Catarina, Paraná, São Paulo and Minas Geraes (Ducroquet et al. 2001). This disease is thought to be the main reason for the decline in cultivated areas of plum since the 1970's (Leite et al., 1997; Hickel et al., 2001). *Xf* can be transmitted by several species of leafhoppers in the subfamily Cicadellinae (Hemiptera: Cicadellidae), commonly known as sharpshooters, and by a few spittlebugs (Redak et al. 2004). Despite the importance of PLS in Brazil, sharpshooters involved in the spread of this disease have not been identified. The only vector survey in this crop was done with sweep nets and Moericke yellow water-pan traps in orchards of a single locality in the State of Santa Catarina, Brazil, indicating the presence of five sharpshooter and two spittlebug species (Hickel et al. 2001). However, little information on activity and abundance of these potential vectors in plum orchards is available. Because there is virtually no specificity for transmission of *Xf* within Cicadellinae (Almeida et al. 2005), any sharpshooter species that visit host plants of this bacterium is a potential vector. Indeed, three sharpshooter species found by Hickel et al. (2001) in plum orchards [*Bucephalagonia xanthophis* (Berger), *Ferrariana trivittata* (Signoret) and *Plesiommatia corniculata* Young] have been reported as vectors of *Xf* in citrus (Redak et al. 2004). In order to identify potential vectors of *Xf* in plum, we carried out a faunistic analysis of Cicadellinae species in two orchards of the 'Serra Gaúcha', a major production region in State of Rio Grande do Sul, Brazil.

### OBJECTIVES

1. Determine the composition of sharpshooter (Cicadellinae) species in plum orchards of Rio Grande do Sul, Brazil.
2. Identify predominant species based on faunistic indices.
3. Determine potential vectors of *Xf* based on prevalence in the orchards and activity on the plum canopy.

### RESULTS

We sampled sharpshooters in two plum orchards located in the municipalities of Farroupilha and Bento Gonçalves, State of Rio Grande do Sul, from September 2006 to September 2008. In Farroupilha, the survey was carried out in 1-half orchard of European plum (*Prunus domestica* L.) cv. Italianinha, located in a hilly area surrounded by woody vegetation (29° 08' 47'' S, 51° 23' 21'' W). The orchard was five years old when the survey started, and >20% trees showed PLS symptoms. In Bento Gonçalves, the experimental area (1 half) was a four-year old orchard of *P. domestica* cv. Italianinha and Rubimel, located in the District of Pinto Bandeira (29° 07' 43'' S, 51° 26' 58'' W). The orchard was neighbored by a road in one edge and by peach orchards in the other edges, and showed 10% trees with PLS symptoms. The ground vegetation of these two orchards was comprised mainly by grasses [*Digitaria sanguinalis* (L.) Scop., *Paspalum conjugatum* P.J. Bergius and *Brachiaria plantaginea* (Link) Hitchc.] and herbs of the Polygonaceae (*Rumex obtusifolius* L.), Asteraceae (*Bidens pilosa* L., *Galinsoga parviflora* H. St. John & D. White), Fabaceae (*Trifolium repens* L.) families. Sharpshooters were sampled by rectangular (8.5 x 11.5 cm) yellow sticky cards (Biocontrole®, Sao Paulo, SP, Brazil). Two cards were installed per sampling point in 10

points that were spaced 35 m apart, in two lines. In each point, one card was attached to a wood stick at a height of 0.5 m above soil, and the other was fixed on the north face of the plum canopy, at a height of 1.7 m. We exchanged the yellow sticky cards every 15 days and the trapping data of all sharpshooter species were submitted to a faunistic analysis. Prevalent species were determined based on higher constancy, frequency, abundance and dominance indices (Silveira Neto et al., 1995). We also calculated indices of diversity of Shannon-Weaver ( $H'$ ), equitability ( $E$ ) and similarity of sharpshooter species for the two experimental orchards.

In Bento Gonçalves, a total of 214 individuals of 18 species of sharpshooter leafhoppers were trapped by yellow sticky traps in 50 sampling dates. Based on the faunistic analysis, eight species were dominant and four of them, *B. xanthophis*, *Erythrogonia dorsalis* (Signoret), *Macugonalia cavifrons* (Stal) and *Molomea lineiceps* Young were classified as predominant because they were also very abundant, very frequent and constant (**Table 1**). In Farroupilha, 899 specimens of 23 species were captured in the 50 sampling dates. Five out of 13 dominant species in this locality were considered predominant, *Dilobopterus dispar* (Germar), *E. dorsalis*, *M. cavifrons*, *Sibovia sagata* (Signoret) and *Spinagonalia rubrovittata* Cavichioli (**Table 2**).

The diversity index ( $H'$ ) was significantly higher in the plum orchard of Farroupilha (2.26) compared with Bento Gonçalves (2.18) ( $P < 0.05$ ), showing that the two areas were distinct regarding the composition of sharpshooter species, with higher diversity in the former one. Species richness was also higher in Farroupilha, but there was no difference in the equitability index between the two locations, indicating uniformity in abundance of sharpshooter species in the two communities. The higher diversity of species in the plum orchard of Farroupilha may be explained by the presence of adjacent woody vegetation with a variety of native trees, which might serve as natural hosts for a larger number of sharpshooter species, as well as a refugium when the orchard does not offer adequate conditions for feeding or reproduction of these insects.

Concerning the sharpshooter activity on the plum canopy, measured by trap catches at 1.7 m above ground, we noticed that most species from tribe Proconiini were captured mainly on the tree canopy, in both areas (**Tables 1 and 2**). Conversely, Cicadellini species (including the very abundant *E. dorsalis*) were usually trapped in much larger numbers at 0.5 m, which suggests a greater activity of these species on the ground vegetation (herbaceous plants); an exception was *D. dispar*, captured mostly (89%) on the tree canopy. Among the predominant species, *D. dispar*, *M. cavifrons* and *M. lineiceps* were the most trapped on the plum canopy. Other less abundant dominant species, e.g. *B. xanthophis*, *Oncometopia facialis* (Signoret), *O. fusca* Melichar and *Paviloma victima* (Germar), were also trapped in significant numbers on the tree canopy. Considering that the probability of *Xf* transmission is enhanced by vector abundance, natural infectivity and preference for the host plant (Purcell 1981), these sharpshooters trapped more frequently on the plum canopy are more likely to play an important role in PLS epidemiology. Two of them, *B. xanthophis*, *O. facialis*, are considered key vectors of *Xf* in citrus orchards, for similar reasons (Lopes, 1999; Almeida et al. 2005). It should be noted, however, that abundant sharpshooter in the ground vegetation of plum orchards, such as *E. dorsalis*, *S. sagata* and *S. rubrovittata* (**Table 2**), may also be important for disease spread if the pathogen colonizes herbaceous weedy hosts. Information on spatial patterns of PLS, possible alternative hosts of the pathogen in plum orchards, as well as on sharpshooters associated with those hosts, will be critical to determine key vectors for PLS epidemiology. Because most sharpshooters tested for transmission of *Xf* to other host plants have been confirmed as vectors (Redak et al. 2004), we expect that sharpshooters considered here as potential vectors will be shown to transmit the causal agent of PLS if tested in future studies.

## CONCLUSIONS

Plum orchards in Rio Grande do Sul, Brazil show a high diversity of sharpshooter species that may serve as vectors of *Xf*, particularly in areas surrounded by native woody vegetation. The faunistic analyses classified five sharpshooter species as predominant in the plum orchards: *B. xanthophis*, *D. dispar*, *E. dorsalis*, *M. cavifrons*, *M. lineiceps*, *S. sagata* and *S. rubrovittata* (**Tables 1 and 2**). Among them, *D. dispar*, *M. cavifrons* and *M. lineiceps* are likely associated with spread of PLS because of their higher activity on the plum canopy.

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**Table 1.** Total number of individuals and faunistic indices of leafhopper species of the subfamily Cicadellinae trapped by yellow sticky traps in a plum orchard of Bento Gonçalves, Rio Grande do Sul, Brazil, from September/2006 to September/2008.

Leafhopper species	Total <sup>(1)</sup>	Faunistic indices				Capture (%) per trap height	
		D <sup>(2)</sup>	A <sup>(3)</sup>	F <sup>(4)</sup>	C <sup>(5)</sup>	0,5 m	1,7m
<b>Tribe Cicadellini</b>							
<i>Bucephalogonia xanthophis</i> (Berg, 1879)*	34	D	va	VF	W	100	-
<i>Caragonalia</i> sp.	1	ND	r	LF	Z	-	100
<i>Diedrocephala variegata</i> (Fabricius, 1775)	2	ND	d	LF	Z	50	50
<i>Dilobopterus dispar</i> (Germar, 1821)	2	ND	d	LF	Z	88	12
<i>Erythrogonia dorsalis</i> (Signoret, 1853)*	30	D	va	VF	W	97	3
<i>Macugonalia cavifrons</i> (Stal, 1862) *	30	D	va	VF	W	73	27
<i>Macugonalia geografica</i>	1	ND	r	LF	Z	100	1
<i>Macugonalia leucomelas</i> (Walker, 1851)	2	ND	d	LF	Z	50	50
<i>Morfo</i> 5	1	ND	r	LF	Z	-	100
<i>Pawiloma victima</i> (Germar, 1821)	9	D	c	F	Y	50	50
<i>Sibovia sagata</i> (Signoret, 1854)	5	ND	c	F	Y	100	-
<i>Spinagonalia rubrovittata</i> Cavichioli 2008	11	D	c	F	W	90	10
<b>Tribe Proconiini</b>							
<i>Molomea consolidata</i> Schoder, 1959	2	ND	d	LF	Z	50	50
<i>Molomea lineiceps</i> Young, 1968*	60	D	va	VF	W	15	85
<i>Molomea personata</i> (Signoret, 1854)	1	ND	r	LF	Z	-	100
<i>Oncometopia facialis</i> (Signoret, 1854)	8	D	c	F	Y	25	75
<i>Oncometopia fusca</i> Melichar, 1925	14	D	c	F	W	52	48
<i>Homalodisca ignorata</i> Melichar, 1924	1	ND	r	LF	Z	-	100

<sup>(1)</sup> Total number of individuals of each species captured in all traps and sampling dates

<sup>(2)</sup> Dominance - D: dominant; ND: non-dominant

<sup>(3)</sup> Abundance - va: very abundant; c: common; d: dispersive; r: rare

<sup>(4)</sup> Frequency - VF: very frequent; F: frequent; LF: little frequent

<sup>(5)</sup> Constancy - W: constant; Y: accessory; Z: accidental

\* predominant species

**Table 2.** Total number of individuals and faunistic indices of leafhopper species of the subfamily Cicadellinae trapped by yellow sticky traps in a plum orchard of Farroupilha, Rio Grande do Sul, Brazil, from September/2006 to September/2008.

Leafhopper species	Total <sup>(1)</sup>	Faunistic indices				Capture (%) per trap height	
		D <sup>(2)</sup>	A <sup>(3)</sup>	F <sup>(4)</sup>	C <sup>(5)</sup>	0,5 m	1,7m
<b>Tribe Cicadellini</b>							
<i>Bucephalagonia xanthophis</i> (Berg, 1879)	37	D	c	F	W	64	36
<i>Caragonalia</i> sp.	1	ND	r	LF	Z	-	100
<i>Diedrocephala variegata</i> (Fabricius, 1775)	11	D	d	LF	Y	90	10
<i>Dilobopterus dispar</i> (Germar, 1821) *	185	D	va	VF	W	11	89
<i>Erythrogonia dorsalis</i> (Signoret, 1853)*	114	D	va	VF	W	95	5
<i>Hortensia similis</i> (Walker, 1951)	1	ND	r	LF	Z	100	-
<i>Macugonalia</i> sp.	9	D	d	LF	Y	100	-
<i>Macugonalia cavifrons</i> (Stal, 1862) *	215	D	va	VF	W	60	40
<i>Morfo 5</i>	5	ND	r	LF	Z	60	40
<i>Morfo 2</i>	4	ND	r	LF	Z	100	-
<i>Morfo 9</i>	1	ND	r	LF	Z	100	-
<i>Morfo 8</i>	2	ND	r	LF	Z	100	-
<i>Pawiloma victima</i> (Germar, 1821)	11	D	d	LF	Y	72	28
<i>Fonsecaiulus</i> sp.	18	D	c	F	Y	72	28
<i>Sonesimia</i> sp.	1	ND	r	LF	Z	-	100
<i>Sibovia sagata</i> (Signoret, 1854) *	74	D	va	VF	W	84	16
<i>Spinagonalia rubrovittata</i> Cavichioli 2008*	77	D	va	VF	W	88	12
<b>Tribe Proconiini</b>							
<i>Aulacizes quadripunctata</i> (Germar, 1821)	1	ND	r	LF	Z	-	100
<i>Molomea consolidata</i> Schoder, 1959	3	ND	r	LF	Z	67	33
<i>Molomea lineiceps</i> Young, 1968	32	D	c	F	Y	3	97
<i>Oncometopia facialis</i> (Signoret, 1854)	61	D	c	F	Y	19	81
<i>Oncometopia fusca</i> Melichar, 1925	26	D	c	F	W	27	73
<i>Tapajosa rubromarginata</i> (Signoret, 1855)	1	ND	r	LF	Z	-	100

<sup>(1)</sup> Total number of individuals of each species captured in all traps and sampling dates

<sup>(2)</sup> Dominance - D: dominant; ND: non-dominant

<sup>(3)</sup> Abundance – va: very abundant; c: common; d: dispersive; r: rare

<sup>(4)</sup> Frequency – VF: very frequent; F: frequent; LF: little frequent

<sup>(5)</sup> Constancy – W: constant; Y: accessory; Z: accidental

\* predominant species

# SEASONAL TRANSMISSION OF *XYLELLA FASTIDIOSA* BY THE GLASSY-WINGED SHARPSHOOTER FROM GRAPEVINES INFECTED FOR VARIOUS LENGTHS OF TIME

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**Reporting Period:** The results reported here are from work conducted July 2007 through September 2008.

## ABSTRACT

This study is part of our larger project aimed at understanding the feeding biology of the glassy-winged sharpshooter (GWSS) as it relates to acquisition and transmission of *Xylella fastidiosa* (*Xf*). GWSS feeding biology was studied in three seasons (summer, fall, winter) on mature Cabernet Sauvignon and Chardonnay grapevines using choice and no-choice studies. When given a choice, GWSS males and females chose to feed on young leaf, petiole, and stem tissue compared to the same tissues on older parts of the cane. However, there was substantial time spent feeding on old stem tissue, a phenomenon that would result in more rapid chronic infection than feeding on young tissue. We also learned that throughout the day, GWSS adults change position frequently between the various tissues, a characteristic that would support the rapid spread of *Xf* that has been associated with GWSS. In no-choice studies, we found that GWSS adults were not able to feed on cordon tissue, regardless of the time of year. They were able to feed on old and young grapevine tissue throughout the year, but the relative amount of feeding on this tissue varied with the season. Future work will evaluate GWSS feeding behavior when confronted with PD-infected grapevines.

## INTRODUCTION

Pierce's disease (PD), a disease of grapes caused by the bacteria, *Xylella fastidiosa* (*Xf*) Wells et al., was described in California in the 1880s during an epidemic in Orange County (Pierce 1882). A second epidemic occurred in Tulare County in the 1930s (Hewitt et al. 1949), and until the mid-1990s, it was considered only a minor problem in vineyards close to riparian areas. In the early 1990s a new vector, the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) (formerly *Homalodisca coagulata* Say), was introduced into the state (Sorenson and Gill 1996), and became associated with a devastating epidemic of PD in the Temecula Valley. Since 1994, at least 1,500 acres of vineyards have been lost to the disease in California; in the Temecula Valley alone, losses have been estimated at \$13 million (Wine Institute 2002). The GWSS has different feeding and dispersal capabilities than native insect sharpshooter vectors and these attributes are thought to have contributed to the increased number of PD-infected grapevines in California (Almeida et al. 2005a, Blua et al. 1999, Redak et al. 2004). Like other insect-borne plant pathogen systems, there are two potential types of pathogen spread: primary or secondary spread. Primary spread occurs when the pathogen is obtained by the vector from sources outside the crop and transported and inoculated into the crop. Secondary spread occurs when the vector acquires the pathogen from infected vines in the vineyard, and subsequently inoculates healthy vines within the same vineyard (i.e. vine to vine spread). It is thought that *Xf* spread with native California vectors was the result of primary spread, but that rapid spread by GWSS may be the consequence of primary and secondary spread (Almeida et al. 2005a, Hill 2006). Understanding details of primary and secondary spread of *Xf* by GWSS can assist in the development of alternatives to the areawide management program. For example, to reduce primary spread, efforts must focus on reducing bacteria-carrying GWSS from entering healthy vineyards, through continued areawide or local treatment programs outside the vineyard, barriers, trap crops, and/or removal of pathogen sources outside the vineyard. Reduction of secondary spread can be accomplished by in-field control of GWSS, finding and roguing infected vines in the vineyard (Varela et al. 2001), and/or minimizing acquisition from infected vines and transmission to healthy vines.

The relationship among time of inoculation, location of inoculation, and disease progression in the vine likely plays a role in determining whether disease becomes chronic and when a vine becomes a source plant for additional spread. When another PD vector, the blue-green sharpshooter, *Graphocephala atropunctata*, infected grapevines early in the season, more persistent infections resulted than from later season infection (Purcell 1981). A potential difference between blue-green sharpshooter transmission and GWSS transmission is that the former is known to prefer feeding at the tips of canes (Purcell 1976), whereas the latter has been reported to feed on older plant parts. Almeida et al. (2005b) demonstrated that GWSS could even transmit *Xf* to dormant vines in the field. However acquisitions from dormant vines in the field were negative. Whether these transmissions and acquisitions are important to disease spread depends on GWSS feeding preferences during the winter months when the vines are dormant. Similarly, it is possible that infection at certain times of the season may not become systemic because infection is pruned out at end of year, or environmental conditions limit bacterial spread (Feil and Purcell 2001, Feil et al. 2003, Hill 2006).

## OBJECTIVES

The objectives of the project are:

1. Document GWSS feeding preference, through the growing season, on established Cabernet Sauvignon and Chardonnay grapevines that either are healthy or have been infected with *Xf* for 2, 3, or 4 years.
2. Evaluate the acquisition by GWSS, through the growing season, from established Cabernet Sauvignon and Chardonnay grapevines that either are healthy or have been infected with *Xf* for 2, 3, or 4 years and determine the subsequent transmission from these acquisitions.
3. Determine the relationship between *Xf* inoculation by GWSS at different times of the year and the development of the vine as a source for further acquisition by GWSS.

In order to proceed with Objectives 2 and 3, we first must determine where GWSS feed on mature vines and this is the focus of the current report.

## RESULTS

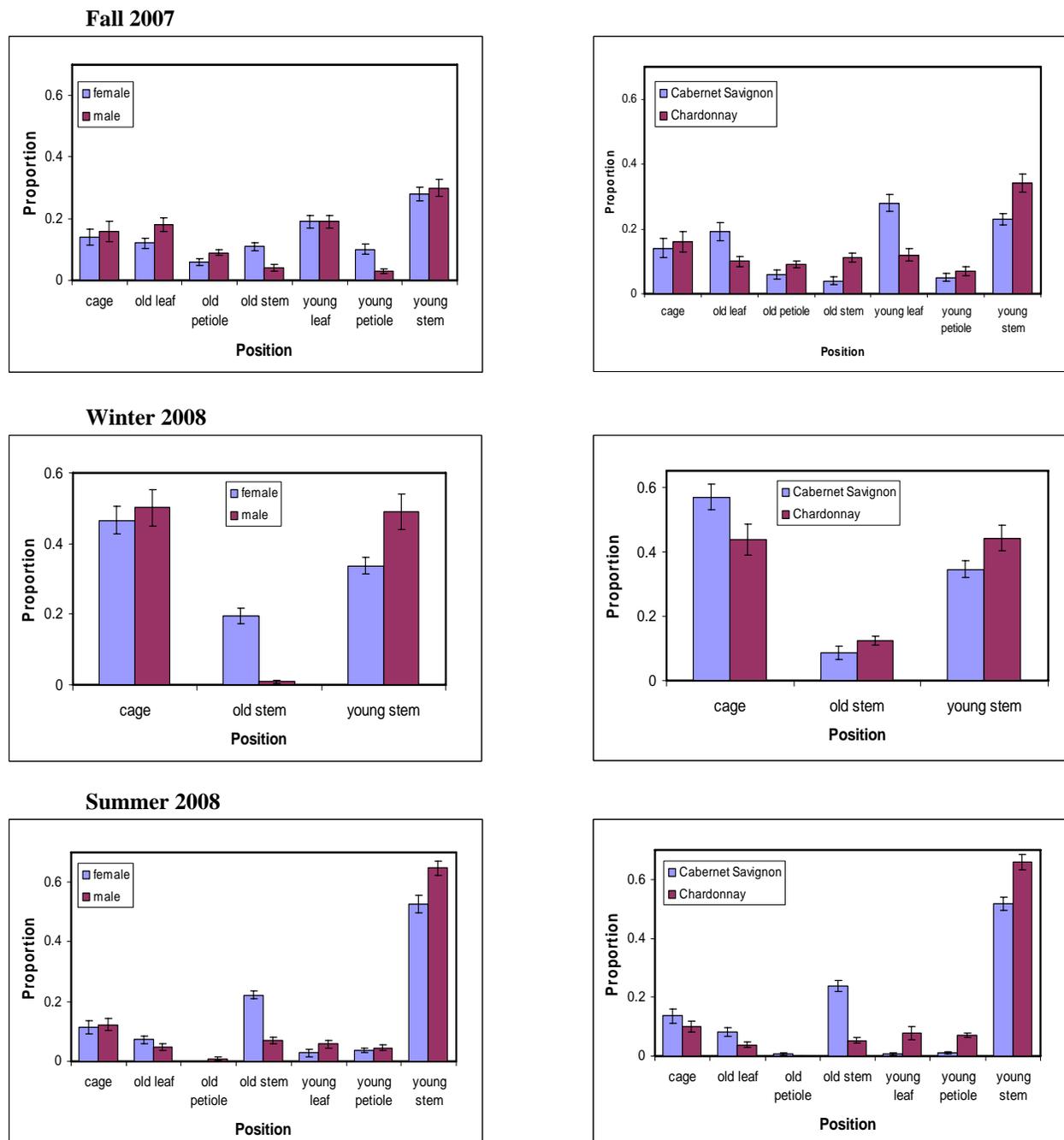
### Choice Tests for Grapevine Tissue Selection

For this research, we placed GWSS adults individually in observation cages fabricated from acetate cylinders (25cm x 17cm diameter) with organically sleeves attached to the ends. The cage was placed over the base of a Cabernet Sauvignon or Chardonnay grapevine cane with the cane terminal looped back into the cage. The ends of the observation cage were sealed giving a single GWSS in each cage access to old and young stems, petioles, and leaves inside the cage. The grapevines were from a mixed field-grown vineyard at the University of California in Riverside (UCR) that was covered with 60% shade-cloth to protect them from PD. We made hourly observations during daylight hours over three consecutive days to determine the location of each GWSS. This experiment was executed twice in the fall of 2007, twice in the winter 2008 and once in summer 2008.

Results of the two fall trials were pooled, as were the results of the two winter trials. In the fall, GWSS were found on the cage in 14% and 16% of our observations on Cabernet Sauvignon and Chardonnay vines, respectively (**Figure 1**). We also found that a high proportion (35%) of GWSS, averaged across variety and gender, switched from one tissue to another each hour (data not shown). Clearly, GWSS moved frequently among the vegetation, important for the spread of bacteria within and among vines. When GWSS were present on the canes, they utilized all tissues with no consistent preference for any type. However, over the course of the trial and averaged across both varieties, GWSS were found more frequently on young tissue (18.2%) than on old tissue (10.7%). Looking further at the data, GWSS were found more frequently on young stems, petioles and leaves (28.5%, 6%, and 20%, respectively) than on old stems, petioles and leaves (7.5%, 7.5%, and 14.5%, respectively). Interestingly, the insects spent the least amount of time on petiole tissue of any age than on any other tissue type. There also were some interesting results with respect to variety. GWSS were found more frequently on leaves (old and young) of Cabernet Sauvignon compared to the leaves of Chardonnay while the reverse was seen for petioles and stems (old and young). These results suggest that the two grapevine varieties vary in the xylem components that are important for GWSS feeding, a result that could impact the location where *Xf* cells are introduced into healthy grapevines. To finish the discussion of this trial, there appeared to be little difference between sexes in their selection of feeding sites (**Figure 1**).

In the winter trial, GWSS were found on the cage walls in 49% of our observations. At this time of year, neither leaves nor petioles were available to the sharpshooters, and GWSS were found on old stems and young stems in 11% and 40% of the observations, respectively. Those tissue preferences differed somewhat among the two varieties and the two sexes (**Figure 1**). The major departure from these numbers was the preference for the old stem among sexes; females and males were on the old stem in 20% and 1% of the observations, respectively. The general preference for the young stem over the old stem was consistent among varieties and among sexes. Changes in GWSS position occurred in 14% of the observations, considerably less than the 35% exhibited in the fall 2007 trials. There was little difference in the tendency of GWSS to change positions among variety or sexes.

The summer trial again offered GWSS young and old leaf and petiole tissue in addition to young and old stems. GWSS were found on the cage wall 12% of the time (**Figure 1**). The general preference for young tissue that was found in the fall and winter also occurred in the summer. GWSS chose young leaves, petioles, or stems in 67% of the observations compared to 21% for the older tissues. The young stem was the preferred tissue, both among varieties and among sexes. However, there were some differences in tissue selection among varieties and among sexes. The old stem was selected 24% of the time on Cabernet Sauvignon but only 5% of the time on Chardonnay. The young leaf and young petiole each were selected in 1% of the observations on Cabernet Sauvignon, while they were selected 8% and 7% of the time on Chardonnay. Among sexes, females chose the old stem in 22% of the observations, but males chose that tissue in only 7% of the observations. Among tissue types of any age, leaves, petioles, and stems were chosen in 12%, 5%, and 83% of the observations, respectively. Changes in GWSS position occurred in 21% of the observations, and that rate of change was consistent among the varieties and among the sexes.



**Figure 1.** GWSS preference on field-grown Cabernet Sauvignon and Chardonnay grapevines in choice experiments initiated on 29 August and 11 September 2007 (Fall 2007), 16 January and 6 February 2008 (Winter 2008) and 1 July 2008 (Summer 2008). Bars represent average proportions of GWSS ( $\pm$ SE) observed on various tissue types for the two varieties and for the two GWSS genders.

No-choice Tests Quantifying Feeding on Grapevine Tissues

No-choice feeding trials were conducted on the same mixed field-grown vineyard at the University of California in Riverside. Individual GWSS were caged on selected grapevine tissue in 50 ml polypropylene centrifuge tubes (Thermo Fisher Scientific Inc., Waltham, MA) by one of two methods. The first method, modified from Andersen et al. (1992), was for use on cordons, stems, and petioles. The cages were made by melting a transverse hole in the side of the tube using hot metal cylinders of diameters similar to the grape tissues. The tube was pressed onto the plant tissue, so the GWSS had access to about 2.5 cm length of the plant through the hole. The cage was affixed and sealed to the tissue by wrapping the tube and tissue with ca. 2 cm wide strips of Parafilm (Pechiney Plastic Packaging, Menasha, WI). The screw cap was tightened, and the cage rested vertically so that excreta collected in the bottom of the tube. The second cage design was for use on leaf tissue. The mouth of an intact 50 ml tube was pressed to the abaxial leaf surface with a piece of coiled spring steel in a clothes-pin like fashion (Blua and Perring 1992). One end of the spring held the 50 ml tube. The other end of the spring had a plastic ring on which was glued a foam pad 1 cm thick by 3 cm in diameter which gently held the leaf against the

polypropylene tube, giving the insect access to leaf tissue of ca. 5.7 cm<sup>2</sup>. This cage, too, was oriented vertically, so excreta drained to the bottom of the cage. Each cage type was loosely covered with aluminum foil in order to shade it from direct sunlight.

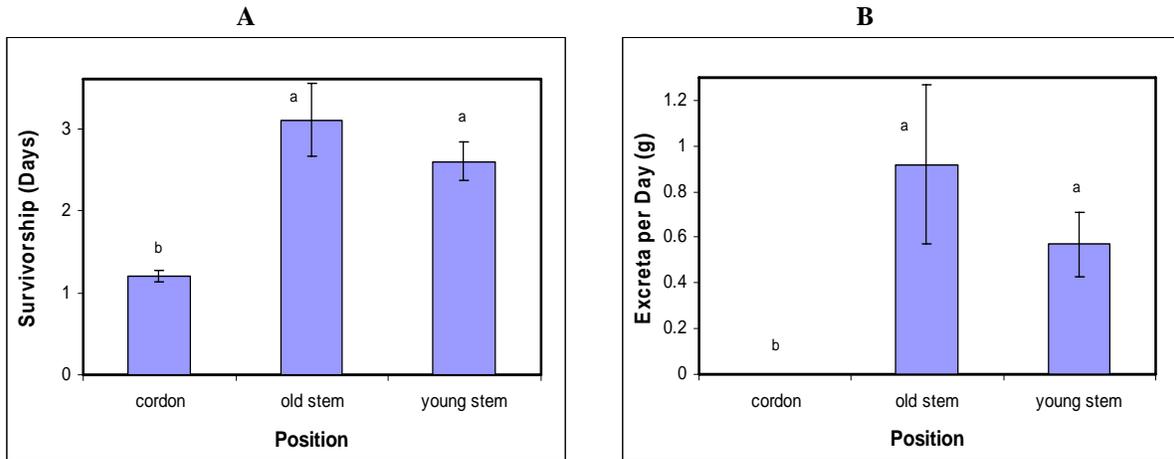
The day before the start of each test, GWSS adults were collected from citrus at Agricultural Operations, UCR, and placed in a cage with a potted rough lemon plant. The following morning, adults were isolated and sexed and then placed individually into the tube cages. Cages were inspected daily and the presence of excreta noted. Cages with dead GWSS were removed, and the amount of excreta was weighed. Up to 1.5 ml of excreta from each cage was frozen for future analysis of chemical content. At the end of the trial, all remaining cages were collected, GWSS mortality was noted, and excreta was weighed.

During the winter trials, GWSS were placed on cordons, old stems, and young stems; leaves and petioles were not available. The overall GWSS feeding rate was 0.37 g of excreta per day, but there was considerable variation among sharpshooters (**Figure 2**). In no case did discernible feeding occur on cordons, tissue several years old with thick dry bark. The old stems were covered with dry, but much thinner bark. Feeding on the old stem averaged 0.92 g of excreta per day and on the young stem, 0.57 g, however those amounts were not significantly different at  $p=0.05$ . There were no significant differences in feeding among varieties or among the sexes. Survivorship in the winter trials averaged 2.04 days, and there were no significant differences in survivorship among varieties or sexes. There were significant differences in survivorship among GWSS on different tissues (**Figure 2**). Of 29 GWSS on cordons, only 6 lived into the second day for an average survivorship of 1.2 days, significantly less than on the other tissues. Among all insects, only one insect that produced no excreta survived as long as 3 days, and only one insect that produced excreta died before the end of the trial.

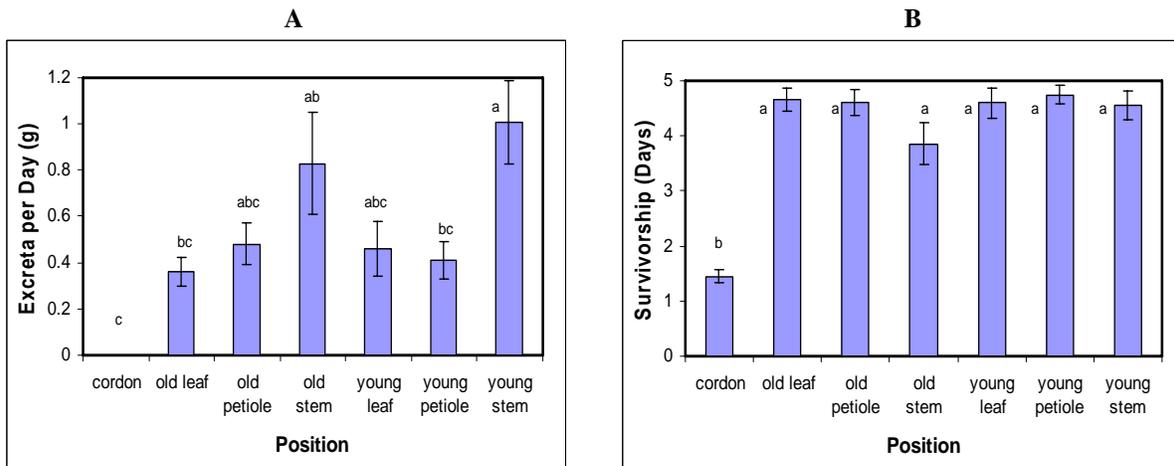
Sharpshooters fed on all tissues except cordons in the summer trial (**Figure 2**), averaging 0.51 g of excreta per day. Feeding on non-cordon tissues was highly variable, but there were some significant differences. Sharpshooters produced significantly more excreta on young stems than on young petioles, old leaves, and cordons. There were no significant differences among varieties, sexes, or tissue age (i.e. old leaves, petioles, and stems vs. young leaves, petioles, and stems). Among tissue types there were significant differences in feeding. Significantly more excreta was produced on stems (0.92 g) than on petioles (0.45 g), leaves (0.41 g), and cordons (0 g), and excreta from the petioles and leaves was significantly greater than from the cordons. Average GWSS survivorship in the July trial was 4.06 days. As in the winter, only survivorship on cordons was significantly less than that on other tissues (**Figure 2**). Other than cordons, there were no significant differences among leaves, petioles, and stems. In addition, survivorship among varieties and among sexes was not significantly different.

In the fall trial, GWSS again fed on all tissues except cordons (**Figure 2**), averaging 0.229g of excreta per day (range 0-1.18g). This was less excreta than that produced by sharpshooters feeding in the winter (0.37g) and summer (0.51g) trials. While we are not sure why this reduction in feeding might occur, it may signal a natural decline in feeding as the sharpshooters enter the winter months. There was substantial variation among GWSS feeding in this trial (**Figure 2**). While it appears that GWSS feeding on old stems and young stems were nearly the same as the other non-cordon tissue, the means in this case are misleading. For the old stem, there were only 2 GWSS that survived longer than 1 day and of these 2 only 1 produced any measurable excreta (0.168g). On the young stems, only 5/20 GWSS survived longer than 1 day, and these insects produced an average of 0.24g of excreta per day (range 0.014-0.779g). This is a contrast to the summer trials, during which the insects survived well on the young stems. We noticed that in the fall trial, the young stem tissue had become hardened and woody, and while GWSS were able to feed on this tissue in the summer, they were not able to do so in the fall. It also is interesting that survival on old stem tissue seemed much better in the winter than in the fall. This may be due to the adaptability of GWSS that were field collected for our trials. In the winter months, GWSS may be better adapted for feeding on woody tissue than populations in the fall. Survival was consistently high on the leaves and petioles and production of excreta was consistent with this survival. The tissue yielding the most excreta was the young petiole (0.311g/day), followed by young leaves (0.233g/day), and old leaves (0.208g/day).

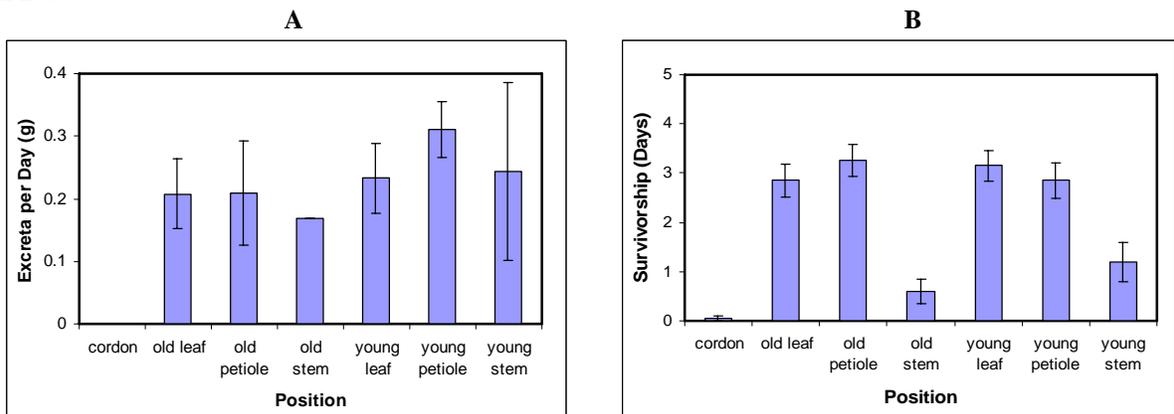
Winter 2008



Summer 2008



Fall 2008



**Figure 2.** GWSS feeding on field-grown Cabernet Sauvignon and Chardonnay grapevines in no-choice experiments conducted in Winter 2008 (26 February, 4 March), Summer 2008 (15 July), and Fall 2008 (19 September). Bars represent A) average amount (g) of excreta per day ( $\pm$ SE) measured from various tissue types, B) average GWSS survivorship (days) ( $\pm$ SE) on the same tissues. Different letters above bars represent statistically significant differences among means at  $p = 0.05$  (ANOVA followed by Tukey's studentized range test for mean separation). At the writing of this report, statistical analyses were not complete on the Fall trial, therefore only means ( $\pm$  SE) are presented.

**CONCLUSIONS**

Vine to vine spread of *Xf* by glassy-winged sharpshooter (GWSS) has been hypothesized as a critical component of devastating PD epidemics that occurred in Temecula and in the General Beale area of Kern County. GWSS landing and feeding behavior and tissue feeding capacity combine with grapevine phenology, and within-vine *Xf* distribution and phenology to make vine to vine spread possible. Our overall goal is to provide information on these various components to

enhance our understanding of vine to vine spread so that strategies can be defined to reduce widespread epidemics in other regions. We have conducted experiments in the fall, winter and summer in which we made hourly observations on the location of individual GWSS adults given access to mature tissue and young tissue on the same cane. Both males and females preferred young tissues (particularly the stems) to mature tissues on Cabernet Sauvignon and Chardonnay grapevines throughout the year. However, GWSS spent a substantial amount of time feeding on old stem tissue (7.5%, 11%, 15% in fall, winter, and spring trials, respectively), where *Xf* could potentially be transmitted leading to chronic infection. A significant finding is that GWSS moved frequently throughout the days of our studies, changing position in 35%, 14%, and 21% of the observations in the fall, winter and spring, respectively. This has serious consequence for moving *Xf* around the vineyard at various times of the year. Further characterization of GWSS feeding behavior was conducted in no-choice studies. We learned that at no time of the year, were individuals able to feed on the cordon tissue. While others have reported observing GWSS feed in this tissue, we were not able to demonstrate it in our trials on mature vines. Aside from cordons, GWSS were able to feed on old and young stems, petioles, and leaves. However, the amount of feeding varied with the season. In the winter and summer, GWSS utilized old stems and young stems, while during the fall they were not able to feed on old stems. In addition, the young stems became hardened and woody, and survival and feeding on the young stems at this time of the year were reduced. Our goal is to integrate the information from the work reported here with planned studies on infected grapevines at different times of the year. Through this work, we will understand the interaction between feeding behavior on specific grapevine tissues that contribute to the spread of *Xf* from infected to healthy vines. With this knowledge, we can direct management strategies to mitigate vine to vine spread.

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## FUNDING AGENCIES

Funding for this project was provided by the CDFR Pierce's Disease and Glassy-winged Sharpshooter Board.

**IDENTIFYING THE SPECIES OF MYMARIDAE REARED IN ARGENTINA AND MEXICO  
FOR POTENTIAL INTRODUCTION TO CALIFORNIA AGAINST THE GLASSY-WINGED SHARPSHOOTER  
AND PREPARING AND SUBMITTING FOR PUBLICATION A PICTORIAL ANNOTATED KEY  
TO THE *ATER*-GROUP SPECIES OF *GONATOCERUS*, EGG PARASITOIDS OF THE  
PROCONIINE SHARPSHOOTERS IN THE NEOTROPICAL REGION**

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**Reporting Period:** The results reported here are from work conducted January 1, 2008 to October 10, 2008.

**ABSTRACT**

The already described Neotropical species of the speciose fairyfly genus *Gonatocerus* Nees (Hymenoptera: Mymaridae) are reviewed and re-diagnosed in the forthcoming publication, which is near completion (Triapitsyn et al. in preparation). 82 valid species are recognized including 10 newly described ones, and an illustrated identification key (based on females) to 77 species is provided (the remaining five species are known from the male sex only). The known distribution ranges and host associations of the included species are indicated, with emphasis on the egg parasitoids of the proconiine sharpshooters (Hemiptera: Cicadellidae: Cicadellinae: Proconiini), all of which belong to the *ater* species group (the *ater* and *morrilli* subgroups) of *Gonatocerus*. Results obtained during the last year of this three-year project (a one-year no-cost extension was granted by the funding agency) are being reported.

**INTRODUCTION**

In the New World, eggs of the proconiine sharpshooters, which are known vectors of *Xylella fastidiosa*, are parasitized by various Mymaridae; their natural biological control is mainly due to the beneficial activity of the numerous species of *Gonatocerus*. A key to the Nearctic mymarid egg parasitoids of the proconiine sharpshooters was published recently (Triapitsyn 2006a). A rationale and a more detailed introduction for this project, which will result in publication of an illustrated, annotated key to the Neotropical species of *Gonatocerus*, were given by Triapitsyn (Triapitsyn 2006b).

**OBJECTIVES**

1. Identification of the numerous species of *Gonatocerus* reared by USDA researchers (G. A. Logarzo) and others in Argentina, Chile, and Peru, colonies of some of which were established in the quarantine facilities in California and Texas, and also of several species reared in Mexico from eggs of *Homalodisca* and other proconiine sharpshooters.
2. Preparation and submission for publication of a pictorial, annotated key to the *ater* species group of *Gonatocerus*, egg parasitoids of proconiine sharpshooters in the Neotropical region, with emphasis on the species targeted for introduction into California (Years 2 and 3).

**RESULTS AND DISCUSSION**

Progress on Objective 1.

Specimen preparation. Due to the enormous volume of the material of *Gonatocerus* from Argentina and Chile (more than 5,000 specimens have already been point-mounted in the course of this project), work on point-and slide-mounting of the specimens and their curation, which began in October 2006, will continue until July 2009.

Specimen identification. Sorting of the new material has continued. We described two new species of *Gonatocerus* reared in Argentina from eggs of *Tapajosa rubromarginata* (Signoret) (Triapitsyn et al. 2007, 2008) and also a new species of *Gonatocerus* from Sonora, Mexico, an egg parasitoid of *Homalodisca liturata* Ball (Triapitsyn & Bernal 2008). Many other

species of *Gonatocerus* egg parasitoids of Proconiini were identified using both morphological and molecular methods (de León et al. 2008; Virla et al. 2008).

#### Progress on Objective 2.

Preparation of the illustrations. High quality digital photographs were taken, using an Automontage system, of all the available types and many non-type specimens of the described *Gonatocerus* spp. from the Neotropical region and also of the new species that are included in the key. All the illustrations have been arranged into plates (more than 100).

Preparation of the key. All keys have been completed and all the already described Neotropical species of *Gonatocerus* have been re-described; descriptions of the 10 new species are under way.

Publications and reports. The project has already resulted in at least 12 scientific papers and reports that either have been published or submitted for publication to the scientific journals (in press). We expect that a review of the described species of *Gonatocerus* in the Neotropical region will be completed in early 2009, and then it will be submitted to *Zootaxa* (Triapitsyn et al. in preparation); it currently has more than 200 manuscript pages.

### **CONCLUSIONS**

A review of the described species of *Gonatocerus* in the Neotropical region is near completion; it also includes descriptions of 10 new species. Additionally, two new species of *Gonatocerus* egg parasitoids of Proconiini were described during the reporting period. Results of this project will be of significant benefit to biological control (especially to the CDFR/PD Biological Control Program) specialists, ecologists, and other researchers that manage the Pierce's disease threat posed by GWSS. When published, this key will make possible identifications of the mymarid egg parasitoids of the proconiine sharpshooters in America south of the USA, differentiation of native vs. introduced species of *Gonatocerus*, and also will provide information on the candidate species of Mymaridae for introduction as part of biological control programs, facilitate surveys for assessing levels of egg parasitism of the proconiine sharpshooters, and indicate all known host associations of the mymarid species important for classical and neoclassical biological control of GWSS and other Proconiini.

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### **FUNDING AGENCIES**

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### **ACKNOWLEDGMENTS**

We thank Vladimir V. Berezovskiy and Jennifer Walker (University of California, Riverside) for technical assistance.

***Section 2:***  
***Vector***  
***Management***





## AGE DETERMINATION AND THE RED PIGMENT IN THE WINGS OF THE GLASSY-WINGED SHARPSHOOTER

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

### ABSTRACT

The glassy-winged sharpshooter, *Homalodisca vitripennis*, has a red pigment that is found in its wings during the final immature stage of its life. Over the course of the sharpshooter's lifespan, the red pigment darkens with maturation and eventually becomes a brown/black color. These pigments are unidentified but believed to be pheomelanin and eumelanin, respectively. The age of the sharpshooter can be determined by analyzing the amount of red pigment found in the wings. In this study, we attempted to identify the red pigment and quantify the amount of red pigment contained in wings via chemical analysis. Ultimately, we found that it was more practical to determine the amount of red pigment compared to brown/black pigment using an image analyzing software (ImageJ) to compare the ratios of each color present.

### INTRODUCTION

In area-wide management studies in California and Texas, glassy-winged sharpshooters (GWSS; *Homalodisca vitripennis*) are collected using yellow sticky cards such as the Trécé Inc. adhesive trap T3306 (Trécé, Inc., Adair, OK). This method works very well for monitoring population numbers and identifying species that occur in the field. However, determining the age of sharpshooter off of traps can be difficult due to the degradation of internal tissues. However, the GWSS's wing color changes from red to black based on its age and this is a static change that is not altered postmortem. This compound is proposed to be pheomelanin which is red-brown and can be converted in to eumelanin (a dark brown/black pigment) (Wakamatsu 2002, Tran 2006).

### MATERIALS AND METHODS

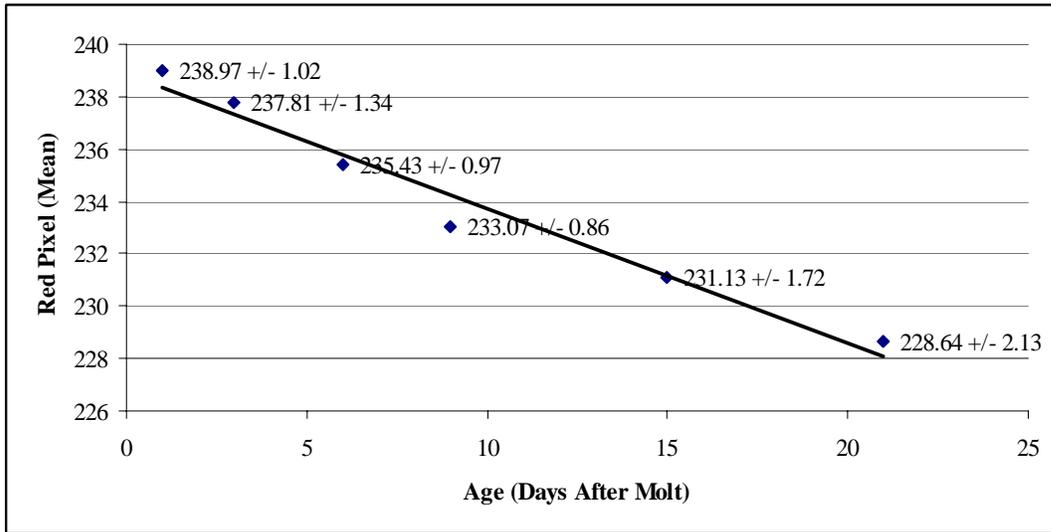
*Determination of red and brown/black pigment.* Age determination was done by scanning the wings using a Hewlett Packard Scanjet 3500c scanner (**Figure 1**). These images were labeled properly and then analyzed by ImageJ software. A color histogram was obtained along with the area of selection in square pixels, and the mean gray value. A numerical value was calculated for each wing ( $x=(R-((G+B)/2))*\text{mean}/\text{area}$ ). These values were used to set up a standard scale using known ages.



**Figure 1.** *Development of Standards and Age Determination for field-collected insects.* GWSS were reared from eggs at the Texas Agrilife Research facility in Fredricksburg, TX. Each day, newly molted adults were collected and transferred to separate cages. These insects were allowed to survive 3, 6, 9, and 15 days. At that time the insects were sacrificed and analyzed for the ratio of red pigment to brown/black pigment as described above. From the yellow sticky traps, GWSS wings were collected and analyzed.

### RESULTS AND DISCUSSION

GWSS collected on different days had significantly different levels of red pigment in their wings (**Figure 2**). We tested a large group of field-collected GWSS and were able to determine the relative age of the insects. However, many of the tested insects were determined to be older than 21 days. Therefore, we need to refine the system and set standards at the max age of the insects.



**Figure 2.** Decreasing levels of red pigment found in GWSS wings over time.

### CONCLUSIONS

The unknown pigment in the sharpshooter wings is believed to be pheomelanin. The other proposed pigments of xanthomattin and erythropterin absorbance spectra are similar in shape to that of other organic pigments unlike the unknown compound. Pheomelanin is commonly found in nature and can be easily converted to eumelanin which is a darker, almost black hue. Pheomelanin (red) can be converted to eumelanin (black) if the solution is lacking a high concentration of sulfur compounds. Therefore if cysteine (sulfur containing) concentration is low, the pheomelanin will readily convert into eumelanin. In the sharpshooter this lack of cysteine would most likely come from a change in diet. Further research is needed to verify that the pigment is in fact pheomelanin.

The age of the sharpshooters can be determined by ImageJ. In an area-wide management program, it is important to understand as many biological factors as possible. Age may have a direct correlation to the vectoral capacity of the insects.

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### 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.

# UNDERSTANDING THE DYNAMICS OF NEONICOTINOID INSECTICIDAL ACTIVITY AGAINST THE GLASSY-WINGED SHARPSHOOTER: DEVELOPMENT OF TARGET THRESHOLDS IN GRAPEVINES

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## ABSTRACT

The impact of systemic treatments of dinotefuran on the adult and egg stages of the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is being evaluated using greenhouse and laboratory scale bioassays. One reason for the use of systemic treatments is that they exploit the xylophagous feeding behavior of the GWSS adult and immature stages. Our current data show that these treatments have an additional contact activity on emerging first instars before they begin feeding. Preliminary data indicate that dinotefuran is inherently more toxic than imidacloprid to first instar GWSS. In bioassays with adults exposed to treated grapevines, we quantify dinotefuran concentrations within the xylem and related mortality. From these bioassays, we expect to generate a value that represents the effective concentration of dinotefuran needed to kill a GWSS adult feeding on a vine. This target threshold can then be used to guide growers in the selection of treatment rates, and as an indicator of the efficacy of treatments and the level of protection their vines are receiving.

## INTRODUCTION

Our research program focuses on the use of chemical insecticides for the management of the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*). We are dedicated to formulating safe and effective treatment programs for California growers, given the almost complete reliance by the grape industry on this method of control. We have conducted extensive trials in Coachella, Napa and Temecula valley vineyards to evaluate the uptake and persistence of three neonicotinoids – imidacloprid, thiamethoxam, and dinotefuran – under the diverse range of climatic, soil, and agronomic conditions associated with these regions. We have an understanding about how the different chemical properties, particularly water solubility, of these neonicotinoids can be exploited to achieve optimum uptake into vines, and we have developed sensitive techniques that allow us to monitor the levels of insecticide present within the vines. To exploit this knowledge further for the benefit of California grape production, we need to ensure that the concentrations of insecticide present within the vines are reaching levels that are effective at rapidly killing GWSS before they can infect vines with Pierce's disease (PD). We also need to understand whether there is a sub-lethal impact of these insecticides on GWSS, since anti-feedant activity may not necessarily eliminate the threat that an infective sharpshooter poses to a vine. Our past and current research projects have established the threshold levels of imidacloprid needed to kill a GWSS at 10 ng/ml xylem fluid, and optimized treatment regimes for growers that will ensure these thresholds are attained following applications via different irrigation methods (drip, sprinkler). In 2007, a new systemic neonicotinoid, Venom (active ingredient dinotefuran), received full registration for use on grapes. An additional systemic neonicotinoid, Platinum (active ingredient thiamethoxam), is scheduled for registration in 2008 (it will soon go through the 30 day posting process according to a personal communication from David Belles, Syngenta). Our work in this area has demonstrated the excellent uptake of these new insecticides following systemic application to vines (Toscano et al., 2007). This is good news for vineyard operators who have experienced problems with imidacloprid. Imidacloprid has been the predominant neonicotinoid in use in vineyards, but our research has shown that its uptake and persistence within vines varies dramatically between regions (Coachella Valley, Napa Valley, Temecula Valley). Despite its apparent poor uptake, growers continue to rely on imidacloprid in many areas. The perception is that the insecticide will work well in all areas given its successful implementation in Temecula vineyards (Byrne and Toscano, 2006). Thiamethoxam and dinotefuran offer a potential solution to overcoming the problems encountered with imidacloprid use – their rates of uptake are faster and they reach higher concentrations at peak uptake than imidacloprid under the more challenging situations. They also exhibit favorable persistence. Having established that the uptake and persistence of these systemic insecticides is superior to imidacloprid in terms of insecticidal titers reached in the xylem, it is important to determine the threshold levels of these insecticides to ensure that the levels attained in the xylem are active against sharpshooters. Comparative data on the efficacies of systemic thiamethoxam and dinotefuran against GWSS are not available.

## OBJECTIVES

1. Determine target thresholds for systemic neonicotinoids against glassy-winged sharpshooters in grapevines.

## RESULTS

We are currently running two sets of experiments. Potted grapevines have been treated with Venom (active ingredient dinotefuran) at recommended field rates (6 oz/acre), and dilutions of this rate. The efficacy of dinotefuran at these treatment rates is being assessed by confining adult sharpshooters on the vines and determining the levels of mortality after one day of

exposure. The concentrations of dinotefuran in the vines are measured after the mortality has been scored so that we can derive a lethal concentration for the insecticide. Xylem fluid is extracted using a pressure bomb and the dinotefuran in the extract is then quantified by ELISA. In the first tests, the use of the field rate resulted in 100% mortality. This is not surprising since the concentrations of dinotefuran in the xylem fluid exceeded 100 ppb, the upper limit we set for the ELISA. We are continuing with the evaluation of lower treatment rates, and will present the results at the annual symposium.

In the second set of experiments, we are evaluating the effect of dinotefuran against the eggs of the GWSS. Adult GWSS are confined in cages with cotton, which is an excellent host for GWSS oviposition. Leaves with egg masses (not older than 24 hours) are cut from the plants and the petioles inserted into vials containing a range of insecticide solutions. The uptake of insecticide into each leaf is allowed to proceed for 24 hours and the leaves are then transferred to leaf boxes. The leaf boxes are maintained under lights until the normal period of embryonic development is completed. Mortality is assessed at the time of emergence of the first instar. In our first set of experiments, we tested 0.1 ppm dinotefuran (prepared from Venom 70SG). After 24 hours, the average concentration of dinotefuran present in the leaves was 7.3 ng/cm<sup>2</sup> leaf. At this concentration, we observed 100% mortality of emerging nymphs. As with imidacloprid, the nymphs developed fully within the egg mass and only succumbed to the effects of contact with dinotefuran during emergence. The high mortality at this concentration is in contrast to our previous data for imidacloprid, where we observed an LC<sub>50</sub> of 39 ng/cm<sup>2</sup> leaf. Our results show that dinotefuran is more toxic to the 1<sup>st</sup> instar than imidacloprid.

## **CONCLUSION**

In previous work, we showed that the rate of uptake of dinotefuran into grapevines was faster than imidacloprid. Also, concentrations of dinotefuran at peak uptake were higher. The results we are generating from this project are encouraging from two standpoints. First, we have shown that dinotefuran is highly toxic to GWSS adults, indicating that it will be an effective product for the control of the insect in vineyards. The use of dinotefuran will provide growers with a product that acts effectively against sharpshooters, particularly in situations where growers must respond quickly to an infestation to prevent the potential transmission of PD. When we conclude our bioassays, we will generate a threshold level of dinotefuran necessary to kill a sharpshooter quickly once it feeds from the xylem. We will then be able to determine the level of persistence that a treatment will provide. And second, dinotefuran is highly toxic to emerging 1<sup>st</sup> instars. Systemic treatments exploit the xylophagous feeding behavior of the GWSS adult and immature stages. We now know that these treatments have an additional impact on emerging 1<sup>st</sup> instars before they begin feeding.

The systemic neonicotinoids imidacloprid and dinotefuran are effective insecticides that growers can use for long-term management of GWSS populations. Because of the contrasting chemical properties of these insecticides, growers can now choose the most suitable product to meet their pest management needs.

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## **FUNDING AGENCIES**

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# RNA-INTERFERENCE AND CONTROL OF THE GLASSY-WINGED SHARPSHOOTER AND OTHER LEAFHOPPER VECTORS OF *XYLELLA FASTIDIOSA*

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**ABSTRACT**

Here are presented the progress on a collaborative biotechnological work aimed to develop an RNA interference (RNAi) strategy designed to control sharpshooter vectors of *Xylella fastidiosa*, the causal agent of Pierce's disease. In the year 2007-2008 we constructed cDNAs corresponding to specific genes of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, and evaluated dsRNAs for their ability to induce RNAi effects against GWSS. We performed injection experiments in GWSS insects and evaluated effects by assessing target RNA degradation. Identified genes will be used to develop transgenic basil plants such that dsRNAs are expressed in xylem tissues via EgCAD2, a xylem-specific promoter. Transgenic plants will be evaluated for their ability to induce RNAi effects on GWSS.

**INTRODUCTION**

Pierce's disease (PD), caused by the xylem-limited bacterium, *Xylella fastidiosa* (*Xf*), is an important threat to the California grape industry ([http://www.aphis.usda.gov/lpa/pubs/fsheet\\_faqs\\_notice/fs\\_phglassy.html](http://www.aphis.usda.gov/lpa/pubs/fsheet_faqs_notice/fs_phglassy.html) [http://orsted.nap.edu/openbook.php?record\\_id=11060&page=21](http://orsted.nap.edu/openbook.php?record_id=11060&page=21)). The most important recent epidemic of PD in California was found to be associated with the introduction of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, an invasive sharpshooter leafhopper known to be indigenous to parts of the Southeastern United States (Blua et al., 1999). The GWSS is a large, robust leafhopper with a broad host range including many native, ornamental and crop plants. The combination of this new PD vector species, its wide host range, abundance of host plants, its affiliation for citrus as a host for reproduction, and its ability for long-distance dispersal (Blua and Morgan, 2003) has raised concerns that PD and GWSS are important threats to the California grape industry beyond the Temecula region.

In addition to being transmitted by GWSS, *Xf* is transmitted to plants by several other species of xylem-feeding leafhoppers (Redak et al., 2004). It is interesting to note that as opposed to phloem-feeding hemipterans, xylem feeders must ingest much greater volumes of plant sap. And it is the ingestion of large volumes of plant sap that offers the potential to deliver toxic molecules to leafhoppers, even if these molecules are produced in low concentration in xylem sap. This is an important component of our strategy.

We propose a new approach, one based on RNA interference (RNAi) directed towards GWSS. RNAi leads to sequence specific degradation of target RNA molecules within the cell cytoplasm, resulting in eliminating or reducing gene expression (mRNA degradation) or antiviral immunity (degradation of viral genomic RNAs) (Lu et al., 2004; Brodersen and Voinnet 2006). There are already several examples of practical implementation of RNAi-based technologies for agriculture. For example, RNAi-based strategies for conferring plant resistance to bacterial, nematode and virus induced plant diseases have been demonstrated, and some have been even used in commercial agriculture (Escobar et al., 2001; Huang et al., 2006; Gonsalves 1998). Recently, particular attention was devoted to RNAi efforts targeting insects. For example in the November 2007 issue of *Nature/biotechnology*, the "news and views" article was entitled "RNAi for insect-proof plants" (Gordon, K.H. and P.M. Waterhouse, 2007) and two research articles in that issue presented current RNAi efforts towards insect pests of plants (Baum, J.A., et al., 2007; Mao, Y.B., et al., 2007). In 2008, in *Trends in Biotechnology* another article was titled "RNAi-mediated crop protection against insects" (Price, D.R. and J.A. Gatehouse, 2008). Of relevance to this proposal is that RNAi offers opportunities for targeting *H. vitripennis* via RNAi-based disruption of essential GWSS genes, thereby resulting in insect deleterious effects.

**OBJECTIVES**

The objectives of our research effort are to develop new and effective, environmentally sound strategies for controlling the GWSS and other leafhopper vectors of *Xf*. Our goal is to develop strategies that are effective and will provide control for PD of grapes, but also have flexibility for use in other important California crops.

The specific objectives of our effort are:

1. To identify and develop RNAi-inducers capable of killing or reducing the survival and/or fecundity of GWSS.
2. To generate transgenic plants capable of expressing and delivering GWSS deleterious RNAi molecules within their xylem.
3. To evaluate transgenic plants for their ability to generate inducers capable of inducing RNAi vs. GWSS.

## RESULTS AND DISCUSSION

For this effort we utilized in vitro and in vivo delivery systems. We assessed RNAi effects in cultured GWSS cells. We also screened dsRNAs in whole leafhoppers by injection, and we are optimizing dsRNA insect delivery by feeding in vitro.

**Rearing GWSS and other PD vectors.** A colony of GWSS insects was collected from Riverside and donated to us by Dr. R. Almeida (UC Berkeley). The colony was transferred into the Controlled Research Facility (CRF) at UC Davis and insects were reared there for more than one year. We were able not only to keep the original colony in good condition, but also to rear continuously new generations of GWSS. Dr. Almeida also provided us with a colony of *Draeculacephala minerva* (*D. minerva*). We decided to rear these insects because they are not quarantined in northern California, they are easy to rear and they are California native vectors of PD. As for GWSS, we were able to maintain and rear colonies of *D. minerva*.

**GWSS cells.** Since GWSS insects are quarantined in Northern California, we decided to test the effect of RNAi in the GWSS cells. We received these cells from the Dr. Bruce Hammock laboratory (Kamita et al., 2005, GWSS cell line Z15). We were able to optimize a cell transfection protocol with an efficiency higher than 50%, as measured using a siRNA fluorescein labeled control. Cell viability was measured with trypan blue staining (data not shown). Alexa-Fluor 488 phalloidin (Invitrogen) was used to label F- actin of GWSS cells grown and fixed on glass slides. This labeling procedure allowed a first evaluation of actin integrity and structural appearance in GWSS cells transfected with actin dsRNA (**Figure 1**).

**Choice of dsRNA inducers:** Fourteen GWSS nucleotide sequences, derived from EST based nucleotide sequences available in GenBank and translatable in putative proteins, were used to design gene specific primers and to generate cDNAs from GWSS cell line Z15. The same approach was used for *D. minerva*. Corresponding sequences were amplified by RT-PCR, cloned and sequenced to confirm their identity.

cDNAs of actin, arginin kinase, *lian 2* (a non-LTR retrotransposon) and *sar1* mRNAs expressed in the GWSS cell line and *D. minerva* insects were cloned in pGMTeasy vector in both orientations relative to the T7 RNA polymerase promoter, and sequenced. The vectors were used for T7 RNA polymerase-mediated in vitro transcription to generate specific dsRNAs (Ambion, dsRNA MegaScript). These dsRNA were delivered via transfection in GWSS cells, and two of them (*sar1* and actin) via injection in GWSS insects.

**Evaluation of RNAi effects in GWSS cells and whole insects.** Realtime RT-PCR primer/probe sets were designed and tested via real time RT-PCR assays of GWSS cell derived RNA.

**RNAi in GWSS cells.** Real time RT-PCR was used to measure the amount of target mRNA in GWSS cells. Actin mRNA was first used as the RNAi target in GWSS cells. Upon cell transfection with actin dsRNA, a reduction of the corresponding mRNA was observed, indicating effective RNAi in cells (data not shown). In a time course experiment, actin dsRNA and actin hairpin loop (cloned in the Gateway pMT-Dest 48 plasmid, Invitrogen) were used in cell transfection. The strongest RNAi effects were observed upon dsRNA delivery at 72 hours post transfection (hpt) (**Figure 2**).

When cells were transfected with actin dsRNA, siRNA and the actin hairpin loop plasmid to identify the best effector for RNAi, the most efficient RNAi inducer proved to be actin dsRNA (data not shown). SiRNA was found to be a good alternative RNAi inducer.

Actin and *sar1* mRNAs were compared as targets for RNAi, via transfection of GWSS cells with actin and *sar1* dsRNA respectively. In these experiments, sets of GWSS cells were also transfected with GWSS arginin kinase and *lian2* dsRNAs as control of genes expressed in GWSS; and *gfp* dsRNA as exogenous control. RNAi was evaluated 72 hpt. Experiments were repeated three times. Of these, twice the cell transfection was performed in three replicates and once in two duplicates. Real time RT-PCR samples were always loaded in duplicates. As result, actin dsRNA seems to be a better RNAi inducer than *sar1* dsRNA (**Figure 3**).

**RNAi in GWSS insects.** RNA interference was experimentally assessed in GWSS insects. Sets of 15 nymphs were injected with 1µg of dsRNAs (*gfp*, actin or *sar1*) or buffer, and groups of 5 insects were sacrificed 1 and 3 dpi. Total RNA was extracted from the insects and the amount of the mRNA was measured by two methods, real time RT-PCR and semi-quantitative RT-PCR. Experiments were repeated three times. Semi-quantitative RT-PCR and real time RT-PCR results were comparable, and both confirmed that injection of dsRNA of corresponding endogenous genes in insects produced a reduction of the mRNA, indicating RNAi in insects (**Figures 4 and 5**).

**Xylem specific promoter cloning.** We cloned the full length EgCAD2 xylem specific promoter from *Eucalyptus gunii* in pGEMTeasy, and we are subcloning this promoter into the binary vector AKK 1431, obtained from Govindarajulu Manjula, (C.G. Tylor lab Donald Danforth Plant Science Center, St. Louis). The plasmid contains the GUS gene. Our constructs will be evaluated in transgenic basil plants and then the GUS sequence will be replaced by an RNAi inducer as identified above.

## CONCLUSIONS

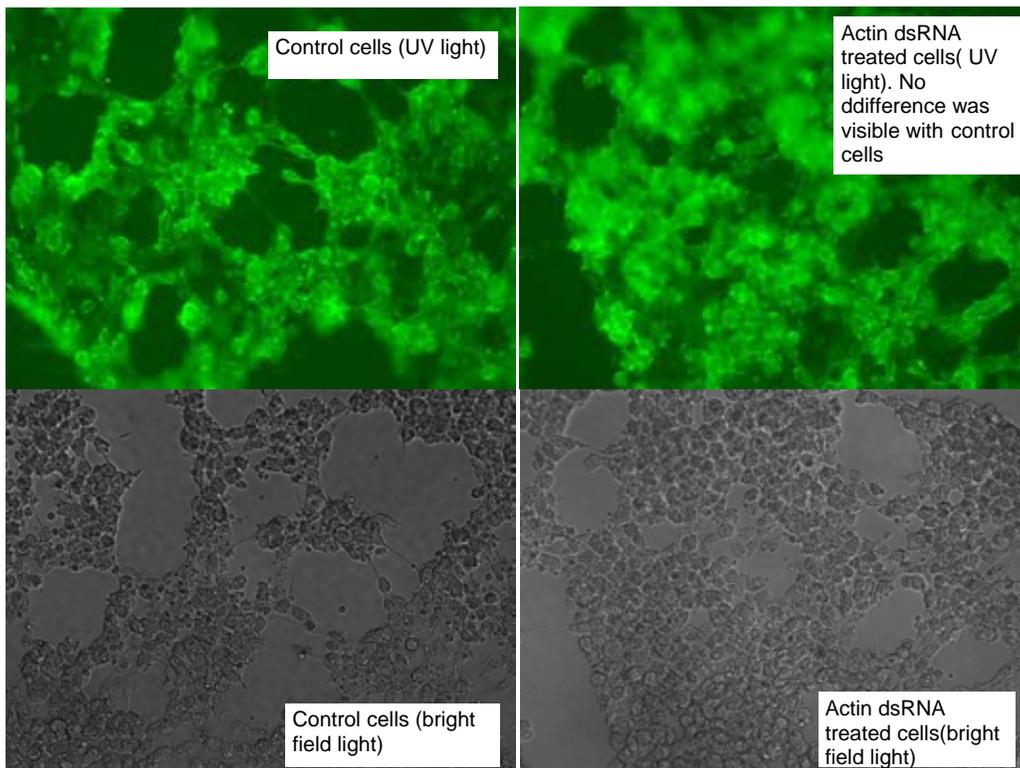
RNAi effects have been demonstrated in GWSS cells and insects after delivery of dsRNA, and GWSS cells can be used to screen candidate gene silencing targets. This study provides the evidence that RNAi might be useful as part of the overall strategy to control *Xf* leafhopper species and to break the cycle between bacterial diseases and their hosts. Future work includes the identification of suitable RNAi targets, the production of transgenic plants expressing dsRNAs in their xylem and the study of the fate of ds/siRNA delivery in insects after feeding.

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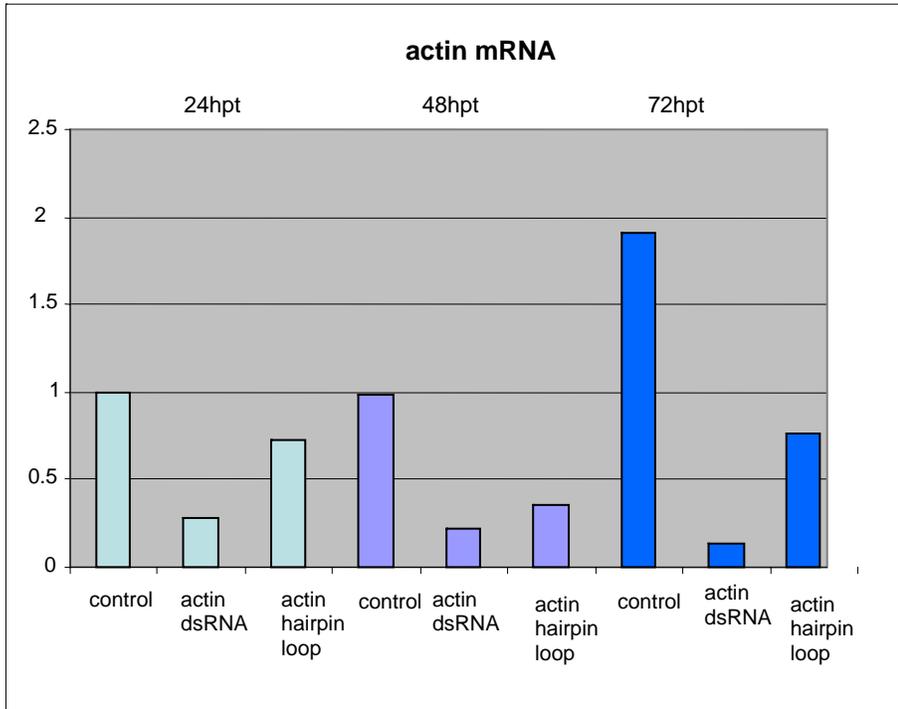
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## FUNDING AGENCIES

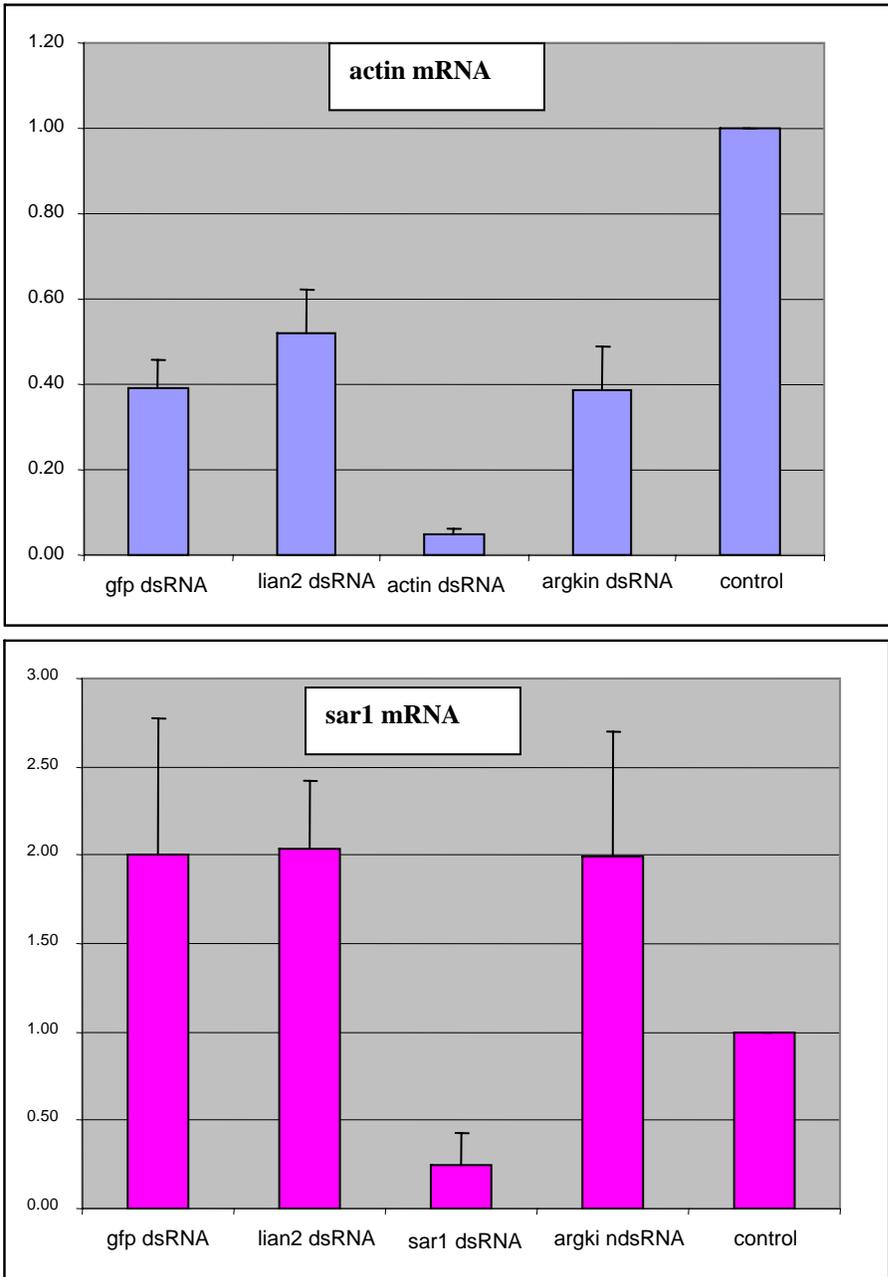
Funding for this project was provided by the University of California Pierce's Disease Grant Program.



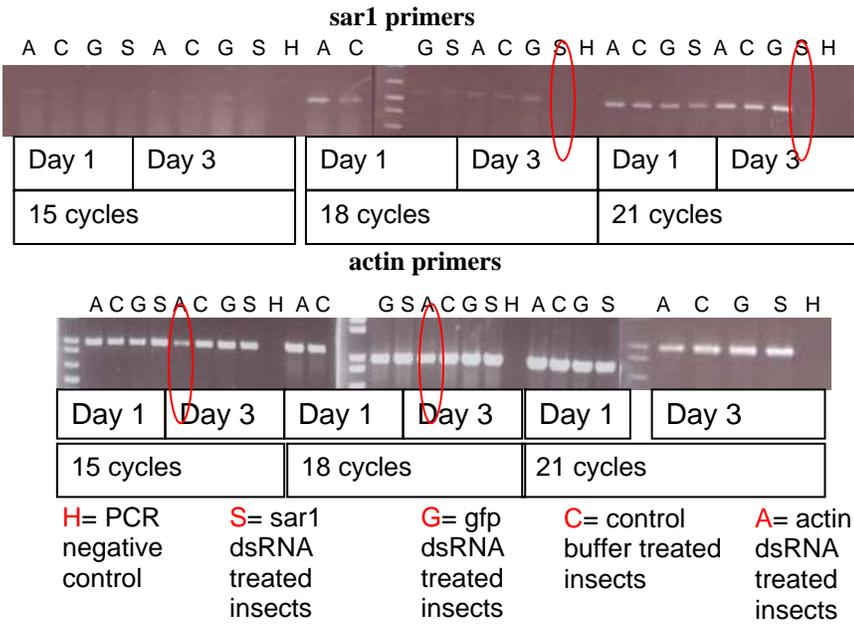
**Figure 1.** Microscope observations at 24 hpt. GWSS cells were grown on a glass slide and treated with Alexa-Fluor 488 phalloidin (Invitrogen).



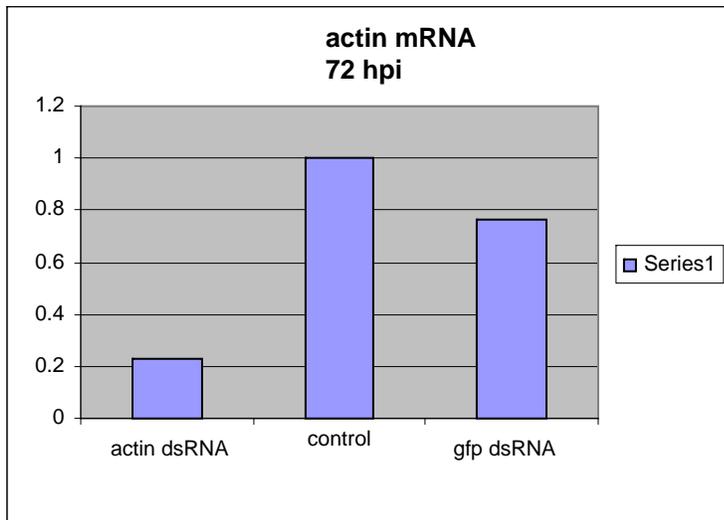
**Figure 2.** Cells transfected with dsRNA actin, or the actin hairpin loop plasmid were harvested 24, 48 and 72 hpt and their actin mRNA levels were quantified by real time RT-PCR. Actin mRNA was reduced the most at 72hpt.



**Figure 3.** Actin, *lian2*, arginin kinase, and GFP dsRNAs (upper panel) and *sar1*, *lian2*, arginin kinase and GFP dsRNAs (lower panel) were transfected in GWSS cells. Cells were harvested 72 hpt and the level of *sar1* or actin mRNAs were quantified by real time PCR. RNAi was reached as proven by the *sar1* and actin mRNA reduction in *sar1* and actin dsRNA transfected cells, compared to cells treated with transfection reagent only (control). Transfection of cells with actin dsRNA generated a better RNAi response compared to transfection of cells with *sar1* dsRNA. Data were generated in three biological independent experiments, with 8 replicates total. Error bars above the columns indicate the standard deviation among the 8 replicates.



**Figure 4.** Gel representative of three separate experiment results. Semi-quantitative RT-PCR results, showing the RNA level in insects injected with 1µg of dsRNAs. Insects were collected 24 and 72 hour post injection. Each RT-PCR was performed using 100 ng total RNA. The PCR was stopped after 15, 18 and 21 cycles.



**Figure 5.** Real time RT-PCR on same samples as above.

# ARE GLASSY-WINGED SHARPSHOOTER POPULATIONS REGULATED IN CALIFORNIA? LONG-TERM PHENOLOGICAL STUDIES FOR GLASSY-WINGED SHARPSHOOTER IN AN ORGANIC LEMON ORCHARD

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**Reporting Period:** The results reported here are from work conducted March 2002 to October 2008.

## ABSTRACT

Glassy-winged sharpshooter (GWSS) population densities have been steadily declining over a 6.5 year period in organic lemons grown in an experimental study plot at UC Riverside Ag. Ops. Peak adult GWSS populations in July 2008 were just 21% of those observed around August 2002. It is uncertain if egg parasitism, which has consistently averaged ~22% per year of GWSS egg masses, is responsible for the observed decline. Density dependent analyses of time series data are planned once data sets are large enough to provide greater insight into factors (i.e., parasitism [density dependent mortality] or weather [density independent mortality]) affecting GWSS population dynamics.

## INTRODUCTION

Data collected from bi-weekly monitoring over the last 6.5 years from organic commercially-managed lemons at Ag. Ops. UC Riverside indicates that glassy-winged sharpshooter (GWSS) populations are declining steadily each year (**Figure 1**). It is uncertain whether parasitism of GWSS eggs by mymarid parasitoids is responsible for this downward population trend (**Figure 2**). In California, there is a guild of natural enemies attacking GWSS. The dominant parasitoid attacking GWSS in California is *Gonatocerus. ashmeadi* followed by *G. morrilli*. *G. triguttatus* from Texas and *G. fasciatus* from Louisiana have been released in California, but widespread establishment and proliferation has not occurred. Other minor parasitoid species include *G. novofasciatus*, *Ufens* sp., and *Zagella* sp. Together, this guild of parasitoids provides an average of ~22% parasitism of GWSS eggs over the entire 6.5 yrs that this study site has been monitored. There are at least four possible reasons for low seasonal parasitism levels in California: (1) competitive exclusion amongst members of the GWSS parasitoid guild is reducing effective biological control; (2) An extremely aggressive and efficacious natural enemy that can outcompete *G. ashmeadi* and completely dominate the system year round to the almost total exclusion of all current parasitoids has not been established in California and is needed for successful biological control of GWSS (this would require exploitation of non-GWSS hosts during long periods of host egg unavailability); (3) The absence of resource subsidies such as nectar provided by flowering plants in agroecosystems may limit parasitoid efficacy because longevity and fecundity is significantly reduced when parasitoids can not access carbohydrates. Understorey management may be an important cultural strategy to benefit GWSS parasitoids if it can be demonstrated not to enhance GWSS and *Xylella* populations; and (4) Climate, in particular, prolonged cool periods over winter when GWSS eggs are unavailable probably has a severe affect on parasitoid reproductive success and the ability of *G. ashmeadi* populations to propagate through the winter. Long-term phenology studies which generate data similar to the project reported on here, can be used to tease out density-dependent and density-independent factors affecting population dynamics to elucidate factors affecting GWSS population growth.

## OBJECTIVES

This project has one objective:

1. Conduct bi-weekly surveys of GWSS eggs, nymphs, and adults, and associated rearing of parasitoids from harvested egg masses from organic lemons at Ag. Ops., UC Riverside.

## RESULTS

The population monitoring study and measures of percentage parasitism clearly indicate that GWSS densities have continued to decline steadily at the long-term monitoring plot (**Figure 1**) and percentage parasitism have remained relatively constant over this time period (**Figure 2**). Detection of density-dependent mortality from sequential census data, such as that presented here, is notoriously difficult and the results of analytical models differ in outcomes depending on assumptions made even when dummy data sets have been constructed to show density dependent mortality. One of the major problems with these types of analyses is serial correlation, where densities at  $N_t$  directly influence the population at  $N_{t+1}$ . Recent developments in analyses of time series data, such as those we are collecting for GWSS, are now providing much more robust tests that overcome autocorrelation problems. The Partial Rate Correlation Function (PRCF) is a relatively new statistical procedure specifically designed for time series analysis of biological populations to detect density dependent feed back. Literature searches so far indicate that PRCF is the best of the extant techniques for analyzing long-term population counts. Consequently, census data collected from GWSS monitoring will be subjected to PRCF once we have data for a minimum of 10 consecutive years to determine if density dependent or density independent feed back is responsible for observed fluctuations from generation to generation. Detection of density dependent mortality will indicate that populations are being regulated, and could suggest that natural enemy populations are responsible. Currently, our data set is too short to

determine if parasitoid activity is providing density dependent mortality and is subsequently responsible for decreasing GWSS densities at the study site.

## CONCLUSIONS

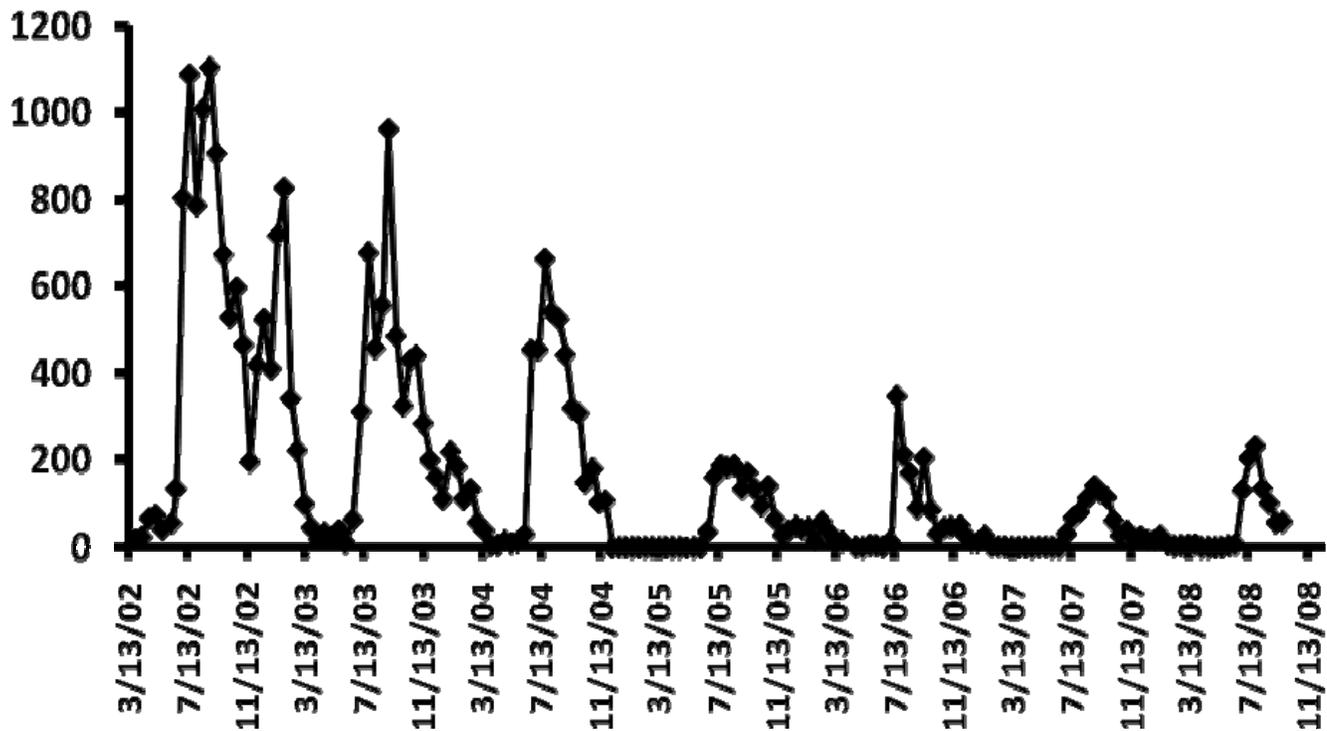
GWSS populations appear to be showing a steady annual decrease in numbers in an organic lemon orchard at the University of California, Riverside. Percentage parasitism of GWSS eggs by mymarid parasitoids, in particular, *G. ashmeadi*, has remained relatively constant from year to year at ~22%. It is unknown if this level of parasitism is sufficient to have caused the steady decline in GWSS numbers observed over the past 6.5 years or whether climatic variables such as wet winters (e.g., 2006), or very cold and dry winters (e.g., 2007) suppressed GWSS population growth, while warmer than normal spring periods (e.g., 2008) accounts for observed rebounds in GWSS populations.

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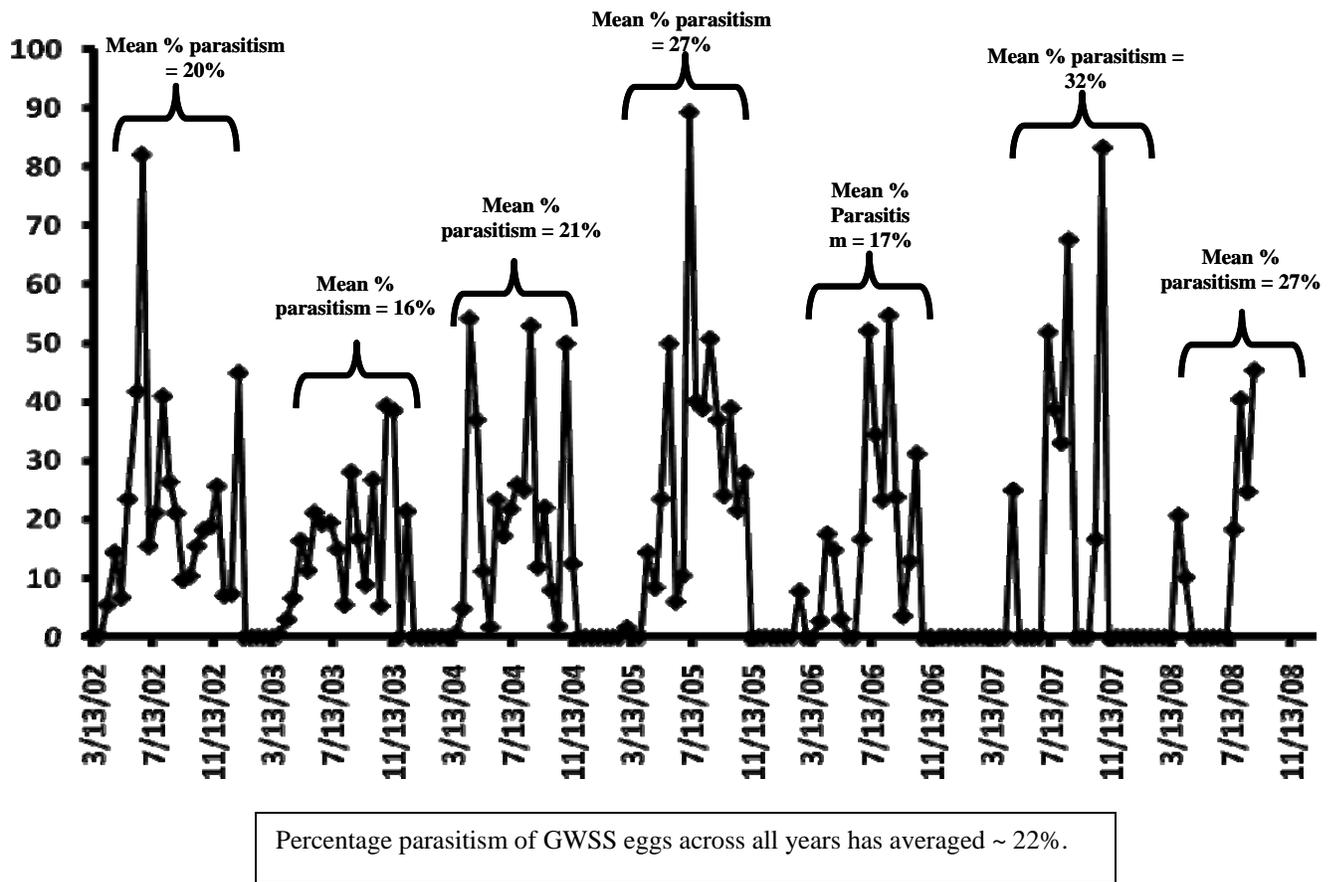
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## FUNDING AGENCIES

Funding for this project was provided in part by the University of California Pierce's Disease Grant Program.



**Figure 1.** Phenology of adult GWSS in organic Eureka lemons. Data are total counts from timed five minute surveys made every two weeks of 10 mature lemon trees at Ag. Ops. University of California, Riverside.



**Figure 2.** Percentage parasitism estimates of GWSS eggs in Eureka lemons. GWSS egg masses are collected from timed five minute surveys made every two weeks of 10 mature lemon trees at Ag. Ops. University of California, Riverside. Harvested leaves are returned to the laboratory, the number of eggs per egg mass are counted and parasitoid emergence and species identity is determined.

# SHOULD NEOCLASSICAL BIOLOGICAL CONTROL AGENTS FROM ARGENTINA BE RELEASED IN CALIFORNIA FOR CONTROL OF THE GLASSY-WINGED SHARPSHOOTER?

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**Reporting Period:** The results reported here are from work conducted July 2008 to October 2008.

## ABSTRACT

To date, we have conducted experiments investigating the egg age preference and competitive ability of clade 1 (Hoddle and Irvin 2007) and clade 2 (presented here), *G. tuberculifemur* against *G. ashmeadi*. Results strongly suggest that *G. ashmeadi* is superior to *G. tuberculifemur* clades 1 and 2 when parasitizing GWSS eggs in “complex” and “simple” experimental conditions with short and long exposure times. The higher rates of parasitism and larger host age range demonstrated by *G. ashmeadi* suggest that this species will be more competitive than *G. tuberculifemur* clades 1 and 2 in the field when competing for GWSS egg masses and will likely prevent the widespread establishment of *G. tuberculifemur* clades 1 and 2. Consequently, it would appear that *G. tuberculifemur* (clades 1 and 2) will contribute little to the biological control of GWSS in California.

## INTRODUCTION

*Gonatocerus tuberculifemur* is a sharpshooter parasitoid from Argentina that attacks glassy-winged sharpshooter (GWSS) egg masses. This parasitoid is being considered for use as a neo-classical or new association biological control agent for GWSS in California. A recent molecular study (de León et al. 2008) has identified two distinct clades within the species currently considered to be *G. tuberculifemur*. Populations of *G. tuberculifemur* collected from San Rafael in Argentina cluster into clade 2 and the rest of the populations for which DNA was analyzed clustered into clade 1. There is substantial uncertainty about the safety of releasing these agents (i.e., unintended spread and non-target impacts) and whether these neoclassical natural enemies would provide additional control of GWSS in California or disrupt the efficacy of the existing parasitoid complex which has been constructed with natural enemies that have evolved to exploit GWSS. The purpose of this work has been to ascertain in quarantine whether these neoclassical biological control agents from Argentina in clades 1 and 2 can outperform the dominant GWSS parasitoid in California, *G. ashmeadi*. To date, we have completed experiments assessing clade 1 and a summary of these results were presented in the 2007 Pierce’s Disease Symposium Proceedings. This report presents experimental results for “*G. tuberculifemur*” that are representative of populations that compose clade 2.

## OBJECTIVES

1. Ascertain oviposition preferences of *G. ashmeadi* and *G. tuberculifemur* clade 2 for GWSS egg masses of different ages.
2. Determine the competitiveness of these two parasitoid species simultaneously foraging for GWSS egg masses in complex and simple environments.

## RESULTS

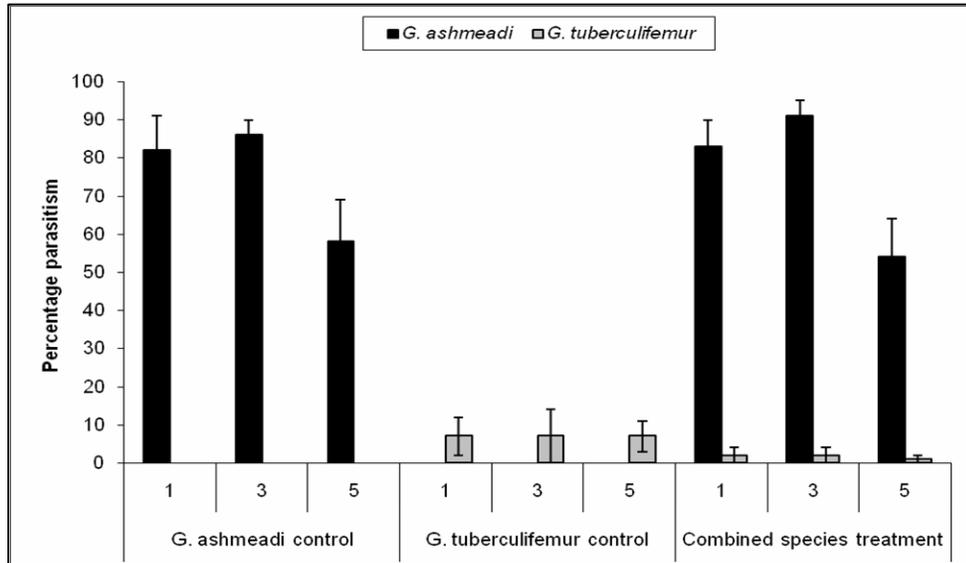
Objectives 1 & 2: Egg age preferences and competitive ability

### Experiment 1 - Complex environmental system:

One mated female *G. ashmeadi* and *G. tuberculifemur* (~24-36 h of age) was presented simultaneously with one GWSS egg mass (composed of ~4-8 eggs) camouflaged amongst four other similar sized lemon leaves in a double ventilated vial. This ‘complex system’ was replicated 15 times for GWSS eggs aged one, three and five days of age. After 60 minutes exposure to foraging parasitoids, leaves with egg masses were placed into individual Petri dishes, labeled and held at 27°C for emergence of parasitoids and GWSS nymphs. The number of emerged and unemerged males and females of each parasitoid species was recorded. Fifteen control vials containing one female parasitoid were set up for each species. Percentage parasitism by *G. ashmeadi* and *G. tuberculifemur* was calculated as the percentage of total eggs.

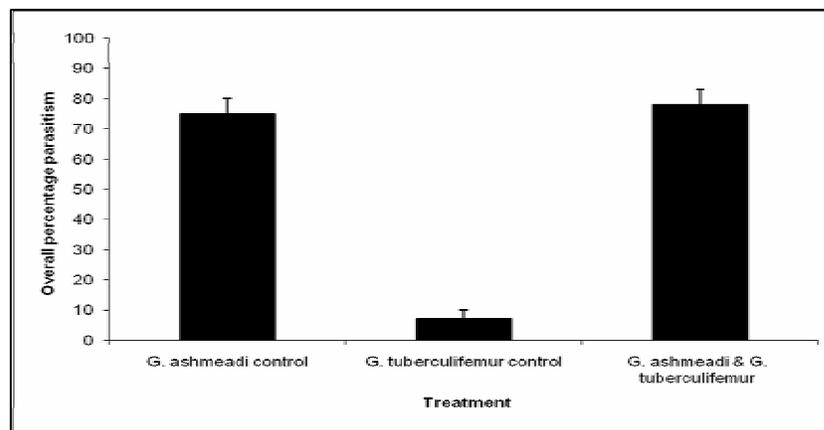
**Figure 1** shows percentage parasitism by *G. ashmeadi* and *G. tuberculifemur* resulting when GWSS egg masses one, three or five days of age were exposed to three different treatments: (i) *G. ashmeadi* control vials consisting of one female *G. ashmeadi*; (ii) *G. tuberculifemur* control vials consisting of one female *G. tuberculifemur*; and (iii) vials containing one female of both *G. ashmeadi* and *G. tuberculifemur* competing for GWSS eggs. Results from vials containing one egg mass exposed simultaneously to one *G. ashmeadi* and *G. tuberculifemur* showed that parasitism by *G. ashmeadi* was consistently 53-81% higher than *G. tuberculifemur* for all three egg ages (one, three and five days of age) tested (**Figure 1**). Results from the control vials (those containing only one female) showed that *G. tuberculifemur* demonstrated no egg age preference, parasitizing 7% of eggs one, three and five days of age (**Figure 1**). Results for the *G. ashmeadi* controls showed that GWSS egg parasitism ranged from 58-82% (**Figure 1**). Statistical analyses are currently underway to determine whether egg age

had a significant effect on parasitism by *G. ashmeadi*. The higher rates of parasitism demonstrated by *G. ashmeadi* suggest that this species will be more competitive than *G. tuberculifemur* in the field when attacking GWSS egg masses.



**Figure 1.** Percentage parasitism by *G. ashmeadi* and *G. tuberculifemur* resulting when GWSS egg masses aged one, three, and five days of age were exposed to parasitoids either alone or in competition with each other.

There was no significant difference in overall parasitism of GWSS eggs between vials containing *G. ashmeadi* only (A) and vials containing both *G. ashmeadi* and *G. tuberculifemur* (AT) for the complex system (**Figure 2**). Female *G. ashmeadi* dominated the mixed parasitoid system and the contributions to overall parasitism by *G. tuberculifemur* were negligible.



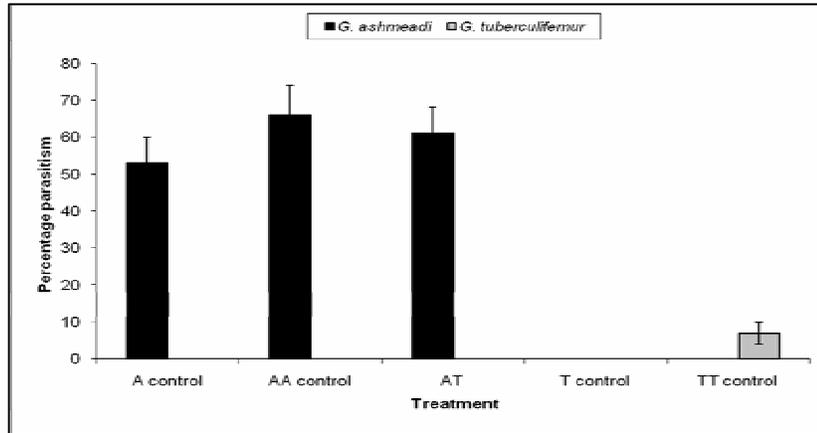
**Figure 2.** Overall percentage parasitism of GWSS egg masses exposed to three treatments in a 'complex experimental system' for 1 h.

Experiment 2 - Simple environmental system:

One mated female *G. ashmeadi* and *G. tuberculifemur* (~24-36 h) was presented simultaneously to one GWSS egg mass (~4-8 eggs, 1-2 days of age) on a single leaf in a double ventilated vial. This 'simple system' was replicated 15 times. Egg masses were not camouflaged amongst four other similar sized leaves. Exposure time of GWSS eggs to parasitoids was 15 minutes and each minute the behavior [searching container (SC), searching leaf (SL), searching egg mass (SE), oviposition (O), resting (R), grooming (G), aggressive chasing (C), antennating conspecific (AC), searching egg mass from top side of leaf (SETS), ovipositing from top side of leaf (OTS), and feeding on honey (F)] of each female was recorded. Fifteen replicates of two types of control vials were also set up for each species. These contained either one female parasitoid or two female parasitoids of the same species to account for interaction effects independent of species.

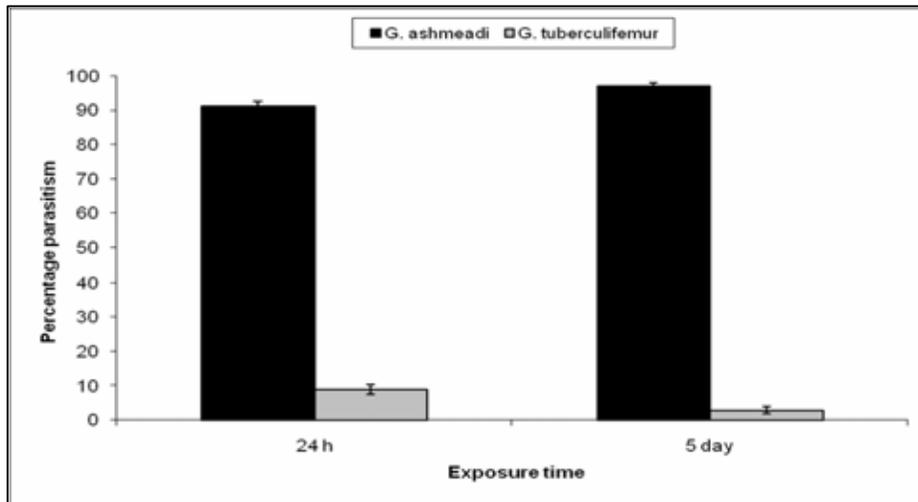
Results from vials containing one egg mass exposed simultaneously to one *G. ashmeadi* and *G. tuberculifemur* in a 'simple experimental system' with just one leaf for 15 minutes showed that parasitism by *G. ashmeadi* was 61% (**Figure 3**), while *G.*

*tuberculifemur* parasitized no GWSS eggs within the 15 minute exposure time (**Figure 3**). Behavioral data are currently being analyzed.



**Figure 3.** Percentage parasitism by *G. ashmeadi* and *G. tuberculifemur* resulting when GWSS egg masses aged 1-2 days of age were exposed to parasitoids either alone or with intraspecific or interspecific competition (A = vial containing one female *G. ashmeadi*; AA = vial containing two female *G. ashmeadi*; AT = one female *G. ashmeadi* and *G. tuberculifemur*, T = one female *G. tuberculifemur*; TT = two female *G. tuberculifemur*).

Experiment 3 - Long exposure time: When ~50 GWSS eggs were exposed to one female *G. ashmeadi* and *G. tuberculifemur* for either 24 hours or five days, parasitism by *G. ashmeadi* was 82-94% higher than *G. tuberculifemur* for both exposure times (**Figure 4**).



**Figure 4.** The mean percentage of *G. ashmeadi* and *G. tuberculifemur* offspring emerging when 50 GWSS eggs were exposed simultaneously to one mated female *G. ashmeadi* and *G. tuberculifemur* for 24 h or five days.

## CONCLUSIONS

*Gonatocerus tuberculifemur* is a sharpshooter parasitoid from Argentina that is being considered for release from quarantine for biological control of GWSS in California. There is substantial uncertainty about the safety of releasing this agent and whether it would provide additional control of GWSS in California or disrupt the efficacy of the existing parasitoid complex which has been constructed with natural enemies that have evolved to exploit GWSS in the home range of this pest (i.e., the southeast USA and northeast Mexico). By studying the egg age preference, competitive ability and functional response of *G. ashmeadi* and *G. tuberculifemur* (clades 1 and 2) we sought to determine which of these three parasitoids is likely to be the

most efficacious.

Results from experiments involving clade 2 of *G. tuberculifemur*, as shown here, suggest that *G. ashmeadi* is superior to *G. tuberculifemur* when parasitizing GWSS eggs in “complex” and “simple” experimental conditions with short and long exposure times. Results from competition experiments where both parasitoids were presented simultaneously to host eggs demonstrated that *G. ashmeadi* outcompeted *G. tuberculifemur* clade 1. These results suggest that *G. ashmeadi* may prevent widespread establishment and proliferation of *G. tuberculifemur* clade 1 in California. This result is similar to Hoddle & Irvin (2007) who showed that *G. ashmeadi* was superior to *G. tuberculifemur* clade 2, and to *G. triguttatus* and *G. fasciatus* under similar experimental conditions (Irvin and Hoddle 2005). Neither *G. triguttatus* or *G. fasciatus* have performed well following mass releases in California where *G. ashmeadi* is present, which suggests that the results of these competitive lab experiments may accurately predict field performance.

Our data thus far suggests that the potential impact of releasing *G. tuberculifemur* clade 2 (and clade 1) in California on the biological control of GWSS may not out-weigh the cost of mass rearing and releasing of this biological control agent. When time and labor costs for large-scale colony maintenance, disruption of existing levels of control achieved with the resident natural enemy guild (especially *G. ashmeadi*), and potential invasion by *G. tuberculifemur* back into the southeast USA where GWSS originated are all considered, there appears to be no quantifiable benefit to releasing *G. tuberculifemur* clade 2 in California for the biological control of GWSS. Similar conclusions have been reached from completed work on *G. tuberculifemur* clade 1 (Hoddle and Irvin 2007).

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#### FUNDING AGENCIES

Funding for this project was provided in part by the CDFA Pierce’s Disease and Glassy-winged Sharpshooter Board.

# IMPROVED DETECTION, MONITORING, AND MANAGEMENT OF THE GLASSY-WINGED SHARPSHOOTER

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**Reporting Period:** The results reported here are from work conducted in 2008.

**ABSTRACT**

Efficient and precise methods for detection of new colony infestations and for monitoring glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) population dynamics on a temporal and spatial basis for IPM related decision-making are lacking. This proposal provides an approach that will address the detection and monitoring needs as well as develop a new strategic approach to management of GWSS.

**INTRODUCTION**

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar), as a vector of *Xylella fastidiosa* (*Xf*) remains a threat to grapes, almonds, stone fruit and oleander and impacts citrus and nursery crops throughout much of California. It remains an important quarantine pest for the Napa and Sonoma Valleys and other uninfested areas. Due to the unique biology and behavior of the xylophagous GWSS which is driven by plant xylem chemistry and nutrition, conventional detection and monitoring approaches may not provide the necessary statistical precision needed by the regulatory and producer community for management decisions. This proposal provides an approach that will address the detection and monitoring needs as well as develop a new strategic approach to management of GWSS.

**OBJECTIVES**

Overall: To determine the most efficient and cost effective trapping system to detect and monitor GWSS population dynamics and the potential to manage GWSS populations.

1. Evaluate and summarize previous sampling and trapping efforts for GWSS.
2. Trap configuration and number: Determine the potential and optimize the number of traps that are most efficient and cost effective in detecting and estimating GWSS populations.
3. Determine the effects of host plants in combination with traps: Determine the potential and the optimization of a combination of GWSS host plants in sentinel plots to detect, estimate and manage GWSS population dynamics.

**RESULTS AND DISCUSSION**

Since the initiation of the Pierce's Disease-Glassy-winged Sharpshooter Research Program, a number of investigators have addressed sampling methodology for estimating population parameters of GWSS. Here we discuss trap and related sampling methodology with potential for the development of effective monitoring and detection of GWSS (**Figure 1**) and summarize the results to date. We do not include all of the literature that used traps to sample GWSS or the literature tangentially related to sampling methodology, such as marking methods for mark-recapture studies. However, new effective methods have been developed within the program (Hagler et al. 2005).

Turner and Pollard (1959) first used sticky board traps (size not described) at 3 heights 0.6, 1.8 and 3 m above ground. Traps at these three heights captured 23, 37 and 42% of the GWSS captures, respectively. Ball (1979) used masonite board traps (27 × 13.5 cm) and screen traps of yellow, white and red covered with either Tack Trap™ or Stickem Special™ in peach orchards in Monticello, FL to trap GWSS and *H. insolita* (Wlk). Traps were placed at 1 and 1.3 m above the ground and data were collected for three years. Yellow boards captured the highest number of leafhoppers of both species. Results relative to brands of stickem were equivalent. The 1.3 m trap height captured 61% of the GWSS, however, the 0.6 m traps captured significantly more *H. insolita*. GWSS populations began to appear on the traps in late March–early April, peaked in June–July and disappeared in late September–early October. Leafhopper populations fluctuated by an order of magnitude from year to year.

Blua et al. (2002) compared yellow sticky cards, beat-net sampling and timed counts to estimate GWSS nymph and adult populations in citrus compared to total tree counts of GWSS obtained with knockdown (pyrethrin canister) pesticide applications. They also estimated trap efficiency as a function of the number of GWSS already on the traps which addressed the physical function of the trap. However, a potential effect on trap attraction due to reduced visual stimulation or trap active distance was not discussed, but may have been involved. A significant inverse relationship ( $Y=6.584-0.0022X-8E-05X^2$ ,  $R^2=0.71$ ,  $P<0.001$ ) was detected between number of GWSS already present on traps and subsequent trap catch. No correlations were observed for nymph or adult GWSS counts between beat-net samples, timed counts or stick card samples. GWSS adult sticky card samples did not correlate with any other sampling method. Absolute counts of GWSS nymphs correlated significantly ( $P=0.035$ ) with timed counts. For nymphs, adults and total GWSS, beat-net samples correlated

significantly ( $P < 0.01$ ) with timed counts. Blua and Redak (2003) compared trapping methods in small citrus trees versus large commercial trees. Adult counts showed significant correlations between all methods tested. In large commercial trees about 50% of variation was explained by a regression of GWSS sticky card counts on total counts. Beat-net and visual counts were less accurate. In small trees, beat-net and visual counts of nymphs had a correlation with total counts near to or greater than 80%.

Blackmer et al. (2002, 2004, 2006) conducted a mark-recapture study using cylindrical traps (40 × 60 cm sheet rolled into a cylinder of 16 cm dia.) at three heights (ground level, 2-3 m and 6-7 m relative to alfalfa and citrus canopies) to determine the dispersal rate and distance of GWSS as well as *H. liturata*. While trap comparison was not part of the studies, a number of parameters that could affect GWSS trap capture rates were evaluated. Pertinent findings included: higher numbers of GWSS were collected on traps at heights below 3 m, 95% of released GWSS were captured within 90 m of the release point in alfalfa and within 31-150 m in citrus, GWSS peak flight activity occurred between 1000 and 1400 h and trap capture was rare at temperatures below 18 °C. Wind speeds above 3 m/sec decreased flight activity. No flight activity was observed between 2200 and 0600 h and this was similar to observations in Florida (Mizell et al. 2008).

Bartels et al. (2002) used Seabright (Seabright Labs, Emeryville, CA) yellow traps (36 cm<sup>2</sup> trapping surface) at 2 m in height to trap GWSS in several types of crop habitats in Kern County, CA. Grape, peach/nectarine, almond and cherry orchards were sampled at 11 locations using a grid approach where traps were placed in 3 transects 24 m apart on the exterior and interior of the habitats. Traps were checked weekly. GWSS were captured throughout the year. An early season peak was observed in citrus but not in the other crops. From July-October most GWSS captures were found in grape and cherry while the organic grape location had the most GWSS. Late in the year GWSS peaked in peach/nectarine.

Hix (2002, 2003) trapped GWSS in wine grape vineyards that were either organically or minimally farmed in Temecula, CA. Commercially-available yellow traps, and plate and nymph traps (not described) in a number of colors were tested and compared against visual counts of GWSS life stages. Adult GWSS trap captures and oviposition in adjacent vegetation (3 sets of 25 vines) were correlated. Also, the number of nymphs found in July-September was strongly correlated to the number of captured female GWSS. The relationship between nymphs and females was described ( $y = b_1(X) - b_0$ ): number of nymphs per search = 3.4 GWSS females - 2.4 ( $R^2 = 0.97$ ,  $F = 379$ ,  $P = 0.003$ ). Yellow plates captured higher numbers of GWSS than commercial yellow traps. Orange plate traps also captured more GWSS than the Seabright trap. Seabright traps configured as a cylinder did not capture more GWSS than regularly configured (two-sided) traps. Four trap sizes (125, 249, 499 and 998 cm<sup>2</sup>) were compared and trap catch increased with size. However, the trap size of 499 cm<sup>2</sup> captured higher numbers on an area/trap basis. Two types of sticky material were also tested but without manifesting differences in trap captures. GWSS phenology in Temecula grapes was characterized with peak numbers occurring in August-September. Using trap color comparisons, it was demonstrated that GWSS were behaviorally attracted to both yellow and orange.

Blua and Morgan (2003) used Pherocon AM/NB (18 × 25 cm, two sides) (Trece', Salinas, CA) to sample GWSS for 21 months in grape-growing areas of southern California. Their methods consisted of trapping the areas between citrus and grape plantings and 0-40 m into the vineyard interiors along with surrounding vegetation consisting of natural coastal sage scrub and riparian areas. They also investigated the effect of trap height on GWSS capture rate. They reported that >97% of GWSS trap captures occurred at trap heights at or below 5 m. Citrus harbored higher numbers of GWSS than the other vegetation types, especially during winter months, but unlike most native leafhopper vectors in CA, GWSS were detected far into the interior of the vineyards. Peak GWSS trap captures were observed in August-September in all vegetation types with citrus exhibiting an additional peak in December.

Castle and Naranjo (2008) compared yellow sticky trap captures of GWSS using four sampling methods: D-Vac, A-Vac, pole-bucket and beat-net for their ability to estimate relative densities of GWSS in citrus. Similar estimates of GWSS distribution and phenology were produced by each of the methods, however, precision, accuracy and relative cost differed between methods. While a male bias was indicated in the sampling method data, female counts correlated well ( $R^2 = 0.95$ ) with yellow sticky trap captures. The pole bucket was judged using precision and costs as the criteria to be the best overall sampling method for both nymph and adult GWSS.

## CONCLUSIONS

With the notable exception of the results reported for citrus by Blua and Redak (2003) and Castle and Naranjo (2008) that compared four sampling methods useful to estimate GWSS population parameters in citrus groves, trapping methods for GWSS remain ill defined with respect to relative cost, accuracy and precision for both regulatory and management purposes. Yellow sticky traps available commercially from either Seabright or Trece' remain the primary method for detection and monitoring GWSS. However, "homemade" or non-commercial traps of various sizes, including dinner plates (Hix 2003), painted a bright hue of yellow or orange have been shown to be behaviorally attractive to and equally effective for the leafhoppers vectors.

The quantification of sampling efficiency and efficacy remains incomplete for crops and habits other than citrus. In grape, Hix (2003) did report significant correlations between trap captures of nymphs and adults at specific times of the season as

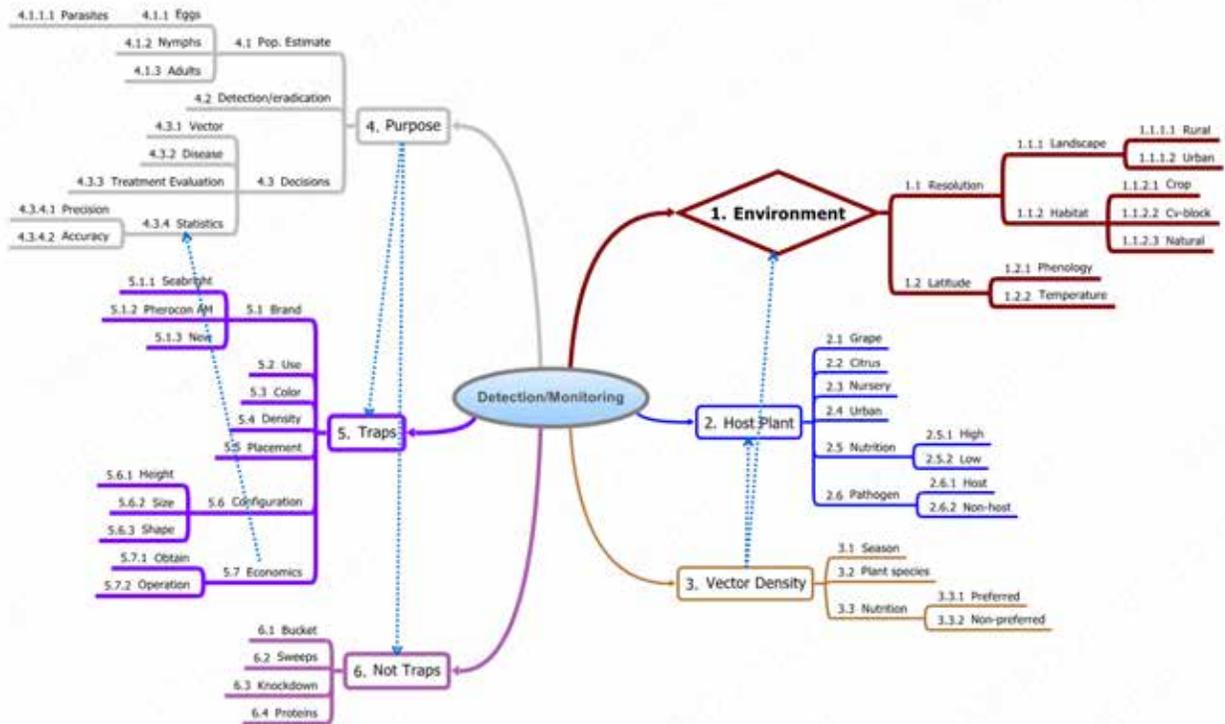
well as correlations of GWSS numbers in the vineyard with those captured on exterior vegetation. Investigations from a number of perspectives with the objective of trap improvement are ongoing. Unlike many other insect pests, GWSS behaviors involved in mating and host plant selection do not appear to be predominantly mediated by olfactory cues. Patt and Sétamou (2007) investigated the response of GWSS nymphs and adults to visual and olfactory cues in the laboratory. They reported that host odors changed the orientation behavior of both nymphs and adults and likely functions as stimulant for enhanced visual attraction to hosts. Potential exploitations of these findings remain to be elucidated. Unfortunately, GWSS is a strong flyer capable of long range dispersal whose nutritional requirements force it to use different host plants over its long lifetime (Mizell et al. 2008). It has demonstrated the ability to spread and establish in non-native habitats in CA and in other parts of the world. Therefore, efficient methods for detection and monitoring remain important tools that need further development to facilitate suppression of this leafhopper vector of *Xf*-caused diseases. A number of behavioral, biological and ecological factors conceptualized in **Figure 1** may perhaps be exploited to further address the problem and these are being investigated.

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## FUNDING AGENCIES

Funding for this project was provided by the CDFFA Pierce's Disease and Glassy-winged Sharpshooter Board.



**Figure 1.** A conceptual "mind map" model of the parameters and potential relationships involved in detection and monitoring of the glassy-winged sharpshooter, *Homalodisca vitripennis*.

## RIVERSIDE COUNTY GLASSY-WINGED SHARPSHOOTER AREA-WIDE MANAGEMENT PROGRAM IN THE COACHELLA AND TEMECULA VALLEYS

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

### ABSTRACT

Riverside County has two general areas where citrus groves interface with vineyards, the Coachella and Temecula Valleys. The Coachella Valley with 10,438 acres of table grapes in proximity to 12,000 acres of citrus and the Temecula Valley with 2,000 acres of wine grapes in proximity to 1,600 acres of citrus are vulnerable to Pierce's disease (PD), caused by the bacterial pathogen *Xylella fastidiosa*. The grapes in the Coachella and Temecula areas of Riverside County are in jeopardy because of the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*), the vector of the PD bacterium, build up in adjacent citrus groves. Citrus is an important year around reproductive host of GWSS in Riverside County, but also one that concentrates GWSS populations over the winter months during the time that grapes and many ornamental hosts are dormant. GWSS weekly monitoring in citrus in grapes began in March 2000 in Temecula Valley and 2003 in Coachella Valley by trapping and visual inspections. Temecula valley GWSS populations in 2008 reached levels not seen prior to the initiation of the area wide GWSS program in 2000. Coachella Valley GWSS populations have decreased dramatically since the treatment program was initiated in 2003.

### INTRODUCTION

The glassy-winged sharpshooter (GWSS) vectors a bacterium that causes Pierce's disease (PD). This insect and bacterium are a severe threat to California's 830,000 acres of vineyards and \$30 billion industry. An area-wide GWSS management program was initiated in Temecula in 2000 to prevent this vector's spread into other California grape-growing regions. In Temecula Valley itself, the wine grape industry and its connecting tourist industry generate \$100 million of revenue for the economy of the area. GWSS/PD caused a 30% vineyard loss and almost destroyed the connecting tourist industry. The area wide GWSS management program initiated in the spring of 2000 saved the industry from a 100% loss. Only a continuation of an area-wide GWSS management program will keep the vineyards viable in Temecula. The table grape industry in the Coachella Valley is represented by 10,465 acres of producing vines, which generate fresh market grapes valued at an average of \$110 plus million annually. The GWSS was identified in the Coachella Valley in the early 1990's. Population increases of this insect in Coachella Valley in the last three years have increased the danger of PD occurrence in this area, as has occurred in similar situations in the Temecula and San Joaquin Valleys. In July 2002, the occurrence of *Xylella fastidiosa*, the PD bacterium, was found in 13 vines from two adjacent vineyards in the southeastern part of Coachella Valley. With this discovery, and the increasing GWSS populations, there was and is a real need to continue an area-wide GWSS/PD management program, to prevent an economic disaster to the work forces and connecting small businesses of Mecca, Thermal, Coachella, Indio, etc. that depend upon the vineyards for a big portion of their incomes. Only a continuation of an area-wide GWSS/PD management program will keep the vineyards viable in Coachella. At present, there are no apparent biological or climatological factors that will limit the spread of GWSS or PD. GWSS has the potential to develop high population densities in citrus. Insecticide treatments in citrus groves preceded and followed by trapping and visual inspections to determine the effectiveness of these treatments are needed to manage this devastating insect vector and bacterium. A total of 106 acres of citrus in Riverside County were treated for the GWSS in February through September, 2008 between a cooperative agreement with USDA-APHIS and the Riverside Agricultural Commissioner's Office under the "Area-Wide Management of the Glassy-Winged Sharpshooter in the Coachella and Temecula Valleys".

### OBJECTIVES

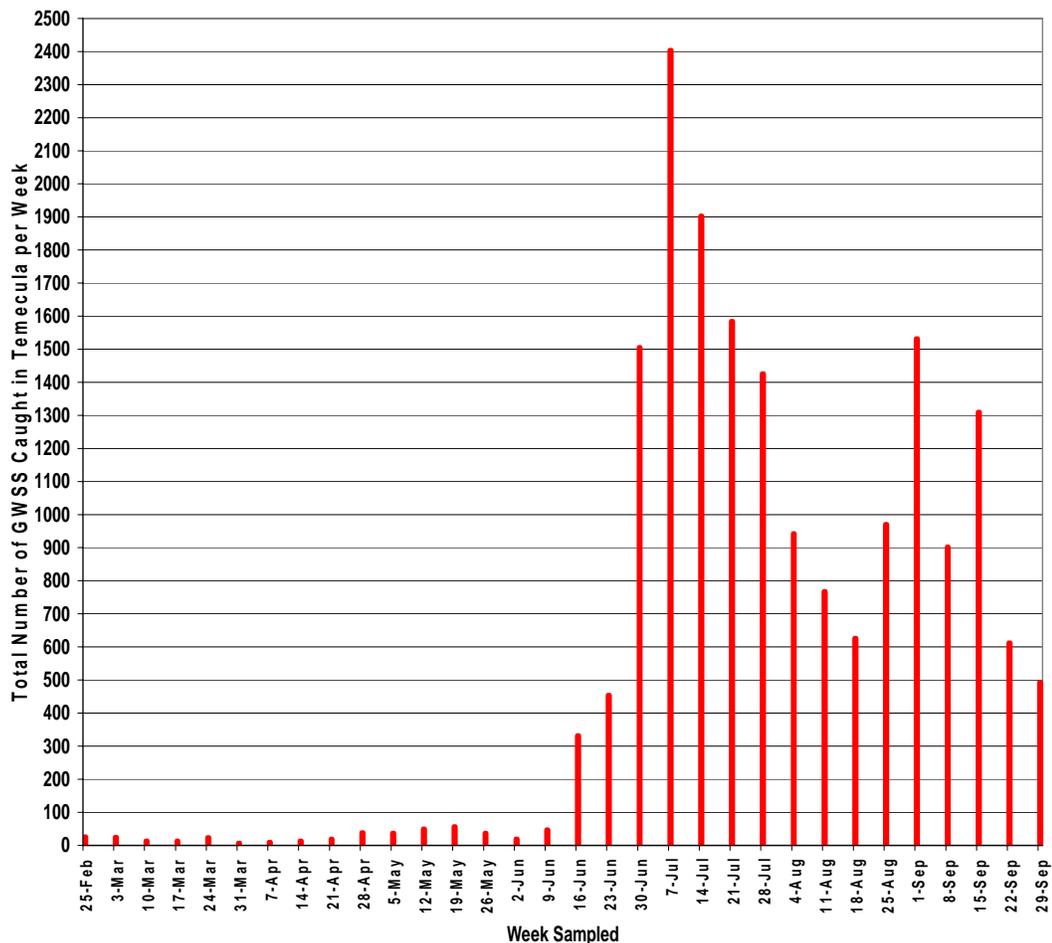
1. Delineate the areas to be targeted for follow-up treatments to suppress GWSS populations in the Temecula and Coachella Valleys for 2009.
2. Determine the impact of the GWSS area-wide treatments to suppress GWSS populations in citrus groves and adjacent vineyards.

## METHODS, RESULTS AND CONCLUSIONS

The programs in Coachella and Temecula were dependent upon grower, pest management consultants, citrus and vineyard manager's participation. The areas encompass approximately 28,000 acres. Representatives of various agencies were involved in the program, they were as follows: USDA-ARS, USDA-APHIS, CDFA, Riverside County Agricultural Commissioner, UC Riverside, UC Cooperative Extension, and grower consultants. Representatives of these agencies meet to review the program. Newsletters are sent to growers, managers, wineries, and agencies with information on GWSS populations and insecticide treatments via e-mail. The information from Temecula is sent weekly, while information from Coachella goes to the various parties monthly.

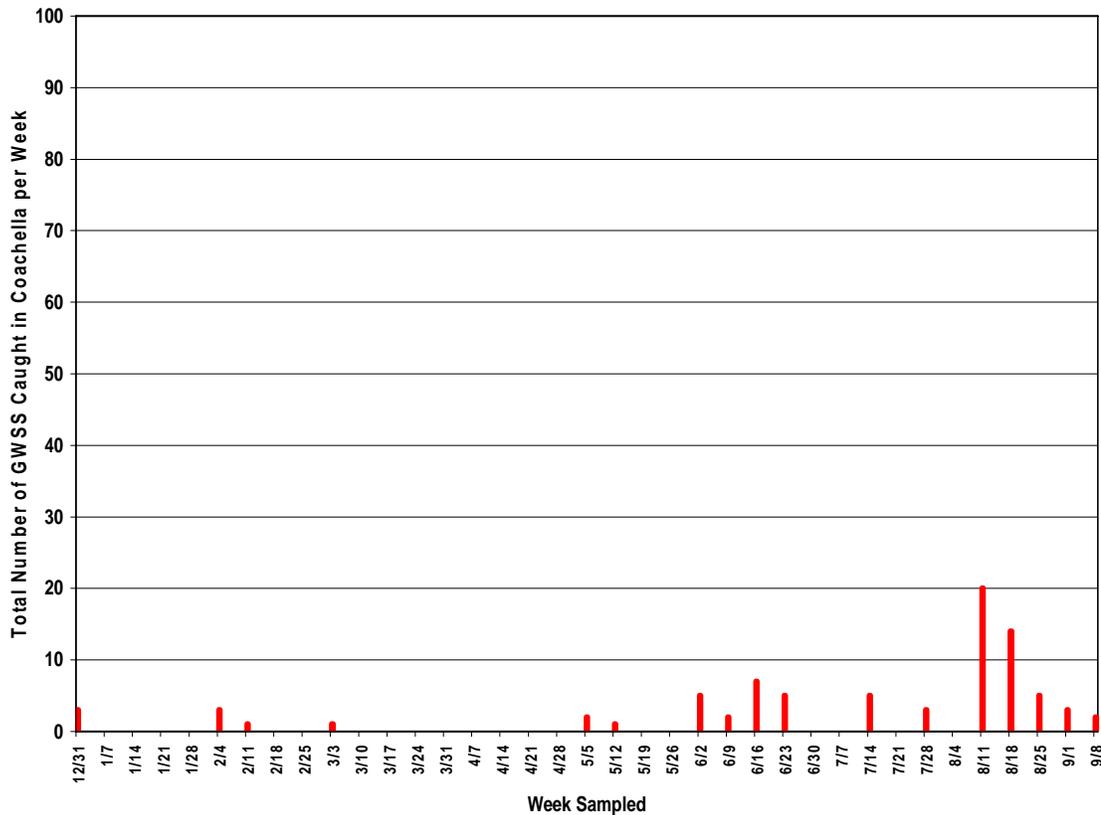
The GWSS/PD citrus groves and vineyards within the GWSS/PD management areas were monitored weekly to determine the need and effect of insecticide treatments on GWSS populations. In August, because of the lack of GWSS trap catches in Coachella Valley, a bi-weekly schedule was initiated. Yellow sticky traps (7 x 9 inches) were used to help determine GWSS population densities and dispersal/movement within groves and into vineyards (**Figures 1 & 2**). Approximately 1,400 GWSS yellow sticky traps are monitored weekly. Based on trap counts and visual inspection, only 106 acres of citrus were treated in Temecula valley for GWSS. No insecticide treatments were needed in Coachella Valley for the management of GWSS in 2008. In Temecula Valley, treatments for GWSS in citrus were initiated when at least 1-2 GWSS adults were found at the same trap location for two consecutive weeks. In Temecula Valley, only the citrus where the GWSS was found were treated. Because of various reasons, some citrus acreage in Temecula should have been treated that was not. These additional acres were not treated because of one of the following three reasons: uncooperative citrus growers, close proximity to homes and the late time of the season that the GWSS appeared. In Temecula Valley, 93 acres of the 106 citrus acres were treated with Admire Pro (imidacloprid) at the rate of 14 oz./acre. In June, 37 of the 93 acres were treated with Lorsban 4E (chlorpyrifos) at the rate of 7 pts./acre. Thirteen acres were treated with PyGanic 5.0 (1.4% Pyrethrins) at 18 oz./acre. PyGanic was used to treat organically grow citrus. On the 13 citrus acres where PyGanic was used to manage GWSS, a follow-up treatments of PyGanic was applied a month after the first application for two consecutive months.

Total Temecula GWSS Catch per Week for 2008



**Figure 1.** In 2008, high numbers of adult GWSS were caught on the yellow sticky traps in Temecula, with populations peaking in July reaching a total of approximately 2,400 trapped.

## Total Coachella GWSS Catch per Week for 2008



**Figure 2.** GWSS populations in Coachella Valley peaked in July with a high of 100 trapped.

### FUNDING AGENCIES

Funding for this project was provided by the USDA Animal and Plant Health Inspection Service, and the California Department of Food and Agriculture.

### ACKNOWLEDGMENTS

We would like to especially thank Ben Drake of Drake Enterprises for his input and counsel and the grape and citrus growers, managers and pest control advisors for their needed cooperation to make the Riverside County GWSS area-wide management program successful. We want to thank Heavenly Clegg for her development of the Temecula GWSS newsletter and Gevin Kenny for managing the Temecula GWSS monitoring and data analysis. We would especially want to thank CDFA's Rosie Yacoub for bar coding of the GWSS sticky traps, which resulted in simplifying our data input and mapping of GWSS populations in Temecula and Coachella Valleys.

# DEVELOPMENT OF A PEST MANAGEMENT PROGRAM FOR THE CONTROL OF LEAFHOPPER VECTORS OF *XYLELLA FASTIDIOSA* IN NORTH CAROLINA VINEYARDS

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**Reporting Period:** The results reported here are from work conducted May 2006 to August 2008.

## ABSTRACT

Three insecticide programs were evaluated in two vineyards in North Carolina from 2006 to 2008 for managing leafhopper vectors of *Xylella fastidiosa* (*Xf*), the bacterium that causes Pierce's disease in grapes. In 2006, the treatments consisted of applications of Assail®, Danitol®, Provado®, or Venom®, in different sequences and frequencies during the growing season. The same insecticides were used in 2007, except Provado®, whereas Admire-Pro®, Belay® and all insecticides used in 2007 were used in 2008. Most insecticides were applied as a foliar spray except Venom which was applied to the foliage and soil and Admire-Pro and Belay which were only applied to the soil. Soil applications were made along the base of the vines. Leafhoppers were monitored in each plot with yellow sticky traps which were changed every two weeks. *Graphocephala versuta* was the most abundant and ubiquitous leafhopper that was trapped in the test plots. Five other species of sharpshooters were identified: *Cuernia costalis*, *Paraulacizes irrorata*, *Oncometopia orbona*, *Homalodisca insolita*, and *H. vitripennis*. However, the latter was only detected in 2007 and 2008. All insecticide treatments significantly reduced the number of leafhoppers trapped compared with the control during the three seasons.

## INTRODUCTION

Several species of leafhopper vectors of *Xylella fastidiosa* (*Xf*), the causal agent of Pierce's disease (PD), are abundant in vinifera vineyards in North Carolina (Myers et al. 2007). PD severely affects *vinifera* grapes debilitating the plants, reducing the productivity life span of vines and increasing costs in vineyards (Purcell and Hopkins 1996). This disease is a limiting factor for the development of the vinifera industry in North Carolina and has been detected in many of the grape growing regions in the eastern two-thirds of the state (Anas et al. 2008). In this paper we report preliminary results of insecticide tests for managing leafhoppers conducted between 2006 and 2008. These tests were conducted in two vineyards located in Wake and Alamance counties. Insecticide classes and rates used are presented in **Table 1**. In 2006, the treatments consisted of single applications of Assail®, Danitol®, Provado®, or Venom®, in different sequences and frequencies during the growing season (**Table 2a**). All insecticides used in 2006 were used in 2007, except Provado® (**Table 2b**), whereas Admire-Pro®, Belay® and all insecticides used in 2007 were used in 2008 (**Table 2c**). Venom was applied to the foliage and soil, whereas Admire-Pro, and Belay were only used as soil applications, and the remainder of insecticides were applied to the foliage. Leafhoppers were monitored with yellow sticky traps placed in the middle row of each plot and replaced every two weeks. All vines in the middle rows of each plot were tested for the presence of *Xf* using an ELISA test kit (Agdia Inc., Elkhart, IN).

## OBJECTIVES

1. To evaluate the effectiveness of insecticide programs on populations of leafhoppers in vineyards of North Carolina.
2. Develop an IPM program for leafhoppers for vinifera vineyards in North Carolina.

## RESULTS AND DISCUSSION

During peak population periods, and throughout most of the growing season, fewer leafhoppers were found in the insecticide treatments than the control in 2006, 2007, and 2008, (**Figures 1 and 2**). *Graphocephala versuta* was the most abundant and ubiquitous leafhopper trapped during the three growing seasons. Significantly more *G. versuta* ( $P < 0.05$ ) were found in the control compared to all the insecticide treatments in 2006, 2007, and 2008 (**Figures 1a, 1b, 1c, and 3a**). A similar trend was observed for all sharpshooters though there was no significant difference among treatments in 2008 (**Figures 2a, 2b, 2c, and 3b**). Five species of sharpshooters were found in the traps: *Cuernia costalis*, *Homalodisca insolita*, *H. vitripennis*, *Oncometopia orbona*, and *Paraulacizes irrorata*. The most abundant were *H. insolita* and *O. orbona*. The glassy-winged sharpshooter *H. vitripennis* was only found in 2007 and 2008 in the Wake Co. vineyard. The peak population of *O. orbona* occurred from mid-June to the first week of July whereas, *H. vitripennis* peaked between mid-July and mid-August. Peak numbers of *G. versuta* were delayed by ~3 weeks in 2007 compared with 2006 (**Figures 1a and 1b**) which may be due to a hard freeze from 6 to 10 April 2007. Similarly, a severe late freeze between 13 and 16 April 2008 may have decreased leafhopper numbers in 2008 compared to the two previous years (**Figures 1, 2 and 3**). These two freeze events may also have affected the survival of *Xf* because the incidence PD in the Alamance Co. vineyard was reduced in 2007 and 2008 (**Table 3**). The Wake Co. grower removed the vines in August 2008 and no PD evaluation was possible. There were no significant treatment effects on PD incidence as measured by ELISA.

**Table 1.** Insecticide classes and rates.

Type	Insecticide (Trade name)	Rate/Ha
Neonicotinoid	acetamiprid (Assail 30 SG )	77.0 g
“	imidacloprid (Provado 1.6 F*)	274.1 ml
“	(Admire Pro <sup>▲◆</sup> )	1024.8 ml
“	dinotefuran (Venom insecticide)	210.0 g
	(Venom insecticide◆)	420.1 g
	clothianidin (Belay <sup>▲◆</sup> )	878.4 ml
Pyrethroid	fenpropathrin (Danitol 2.4 EC)	1460.5 ml

(\*) Provado was used only in 2006 and (▲) Admire Pro and Belay were used only in 2008. (◆) Indicates soil application. In all other cases insecticides were applied to the foliage, unless indicated.

**Table 2.** Insecticide application schedule for (a) 2006, (b) 2007, and (c) 2008.**(a) 2006**

Treat.	15-Apr	3-May	17-May	1-Jun	15-Jun
T1	Danitol	Assail	Danitol	Assail	Venom
T2	Danitol	Provado	Danitol	Provado	Venom
T3	Venom◆	-	Venom	Danitol	Assail
Control	-	-	-	-	-

**(b) 2007**

Treat.	27-Mar	12-Apr	26-Apr	9-May	23-May	5-Jun	19-Jun	3-Jul	17-Jul
T1	Venom◆	-	Venom	Danitol	Assail	Danitol	Assail	-	-
T2	-	Venom◆	-	Venom	Danitol	Assail	Danitol	Assail	-
T3	-	Venom◆	-	Venom	Danitol	-	Assail	Danitol	Assail
Control	-	-	-	-	-	-	-	-	-

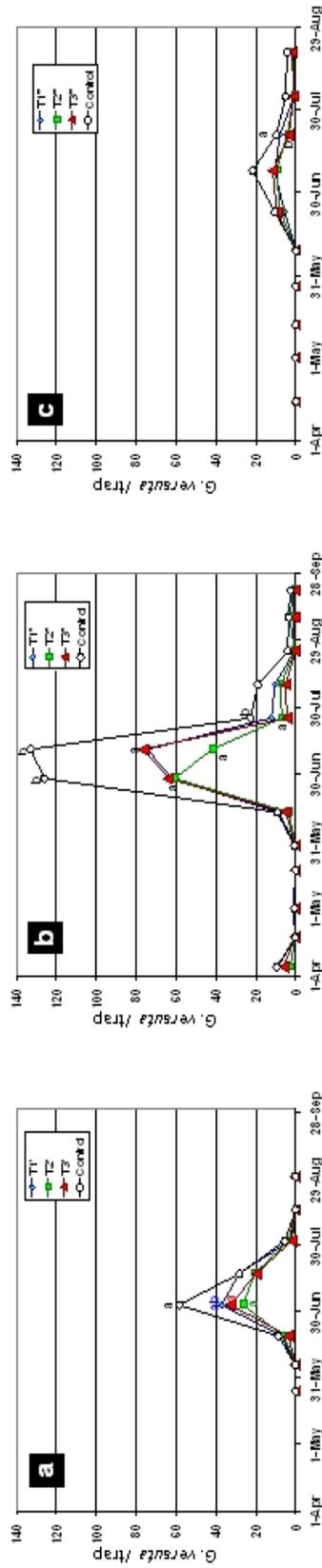
**(c) 2008**

Treat.	2-Apr	15-Apr	29-Apr	12-May	27-May	10-Jun	24-Jun	8-Jul	22-Jul
T1	Venom◆	-	Venom	Danitol	Assail	Danitol	Assail	-	-
T2	-	Venom◆	-	Venom	Danitol	Assail	Danitol	Assail	-
T3	-	Venom◆	-	AdmirePro◆		Belay◆	-	Danitol	Danitol
Control	-	-	-	-	-	-	-	-	-

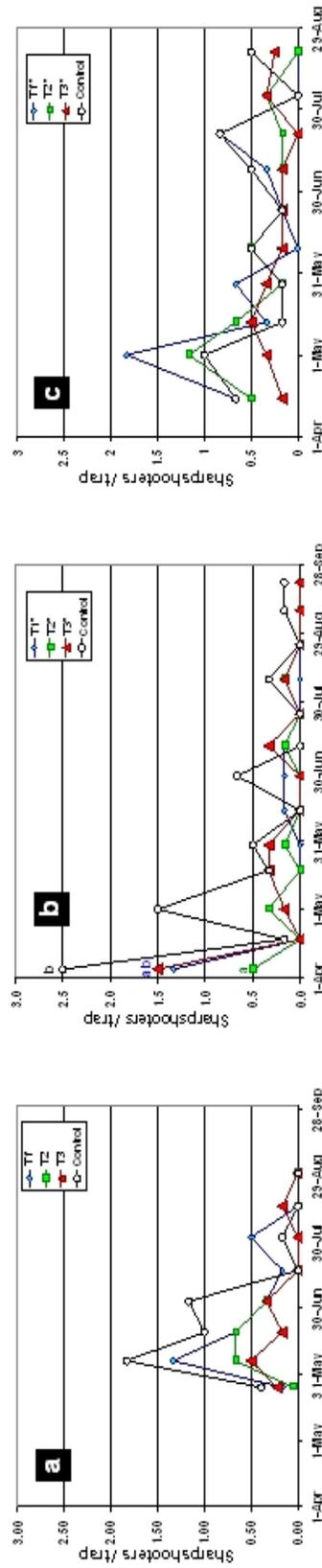
**Table 3.** Percentages of PD in middle row plants (Wake: n=96, Alamance: n=142). The grower removed all plants in the Wake Co. vineyard in 2008. The Alamance Co. grower replaced vines killed by PD in the spring of 2008. If these vines are considered, the percentage vines affected would be 13.1% for 2008.

Year	County Location	
	Wake*	Alamance
2006	24.5%	73.1%
2007	72.2%	11.8%
2008*	n/a	4.2%

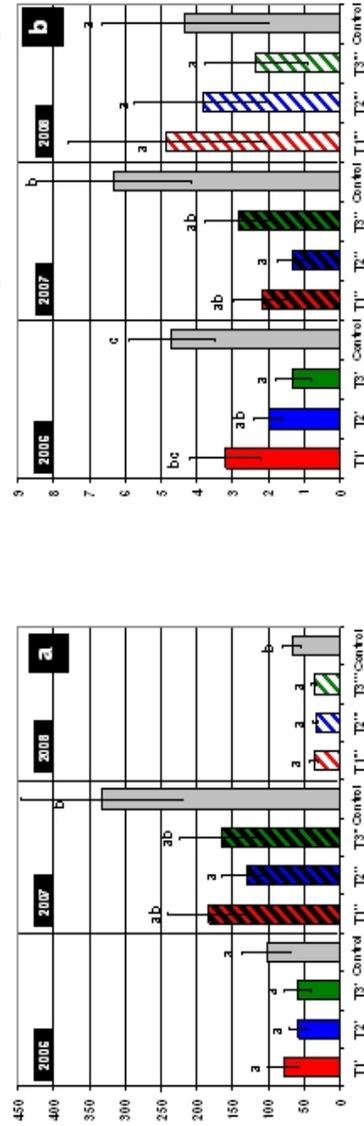
**Figure 1. Effect of insecticide treatments on *G. versuta* populations: (a) 2006, & (b) 2007. Treatments with same designation differed between years. See Table 2.**



**Figure 2. Mean No. of sharpshooters/trap in insecticide treated and control plots in (a) 2006, and (b) 2007. See Table 2**



**Figure 3. Mean total ( $\pm$ SEM) (a) *G. versuta* and (b) sharpshooters per 8 traps in 2006 (15-May to 30-Aug.), per 13 traps in 2007 (22-Mar to 20-Sep.), and per 10 traps in 2008 (Mean separation within year;  $P \leq 0.05$ . See Table 2).**



## CONCLUSIONS

All the insecticide management programs we studied reduced the numbers of total sharpshooters and *G. versuta* in the treated plots compared with the control. In addition, in most cases, the insecticide treatments also reduced the numbers of other leafhopper species (data not shown) during the three-year study. *Graphocephala coccinea*, *Ponana puncticollis*, *Paraphlepsius irroratus*, *Texananus scultus*, *Norvellina seminuda*, *Gyponana*, *Draecucephala* sp., *Scaphoideus titanus*, *Scaphytopius*, and *Agalliota* sp. were some of the species collected in this study and may be potential vectors *Xf*. Some of these species had been reported as carriers of *Xf* (Purcell 1979, Myers et al. 2007) but have not been shown to transmit *Xf*. Based on our tests, North Carolina vinifera grape growers have several options that will suppress populations of leafhoppers. However, the program still needs to be refined to be more cost effective. Additionally, the effect of these programs on reducing vines infected with *Xf* needs further evaluation. Although we did not see any differences in the incidence of PD between treated and non-treated vines in our small test plots, we have some evidence from a vineyard trial that insecticide applications will reduce the incidence of PD. In 2008, we initiated a test in a newly planted vineyard to assess the effectiveness of one of our programs (T3, 2008) compared to the grower's program. When our program was used in a vineyard planted in 2008 (n=592), PD symptoms were observed in only 4.4% of vines in late September whereas in an adjacent vineyard with replanted vines (n=74) where our program was not used, 44 plants (59.4%) had PD symptoms. Only one vine in each plot tested positive for *Xf* using ELISA. Visible symptoms (marginal leaf scorch) occurred late in the season consequently the titer of *Xf* may have been too low to detect using ELISA. This test will be continued to evaluate the long-term effectiveness of the program.

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## FUNDING AGENCIES

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## ACKNOWLEDGMENTS

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***Section 3:  
Pathogen Biology  
and  
Ecology***





## BIOLOGY OF THE *XYLELLA FASTIDIOSA*-VECTOR INTERFACE

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**Reporting Period:** The results reported here are from work conducted March 2008 to September 2008.

### ABSTRACT

The interactions between the economically important plant pathogenic bacterium *Xylella fastidiosa* (*Xf*) and its leafhopper vectors have been poorly characterized. We used different approaches to determine how *Xf* cells interact with the cuticular surface of the foregut of vectors. We demonstrate that *Xf* binds to different polysaccharides with variable affinities, and that these interactions are mediated by cell surface carbohydrate-binding proteins. In addition, competition assays showed that N-acetylglucosamine inhibited bacterial adhesion to vector foregut extracts and intact wings, demonstrating that attachment to leafhopper surfaces can be affected in the presence of specific polysaccharides. In vitro experiments with several *Xf* knockout mutants indicated that hemagglutinin-like proteins were associated with cell adhesion to polysaccharides. These results were confirmed with biological experiments, when hemagglutinin-like proteins mutants were transmitted to plants at lower rates when compared to the wild type. Furthermore, although these mutants were defective in adhesion to the cuticle of vectors, their growth rate once attached to leafhoppers was similar to the wild type, suggesting that these proteins are important for *Xf* initial adhesion to leafhoppers. We propose that *Xf* colonization of leafhopper vectors is a complex, stepwise process, similar to the formation of biofilms on surfaces. Results presented here and in the 2007 report have been combined and submitted to publication.

### INTRODUCTION

The interaction of *Xylella fastidiosa* (*Xf*) with the foregut cuticle differ from other xylem-limited bacteria such as *Leifsonia xyli* which can be acquired from plants but are not transmitted by insects (Barbehenn and Purcell 1993). Only two studies with *Xf* knockout mutants have addressed aspects of vector transmission (Chatterjee et al. 2008, Newman et al. 2004). However, both studies focused on *Xf*'s cell-cell signaling system, which regulates cascades of genes and pathways, thus allowing the identification of target genes, but not identifying specific interactions between vector and pathogen. The *rpfF* gene (Regulation of Pathogenicity Factors *F*) encodes an enzyme that synthesizes the signaling molecule DSF (diffusible signaling factor), whereas *rpfC* is part of a hybrid two-component DSF sensor (Chatterjee et al. 2008). An *rpfF*- mutant is not transmissible by insects because it does not colonize the foregut of vectors (Newman et al. 2004), while *rpfC*- colonizes insect's foregut but is transmitted at lower rates compared to the wild type (Chatterjee et al. 2008). In vitro adhesion assays indicated that *rpfF*- did not form biofilms, while *rpfC*- adhered to surfaces more strongly than did the wild type. Targeted gene expression analyses of *Xf* adhesins indicated that hemagglutinin-like proteins (afimbrial adhesins) and type I pili were associated with adhesion and transmission of these knockout strains, but type IV pili were not (Killiny and Almeida submitted). Thus, indirect evidence allowed us to hypothesize that some adhesins are important for *Xf* attachment to and colonization of vectors, and subsequent inoculation into susceptible hosts, while other adhesins are putatively of little or no role in this process. In this study we sought to determine the nature of *Xf*-vector interactions using biochemical, molecular and biological assays.

### OBJECTIVES

1. Determine the nature of the *Xf*-vector interactions.
2. Identify *Xf* surface proteins involved in the transmission process.
3. Develop an artificial diet system to study *Xf* transmission.
4. Identify molecules that disrupt *Xf* adhesion to vectors.

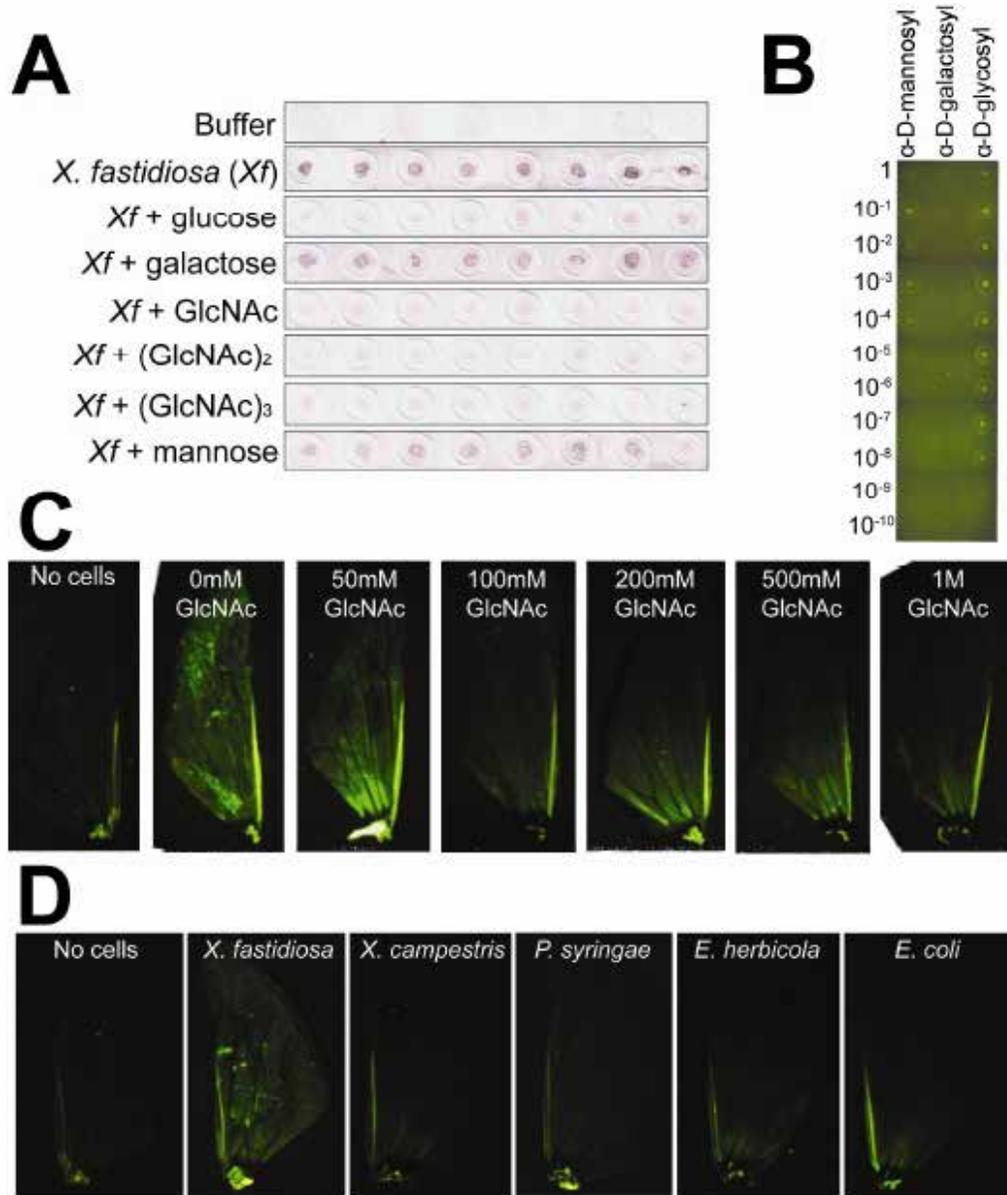
Here we report on the first two objectives mentioned above.

### RESULTS AND DISCUSSION

#### ***N*-acetylglucosamine blocks *Xf* adhesion to insect surfaces.**

To determine the affinity of *Xf* cells to different sugars we used a competition assay based on the concept that polysaccharide-binding proteins on the surface of *Xf* can be saturated by exogenous molecules, reducing overall cell attachment to leafhopper foregut extracts. D(+)-galactose did not interfere with the binding of *Xf* to foregut extracts while D(+)-mannose had a small effect (**Figure 1A**). However the monomeric moiety of chitin, *N*-acetylglucosamine, as well as its dimmer chitobiose and its trimer chitotriose, as well as its core molecule glucose, blocked cell adhesion to leafhopper foregut extracts (**Figure 1A**). Thus, specific carbohydrates inhibit *Xf* adhesion to extracts from leafhopper vectors. The affinity of *Xf* carbohydrate-binding proteins to sugars was also tested using synthetic copolymers as described by (Chadli et al. 1992). Our goal was to eliminate potential sources of error on the competition assays, as the previous experiment was performed using

leafhopper extracts mimicking *in vivo* conditions that could have other factors affecting the tests. We also used GFP (green fluorescent protein)-labeled *Xf* (Newman et al. 2003) to limit sample processing. We determined that cells specifically bound to the glucosyl ligand “poly (O- $\alpha$ -D-glucopyranosylacrylamide) copolymer”. A negligible interaction was obtained with the galactosyl ligand, while binding of *Xf* to the mannosylated copolymer was detected half way through the dilution series used (**Figure 1B**).



**Figure 1.** Carbohydrate-mediated inhibition of *Xf* cell attachment to surfaces. A) Carbohydrate inhibition of *Xf* attachment to leafhopper foregut extracts spotted on nitrocellulose membrane, indicating that cell surface adhesins can be saturated if incubated with certain molecules (GlcNAc - *N*-acetylglucosamine). B) Adhesion of GFP-labeled *Xf* to carbohydrate-acrylamide copolymers (O-glycosylacrylamides) dilution series. C) Dilution series of *N*-acetylglucosamine inhibition of GFP-labeled *Xf* attachment to leafhopper hindwings. D) Specific adhesion of *Xf* to insect hindwings compared to other plant pathogenic bacteria and *Escherichia coli*.

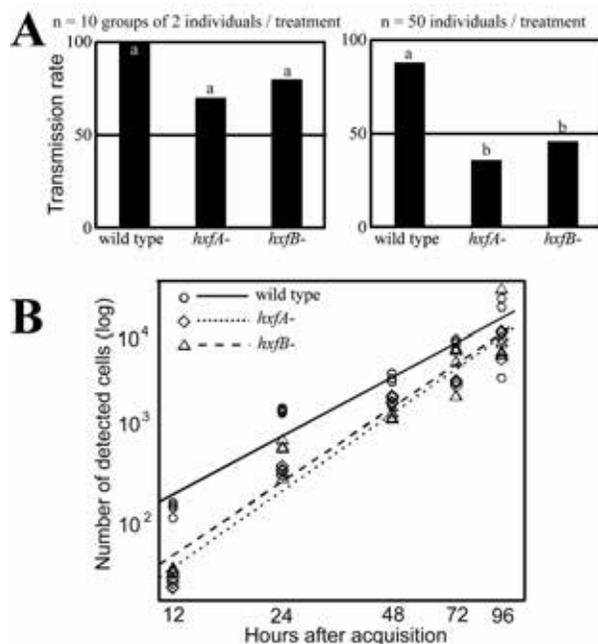
In order to compare our *in vitro* observations to *in vivo* cell adhesion to leafhoppers we used the hindwings of insect vectors to mimic the cuticular surface of the foregut canal that *Xf* colonizes. The entire exoskeleton of insects is generally assumed to have similar chemical composition, although details are lacking for this specific system. We used *N*-acetylglucosamine as competitor molecule in assays testing for GFP-labeled *Xf* cell attachment to hindwings. Attachment diminished as *N*-acetylglucosamine concentration increased in the dilution series (**Figure 1C**). These results indicate that *Xf* binding to polysaccharides *in vitro* is similar in its characteristics to its binding to the cuticle of leafhoppers. Lastly, in order to test the specificity of bacterial adhesion to leafhopper hindwings, we tested if other GFP-labeled bacteria, including the plant pathogens *Pseudomonas syringae*, *Xanthomonas campestris*, and *Erwinia herbicola*, and *Escherichia coli* attached to that surface (**Figure 1D**). Interestingly, only *Xf* cells attached to the wings. Thus, *Xf* cells have surface proteins with affinity to

polysaccharides on the surface of insects wings and glucosylated molecules, which can be saturated by *N*-acetylglucosamine and similar molecules.

### Transmission of *hxfA*- and *hxfB*- mutants.

In previous reports, we presented biochemical results indicating that the hemagglutinin-like proteins (HxfA and HxfB) were associated to cell adhesion to insect surfaces and polysaccharides *in vitro*. Thus, we conducted two experiments to determine the role of *hxfA* and *hxfB* in *Xf* transmission by sharpshooters to plants. In the first experiment, we confined non-infected *G. atropunctata* on plants mechanically inoculated with the wild type, *hxfA*- and *hxfB*- cells, after which groups of two individuals were moved to healthy plants for four days as an inoculation access period. Transmission occurred in all treatments, with *hxfA*- and *hxfB*- being transmitted less than the wild type (70, 80 and 100%, respectively), albeit not with any statistical difference ( $X^2$  test,  $P = 0.1864$ ). In a second experiment we used individuals instead of pairs to more precisely estimate single insect transmission efficiency. With this more discriminating approach we found that *hxfA*- and *hxfB*- mutants were transmitted at lower rates than the wild type (36, 46 and 88%, respectively  $X^2$  test,  $df = 1$ ,  $P < 0.001$ ). Because *Xf* transmission rates are correlated with bacterial population in plants, we quantified the infection level in plants used in these tests. Plants infected with *hxfA*- and *hxfB*- mutants used for the transmission tests had populations ~10-fold higher than the wild type (data not shown, results similar to Guilhabert and Kirkpatrick 2005), suggesting that *hxfA*- and *hxfB*- mutants were transmitted less than the wild type because of their impaired interactions with insects rather than because of lower populations in source plants.

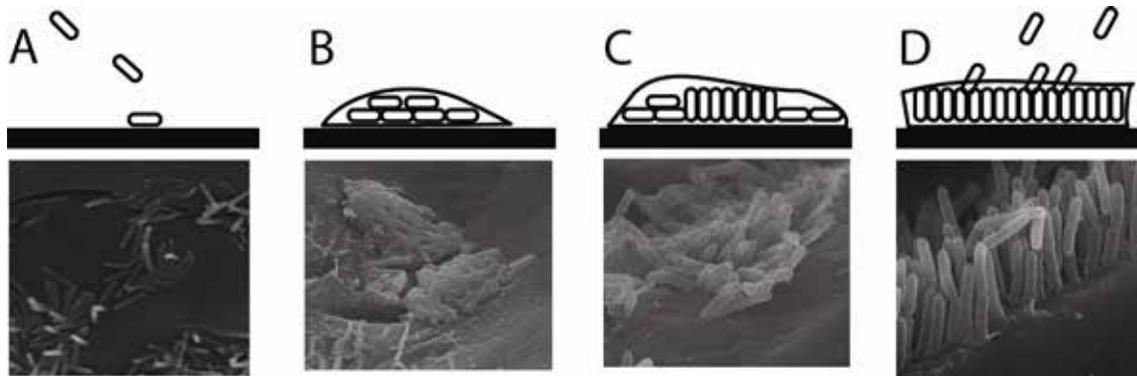
We hypothesized that the reduced transmission rate of *hxfA*- and *hxfB*- mutants was due to limited colonization of vectors early in biofilm formation. In order to test this hypothesis we conducted another experiment and quantified the number of *Xf* cells in the head of vectors over time after a 12-hour pathogen acquisition access period. Overall, 80% of insects that fed on grapevines infected with the wild type were positive for *Xf*, whereas only 38% and 42% of those fed on *hxfA*- and *hxfB*- mutants were infected, respectively ( $X^2$  test,  $df = 2$ ,  $P < 0.001$ ). We quantified the number of cells of these strains within vectors (positive samples only). There were significant effects of strain ( $F_{2,54}=23.229$ ,  $P < 0.0001$ ), time ( $F_{1,54}=803.341$ ,  $P < 0.0001$ ), and an strain by time interaction ( $F_{2,54}=5.362$ ,  $P=0.0075$ ). Populations of the two mutants soon after leafhopper access to infected plants were similar to each other but statistically different from the wild-type (**Figure 2B**). Twelve hours after acquisition we found insects fed on the wild type averaged 415 detectable cells, whereas average of 96 and 120 cells were detected in leafhoppers fed on *hxfA*- and *hxfB*- plants respectively. However, after 96 hours the bacterial populations of all three strains were similar to each other (**Figure 2B**). It was interesting to find that the slopes for the 2 mutants were similar (**Figure 2B**), suggesting that *hxfA* and *hxfB* may have redundant roles in relation to vector transmission and that, importantly, the knockouts were impaired in early attachment to insects, but after they attached, their patterns of foregut colonization (i.e. population growth) were similar to the wild type (slope of regressions). Testing of a *hxfA*-/*hxfB*- double mutant is necessary to determine if these proteins have redundant roles on cell attachment to vectors as our data suggest. However until recently, there were no protocols available for complementation studies with *Xf*, or to generate double mutants, prohibiting this test here (Reddy et al. 2007).



**Figure 2. A)** Transmission of *X. fastidiosa* by leafhopper vectors. Both experiments show that *hxfA*- and *hxfB*- were transmitted less often than the wild type, but results from larger experiment using individuals instead of groups were statistically significant. Different letters on bars indicate statistically difference ( $P < 0.05$ ). **B)** Bacterial populations within leafhopper vectors over time after a 12-hour pathogen acquisition access period. Wild type (solid regression line), *hxfA*- (dotted regression line) and *hxfB*- (dashed regression lines). Note values immediately after acquisition (12-hour period) and 4 days afterwards. Fewer *hxfA*- and *hxfB*- cells adhered to vectors, but after a few days populations were of equal size.

## CONCLUSIONS

We propose that *Xf* colonization in vectors is similar to the formation of biofilms on surfaces. Scanning electron microscopy observations we have made support this hypothesis (Almeida and Purcell 2006). We hypothesize that cells initially adhere laterally to the foregut cuticle via carbohydrate-binding proteins, such as HxfA and HxfB (**Figure below A and B**). As these proteins are assumed to occur throughout cells, adhering laterally increases the cell surface area in contact with the substrate and streamline the bacteria to the flow of xylem sap ingested by the insect vector. After initial adhesion, cells may produce large quantities of EPS that can result in the concentration of resources and DSF in microcolonies. As the colony size increases, cells at the center of the biofilm became polarly attached (step C, below), potentially through polar short type I pili, increasing surface area for nutrient absorption. Lastly, a typical mature *Xf* biofilm within vectors is formed, with all cells polarly attached (step D). At this stage, newly divided cells are not anchored on the cuticle of insects and may be occasionally detached from vectors and inoculated into plants. This hypothesis may be useful to guide future studies on this system by providing testable questions, as up until now no data on these interactions, with the exception of microscopy observations, were available.



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## ACKNOWLEDGMENTS

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## EVOLUTION OF *XYLELLA FASTIDIOSA* AVIRULENCE

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### ABSTRACT

The goals of this objective are to quantitatively and qualitatively determine how and when *Xylella fastidiosa* (*Xf*) loses pathogenicity and potentially vector transmissibility, after serial passages in vitro. We replicated *Xf* in vitro for one year (80 passages on solid rich medium), creating parallel populations that have phenotypes in vitro that differ from the ancestral isolate. We are now studying host plant colonization and insect transmission for selected populations (several passages were frozen at -80°C for 10 lineages). Once phenotypes of interest are identified (reduced pathogenicity or transmissibility, results pending), we will compare these *Xf* populations with the original isolate and search for other phenotypic and molecular differences. We will also be able to quantify the rate of genetic change in these populations, providing a molecular calibration data for researchers interested in *Xf* evolution, diversity and ecology.

### INTRODUCTION

Hopkins (2005) demonstrated the potential of avirulent *Xylella fastidiosa* (*Xf*) as a tool to control Pierce's disease (PD). He also illustrated the challenges of such an approach. For example, not all weakly virulent or avirulent isolates resulted in similar degree of control, and in most cases plants eventually become symptomatic. Understanding the biology of avirulent isolates and by which mechanisms they may reduce disease symptoms is of importance if this approach is to be widely adopted. This project tackles those questions by comparing evolved avirulent isolates with a parent pathogenic isolate. Being able to retrospectively compare these isolates using high resolution tools and biological assays will allow us to determine when, and how, *Xf* loses avirulence. We will also shed light on the mechanism how avirulent isolates suppress pathogenic ones. This proposal seeks to understand the evolution of avirulent *Xf* through serial passages in vitro. Such isolates have recently been shown to have potential to biologically control PD. We are studying how avirulent isolates evolve, biologically and genetically, and will test their potential as control agents of pathogenic *Xf*.

We have different *Xf* populations in the laboratory maintained under a selection protocol to obtain lineages that are avirulent in plants. This process is finished and now we are characterizing the phenotype of four out of 10 lineages we have created. We are comparing the evolved populations with the original one to determine if they have different phenotypes.

### OBJECTIVES

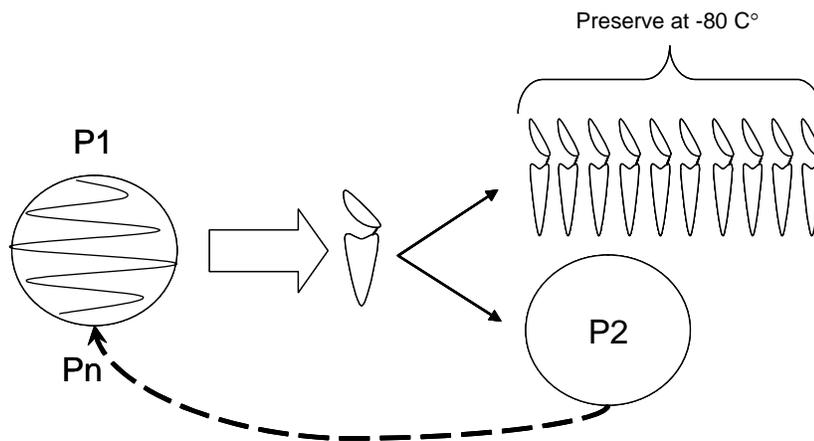
Original objectives in the proposal submitted in 2007 were:

1. Generation of in vitro evolved populations.
2. Phenotypical characterization of populations.
3. Molecular characterization of populations.
4. To test avirulent populations as biological control agents.

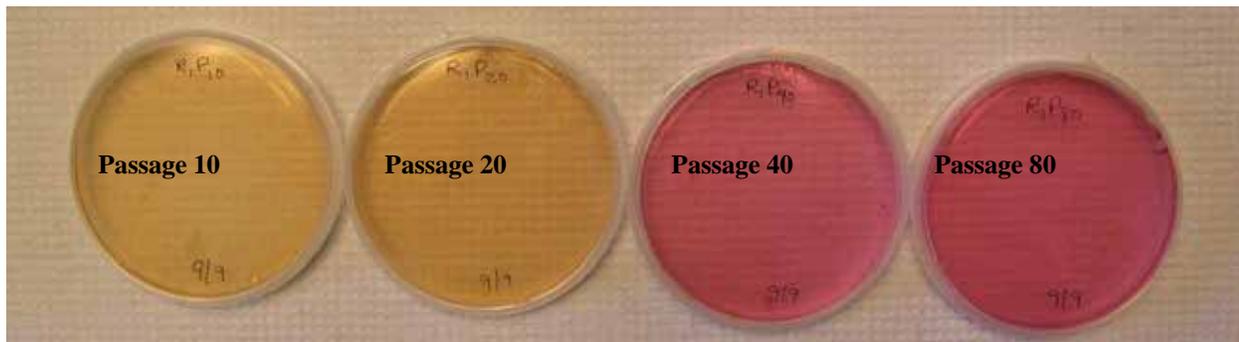
Here we report on the first two objectives mentioned above. This project was funded for one year.

### RESULTS AND DISCUSSION

We have started to work on Objectives 2-4. Objective 1 has been finished. For every 10 passages of the populations in rich medium (PWG), we stored a sample in a -80°C freezer. We have recovered some of those for phenotypical and molecular characterizations. We have a total of 80 passages in this experiment, totaling eight frozen populations per lineage. We are using four randomly selected lineages and passages 0, 10, 20, 40 and 80 for our characterization studies. The general protocol is illustrated in the **Figure** below:



Although we do not have final data on the phenotype of these populations, we have noticed that on solid medium they are growing approximately twice as fast as the original population from which they derived, suggesting fast adaptation to new environmental conditions under selective pressure. One indirect measurement of growth on PWG medium is a change of pH to basic conditions, indicated by a pink color. Below, populations from selected passages were plated at the same time on PWG, illustrating the change in pH for later passages (40 and 80) that have been subject to the selection process longer than passages 10 and 20. Other factors may be increasing the pH on these plates, although we have not started to investigate this process at this point. To measure the growth rate of different passages we have tested different liquid medium-based approaches. However, we noted that cell attachment to surfaces and clumping varies significantly among these populations. Therefore, we are testing alternative protocols to determine the growth rate of representative populations. Ongoing experiments for phenotypic characterization of lineages include: growth rate, adhesion, biofilm formation, gum and protein production.



We have also inoculated these lineages/passages into plants (final results pending). Passages 0, 10, 20, 40 and 80 40d 80 from four parallel lineages were inoculated into almond plants. We will determine movement and multiplication of these populations by culturing from samples two and four months after inoculation (15 cm above inoculation site). From our two-month samples, we have determined that early passages were recovered in higher frequency than later passages. The proportion of infected plants for each passage (different lineages combined) 2 months after inoculation were: passage (P) 0 - 25%, P10 - 9.4%, P20 - 12.5%, P40- 0%, P80- 3%. We interpret these preliminary results as a gradual loss of pathogenicity by evolved populations, here interpreted as reduced movement and multiplication compared to ancestral population. We note that additional sampling will be performed on this experiment and we will repeat these assays next Spring.

Because we have noticed dramatic changes in growth rate of the lineages on rich solid media, we are also looking into potential protocols for identifying mutations in these clones if some of them are not pathogenic to plants. This would possibly identify spontaneous mutations and new pathogenicity factors in *Xf*, which could be used as targets for disease control. Lastly, we are conducting a multilocus sequence typing study to determine how fast these loci vary over time and to confirm the identity of the isolate we started our experiments with.

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**ACKNOWLEDGMENTS**

We thank Nabil Killiny and Mate Lopes for technical assistance.

## GENETIC DIVERSITY ANALYSIS OF *XYLELLA FASTIDIOSA* STRAINS USING MULTIPLE *TONB* GENES AND THE *ZOT* GENE

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

### ABSTRACT

Multiple subspecies of *Xylella fastidiosa* (*Xf*) exist which are differentially pathogenic. Previously, DNA sequence analysis of the *mopB* and *gyrB* gene has been used to separate *Xf* strains into their subspecies groups. The *TonB* gene family can be used to confirm this genetic diversity between *Xf* strains and regions within these genes can be used to separate strains beyond subspecies due to increased variability. *TonB* protein is a cytoplasmic outer membrane protein that can be found on gram negative bacteria, such as *Xf*. The protein functions as an energy transducer to support a variety of transport events across the outer membrane and interacts with outer membrane receptor proteins which carry out high-affinity binding and energy-dependant uptake of specific substrates into periplasmic space. In this study, five different *TonB* genes (*TonB*-a through *TonB*-e) were used to differentiate between three *Xf* strains (grape, ragweed, and oleander). The results of this study were consistent with genotype differentiation using conserved *mopB* and *gyrB* genes. Additionally, sequencing of another gene, analogous to the zonula occludens toxin (ZOT) gene, was used to separate groups below the strain level. The discovery of new variable genes provides another genomic location to be exploited for the improvement of diagnostics to aid in the management of Pierce's disease.

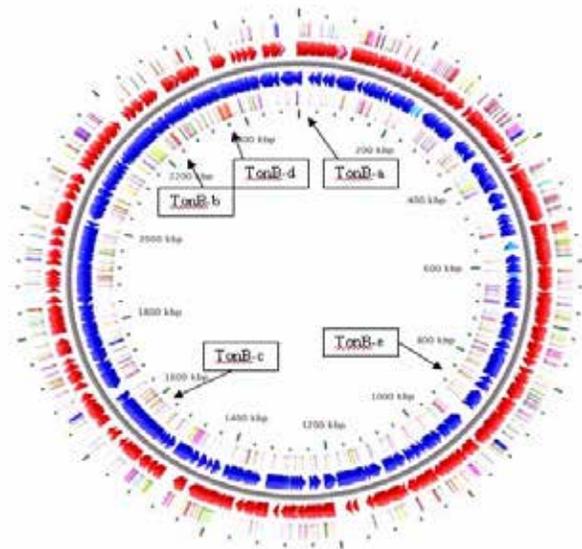
### INTRODUCTION

*Xylella fastidiosa* (*Xf*) is a fastidious, xylem-limited, Gram negative bacterium that resides in the xylem tissue of many plants. This bacterium, which contains multiple subspecies can disease in multiple plants taxa, such as Pierce's disease (PD) on grapevine, oleander leaf scorch (OLS), and citrus leaf chlorosis (CVC) (Schaad 2004). One unique structure to Gram-negative bacteria is the outer membrane. This membrane has distinctive permeability process and possesses active transport system called *TonB*-dependent transport system. This system has high affinity and specificity for binding and transporting scarce nutrient across the outer membrane (Cadieux and Kadner 2003). *TonB* is an outer membrane protein that localized in the cytoplasmic membrane by its uncleaved amino-terminal signal sequence, with the bulk of the protein extending into the periplasmic membrane (Skare and Postle 1991). The function of this protein is as an energy transducer to couple cytoplasmic protonmotive force to active transport of nutrients and metabolic products across the outer membrane. *TonB*-dependent transport system consists of high affinity membrane receptor, a periplasmic binding protein, and a cytoplasmic membrane transporter homologous to other traffic ATPases (Skare 1993). *TonB* genes, which encodes for *TonB*, can be used to differentiate different *Xf* strains due to multiple single base pair alterations. A search of the *Xf* genome determined that multiple *TonB* genes were present in the genome.

The zonula occludens toxin has been suggested as a new potential virulence factor in the CVC system (da Silva 2004). This protein is similar to one found in *Vibrio cholerae* which has been linked to disruption of tight junctions (Johnson 1993). DNA sequence variation in this gene may be useful in separating strains from one another and potentially separating populations with in a strain group.

### MATERIALS AND METHODS

Extracted DNA from grape and ragweed strains of *Xf* were received from Lisa Morano. DNA from oleander strain was cultured Hercules, CA), 6.4- $\mu$ l auctoclaved nanopure water, 0.8  $\mu$ l forward and reverse primers, and 2- $\mu$ l of DNA template. PCR was conducted using initial denaturing step of 3 min at 94°C, the reaction was cycled 35 times under the following parameters: 94°C for 60 s, 65°C for 90 sec, 72°C for 150 s and followed with another extension at 72°C for 7 min. A non-template control (NTC) was also run with each assay as a negative control. The presence of the desired amplicon was determined by agarose DNA gel electrophoresis that was run at 75V for 60 minutes.



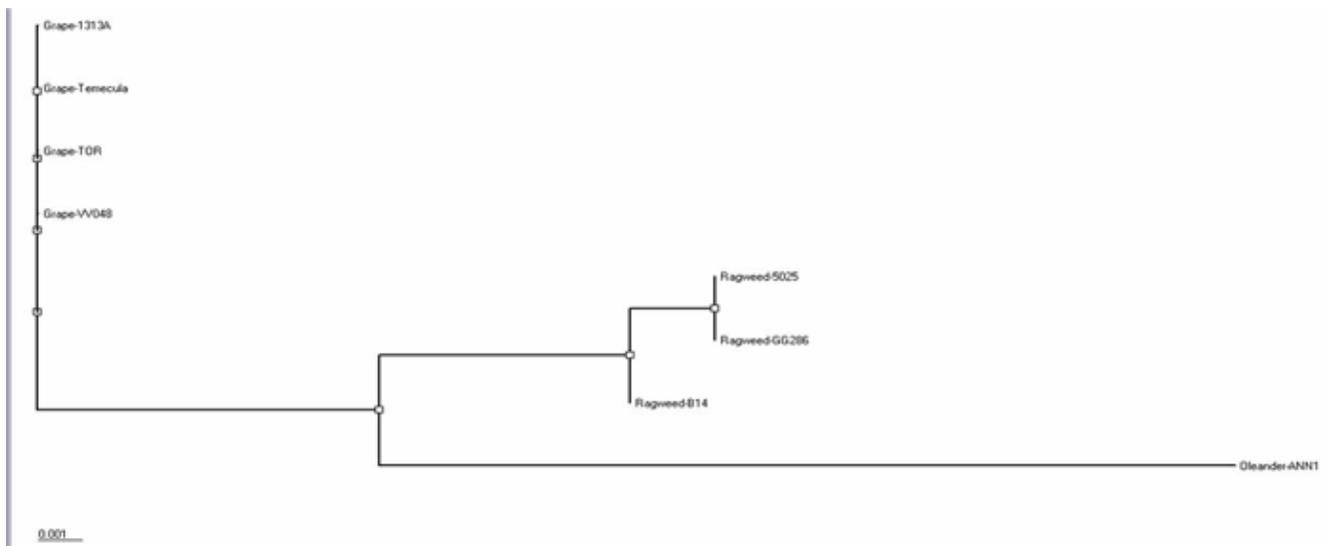
**Figure 1.** Location of multiple *TonB* genes in the *X. fastidiosa* genome.

The positive samples PCR products were purified using QIAquick PCR purification kit (Qiagen, Valencia, CA), using the manufacturer's protocol. DNA sequencing reaction was performed in a I Cycler™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The reaction was carried out in a 10-μL reaction contained 4 μL DTCS Quick Start Master Mix (Beckman and Coulter, Fullerton, CA), 2 μL of either forward or reverse primer, 2 μL autoclaved nanopure water, and 2 μL DNA template. The reaction was cycled 30 times under the following parameters: 95°C for 20 s, 50°C for 20 sec, and 60°C for 4 min, followed by holding at 4°C. DNA samples were precipitated using ethanol precipitation process according to Beckman and Coulter's protocol. DNA pellets were resuspended with 40 μL of sample loading solution (Beckman and Coulter, Fullerton, CA) and transferred to the appropriate wells of a sample plate and loaded into a CEQ™ 8000 Genetic Analysis System (Beckman and Coulter, Fullerton, CA) for DNA sequencing. Sequences were retrieved and analyzed in BioEdit and compared to GenBank database (<http://www.ncbi.nlm.nih.gov/>).

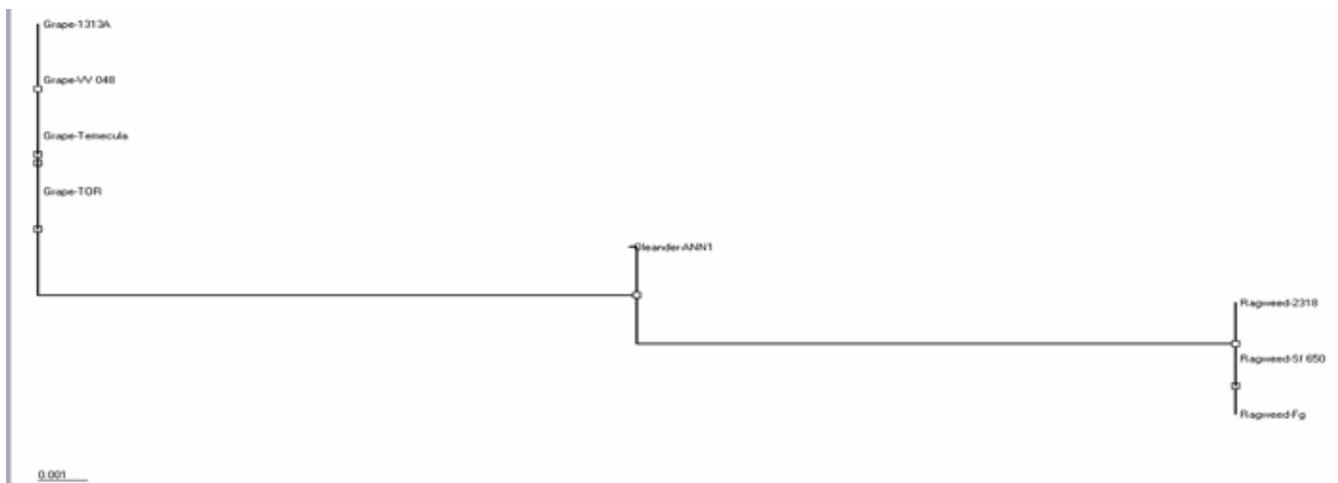
Sequence data is currently being collected for nearly 2,000bp of the ZOT gene for these strains. The same procedures apply.

## RESULTS AND DISCUSSION

BLAST search shows alignment of grape, ragweed, and oleander strains with *Xf* Temecula and *Xf* 9a5c. Alignment of grape, ragweed, and oleander strains using BioEdit software shows multiple single base pair alterations between grape, ragweed, and oleander strains. From these multiple base pair alterations, oleander strain shows partial alignment to both grape and oleander strain (**Figure 2 and 3**).



**Figure 2.** Alignment of *TonB* sequences from multiple *Xf* strains from Texas.



**Figure 3.** Alignment of *gyrB* sequences from multiple *Xf* strains from Texas (also analogous to *mopB*).

The multiple single-base alterations in oleander strain and single base alteration in ragweed strain result in different polarity and acid-base properties of amino acids to be translated. For instance, single base alteration in ragweed strain causes neutral-polar amino acid (serine) to be translated instead of neutral-non-polar amino acid (proline). The multiple single-base alterations in oleander strain cause basic-polar (histidine), neutral-polar (serine and alanine), and neutral-non-polar (proline) amino acids to be translated instead of neutral-polar (serine and threonine), and basic-polar (arginine) amino acids. These will have an effect on absorption properties, different reactions with other amino groups, and different functionality since different proteins will be produced.

We are currently working through the ZOT gene for all of our strains. Within this gene, we have found a conserved domain (most likely a beta barrel associated with attachment to the *Xf* membrane) and another domain that appears to be hypervariable. Analysis of this preliminary sequence data indicates that the conserved domain follows the same separation as *gyrB*, *mopB*, and *tonB* (i.e. separation by strain). However, the hypervariable region may be useful for separation beyond strain.

## CONCLUSIONS

Sequencing using *TonB* genes and the ZOT gene highlights genetic variability between three different strains of *Xf* (grape, ragweed, and oleander strains). The results of this study are consistent with genotype differentiation using conserved *mopB* and *gyrB* genes. The discovery of this variable gene provides another genomic location to be exploited for the improvement of diagnostics to aid in the management of PD.

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# THE SIGNIFICANCE OF *XYLELLA FASTIDIOSA* TYPE I AND TYPE IV PILI IN BIOFILM STRUCTURE, BACTERIAL SURVIVAL IN BIOFILMS, AND DNA SECRETION AND UPTAKE

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

## ABSTRACT

We have determined that type I and type IV pili of *Xylella fastidiosa* (*Xf*) play essential roles in biofilm development, based on their individual contributions to cell adhesion and motility. Our recent studies with grape xylem sap indicate that it triggers the development of a more robust and structured biofilm than caused when the bacterium is grown in PD2 medium. Cell viability in the *Xf* cell matrix showed the interior of biofilms to contain a high level of dead cells whereas the outer periphery consists of mainly live cells. We also found that the signal molecule DSF inhibits twitching motility as does the chemical stressor EDTA.

## INTRODUCTION

Biofilm formation is recognized as a major virulence factor of *Xf*, being essential for bacterial survival *in planta* and disease development (Newman et al., 2004; Koide et al., 2004; Li et al., 2007). Maintaining *Xf* in axenic culture over several passages gradually changes the expression of pathogenicity factors and leads to loss of virulence (de Souza et al., 2003). It has been shown previously that when *Xf* is grown in media that are intended to mimic xylem fluid chemistry growth, biofilm formation and aggregation are affected. (Andersen et al., 2007). Here we show that culturing *Xf* in xylem sap is more suitable for biofilm studies than standard culture media broadly used, such as PD2 and PW, given it enhances the adhesive characteristic of the bacterium and induces a more realistic *in planta* phenotype.

We have demonstrated that two distinct classes of *Xf* pili are associated with the cell's ability to move in grapevine xylem (via twitching motility) and to form biofilms and cellular aggregates (Meng, et al., 2005; Burr and Hoch 2006, Li et al., 2007). Whereas wild-type *Xf* is able to move against the transpiration stream within grape to colonize vines, mutants without type IV pili were unable to move (Meng et al., 2005). Mutants lacking shorter, type I pili, moved faster than the wild-type indicating that type I pili serve to anchor and slow movement (De La Fuente et al., 2007b). This scenario is supported by the fact that mutants with only type I pili form biofilms that have a more spreading phenotype on surfaces as compared to the wild-type and to mutant strains that have only type IV pili (Burr and Hoch 2006, Li et al., 2007). Biofilms formed by the wild-type have a denser-appearing phenotype and therefore we hypothesize that type IV pili function in secondary structure. Mutants that do not produce type I pili form biofilms that are sparse but appear to be made of dense clusters of cells again suggesting a role for type IV pili in cell-cell attachment and secondary structure of biofilms.

The signal molecule DSF (Diffusible Signal Factor) produced by *Xf* has been recently identified and shown to be required for insect colonization and to reduce its virulence to grape (Simionato et al., 2007; Chatterjee et al., 2008).

## OBJECTIVES

1. Assess and understand the biology and role of *Xf* biofilms in Pierce's disease. For this objective, we will be particularly interested in:
  - a. Understanding the developmental stages and architecture of biofilm formation.
  - b. Determine how the presence of type I and type IV pili affect biofilm morphology and integrity.
  - c. Assess the viability of *Xf* cells temporally and spatially in biofilms.
  - d. Determine whether *Xf* secretes DNA into the extracellular environment and how it affects biofilm morphology and integrity.
  - e. Evaluate effect of DSF on motility and biofilm regulation.
2. Determine how the stage of biofilm development and structure (dependent on pili) influence *Xf* sensitivity to chemical and environmental stresses.

### 3. Determine the role of type IV pili in *Xf* uptake of extracellular DNA (natural transformation).

## RESULTS

### Developmental stages and architecture of biofilm formation.

Whether or not *Xf* forms a structured community with specialized cell functions or if it is merely a consequence of cell to cell aggregation has been a point of interest of our group. We recently started growing *Xf* in pure grape sap, allowing a more “natural” environment for growth. This was done by initially transferring the cells growing in PD2 medium to a mixture containing 50% sap and then gradually increasing the sap concentration in each passage. The supplementation with sap not only induced faster growth (Hoch and Burr et al., 2008), but also increased the attachment of cells to glass surfaces, as assessed in test tubes and on microscope slides (Hoch and Burr et al., 2008). Besides the greater biomass visible in both assays, the biofilm formed in tubes containing 100% sap or sap:PD2 mixes were denser and difficult to disrupt by vortexing (Hoch and Burr et al., 2008). We are quantifying this by crystal violet staining and spectrophotometry.

To study spatial-temporal biofilm development we are using microscope slides fixed inside 500mL jars that receive 20mL of culture media. The jars are shaken at 100 rpm and the biofilm is formed on the slide surfaces at the air-media interface. Biofilm development and architecture were found to be greatly influenced by medium i.e. PD2 or grape sap.

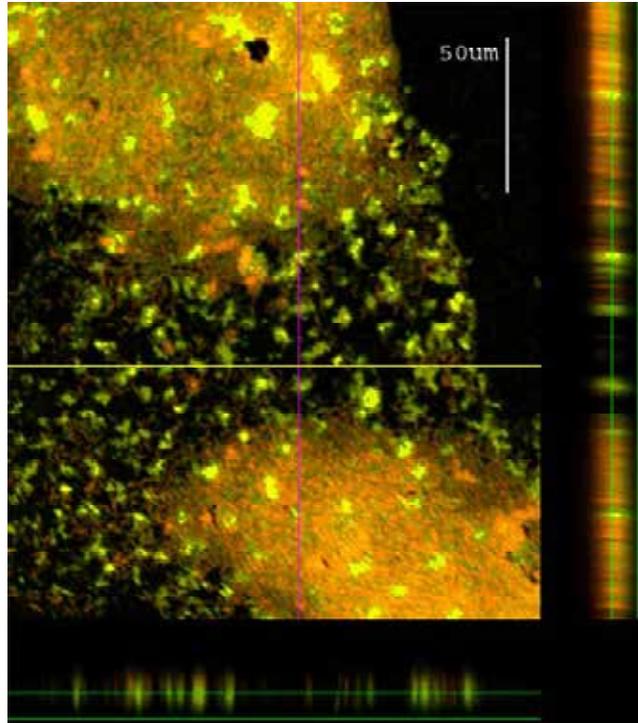
Preliminary results using light and laser scanning microscopy indicate that aggregates up to 20 $\mu$ m in height were observed in PD2 whereas in a mixture of sap and PD2 (90% sap, 10% PD2) aggregates of 100 $\mu$ m in height were commonly seen. This might be related to a quicker aggregation of cells that appears to occur in sap. Whether in PD2 or sap, *Xf* characteristically forms small aggregates that retain mobility and eventually merge together. We observed that such aggregates are retained even when the extracellular matrix accumulates in later developmental stages. These denser cell aggregates consist mainly of live cells and are surrounded mostly by dead cells within the large aggregates (**Figure 1**). At the substrate surface level, there is a higher proportion of live cells at the periphery of the biofilm.

Another distinctive feature of biofilm formation in sap is what appears as “fluid channels” throughout the matrix. They might function in increasing exchange rates of nutrients and toxic by-products of metabolism, enabling biofilms to develop as a functional unit. Initial investigations reveal what appears to channel formation in biofilms formed in microfluidic chambers and on surfaces of microscope slides (Hoch and Burr et al., 2008).

Further studies will use the fluorescence-tagged strain KLN59.2 (Newman et al., 2003) to study biofilm architecture in different types of devices and *in planta*. This strain displays attachment and motility on surfaces similar to wild-type Temecula.

### Influence of type I and type IV pili on biofilm morphology and integrity.

We have been conducting studies to investigate how the type of pili (I and IV) affect biofilm development and structure. Mutant cells lacking either type I (*fimA*) or type IV pili (*pilB*) showed reduced biofilm formation, consistent with reduced adherence to surfaces among *fimA*<sup>-</sup> cells; probably due to the lack of the strong anchoring character conferred by type I pili (De La Fuente et al., 2007a; 2007b). Mutant cells deficient for both type I and IV pili (*fimA*, *pilQ*) did not form a biofilm on the glass surface (Li et al., 2007), and as a result generally remained in a planktonic state. Our results suggest that the type of pili affects cell clustering and biofilm morphology. We are also studying a chemosensory system of *Xf* involved in motility and biofilm formation (Burr and Hoch, 2008).



**Figure 1.** Cell viability within biofilm formed by *Xylella fastidiosa*. Cells were stained with SYTO-9 (green) and propidium iodide (red), equivalent to a “live and dead assay” (Boulos et al., 1999). The figure shows a portion of a mature biofilm formed after 7 days in PD2 medium. Small compact cellular aggregates can be seen embedded in and outside the biofilm matrix. The yellow and magenta lines indicate cross-sections in the Z-plane, shown on the bottom and right side of center image. The green lines on border images indicate the position of the plane shown on the center image. Obtained with confocal imaging.

### Effect of DSF on motility and biofilm regulation.

The effect of Diffusible Signal Factor (DSF) (Newman et al., 2004) on *Xf* twitching motility is being studied by our group in collaboration with Steve Lindow (UC, Berkeley). Purified DSF produced by *Xf* was obtained from the Lindow laboratory and re-suspended in 60% methanol. The DSF suspension (approximately 1 unit/ $\mu$ l) was used to supplement culture media. The effect of DSF on *Xf* movement has been observed using three different approaches:

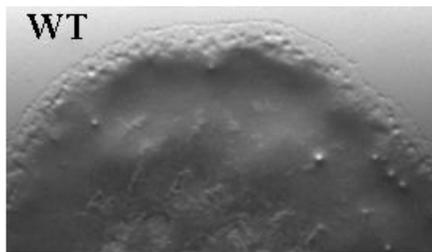
(i) **Agar plates diffusion assays:** a 5mm-diameter well was made in the center of each plate and filled with the DSF solution referred above (10, 20, 30, 40, 50 and 60 $\mu$ l) (**Figure 2**). Bacterial colonies of *Xf* WT and a *fimA* mutant (Meng et al., 2007) were spotted at two different distances (8 and 15mm) from the center of the plate (**Figure 2**).

We observed an absence of peripheral fringe in WT colonies spotted closest to the DSF (8mm distance) and specifically the colony edge facing the DSF-containing well. Normal colony fringe was observed on the edge of the colony opposite to the center (15mm).

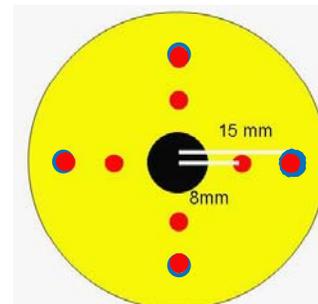
(ii) **Microfluidic chambers: addition of DSF to culture media:** Dual channelled microfluidic chambers (De La Fuente et al., 2007a) were used to microscopically observe the direct effect of DSF on twitching movement. The feeding syringes were interchanged every 1-2 days, thus exchanging between fresh and supplemented media. Whenever DSF was introduced in the chambers, the twitching movement was greatly reduced after 8-12h. We observe only a few cells moving short distances in the presence of DSF. Control cells in PD2 supplemented with methanol, showed normal twitching activity.

(iii) **Twitching movement of DSF non-producing mutant:** A mutant deficient in the production of DSF (*rpfF*-DIF2) was obtained from the Lindow lab. Preliminary observations on solid media (**Figure 3**) and in microfluidic chambers showed that the speed of twitching movement of the *rpfF* mutant is slightly higher than the WT. The speed was calculated as 0.98 $\mu$ m/min (as compared to the reported 0.86 $\mu$ m/min for the WT strain) (De La Fuente et al., 2007b).

The characterization of this mutant is still ongoing in our laboratory. Other observations indicate that aggregation of the *rpfF*-DIF2 mutant in chambers resembles the phenotype of mutants reduced in biofilm formation, such as *fimA*<sup>-</sup> (see above). These results suggest that the presence of DSF reduces movement in *Xf*. We are continuing to investigate the effect of DSF on biofilm formation.



**Figure 3.** Colony fringe of WT and mutant *rpfF*-DIF2 deficient in DSF production.



**Figure 2.** Schematic of the agar diffusion assays. The circle in the center represents the well where DSF or the control solvent were added. The smaller red circles indicate where bacterial colonies were spotted. The blue zones represent the fringes observed

### Effect of chemical and environmental stresses on movement and biofilms.

Previous studies with other microorganisms have shown the importance of biofilm in increasing resistance to detergents, toxins and environmental stresses like salinity, acidity and low humidity (Xavier et al., 2005). The protectiveness conferred by the biofilm can be studied by comparing cell viability between planktonic cells to those within biofilms after exposure to the stresses.

We have found that a chelating agent, such as EDTA has an effect on WT *Xf* cell movement. Whenever EDTA was added to the cells (8, 6, 4, and 2 mm) they slowly reduced movement and eventually stopped their displacement. Nevertheless, the presence of the chelating agent did not affect cell division or growth. We are now investigating the effect of EDTA on *Xf* cell aggregates. Based on the fact that movement affects morphology of cell clusters, we expect to see an effect of EDTA on biofilm formation.

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## DIFFERENTIAL EXPRESSION OF GENES OF *XYLELLA FASTIDIOSA* IN XYLEM FLUID OF CITRUS AND GRAPES

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

**ABSTRACT**

Understanding regulatory pathways controlling microbial pathogenesis can lead to a better understanding of virulence factors as potential targets for disease control strategies. Preliminary research has shown that citrus is tolerant or resistant to Pierce's disease (PD) strains of *Xylella fastidiosa* (*Xf*) but may serve as a reservoir for the bacterium. Commercial citrus (lemon, orange and grapefruit) orchards in proximity to vineyards in Temecula Valley were selected to determine the effect of xylem fluid on *Xf*. Current results revealed that pure xylem fluid of grapefruit, orange, and lemon caused the bacterial cells to form aggregations of large whitish clumps, whereas the xylem fluid of grape caused them to form a visible thick biofilm. Macroarray analysis was conducted with 111 genes, predicted by the *Xf* genome sequence to have roles in virulence, as well as nucleic acid and protein metabolism, cellular transport and stress tolerance. There were 28 genes with greater expression in xylem fluid of grape, vs. that of citrus. The virulence regulator *xrvA*, transcriptional regulators *algU*, *agmR*, *gcvR*, two-component regulators *gacA* and *colS*, and posttranscriptional regulator *hsq*, were expressed at higher levels in grape xylem fluid. Other genes that were over expressed in grape xylem included *hsf*, *xadA*, *fimT*, *pill*, *pilT*, *pilU*, and *pilY1*, related to attachment, biofilm formation, and twitching motility of *Xf* within xylem. The data indicated that grape xylem fluid stimulates the expression of virulence genes, likely contributing to PD in grapevine. *Xf* may use gene regulatory mechanisms to respond to changing environments in the xylem of plants, and host range may in part be determined by differential regulation of virulence genes in different host xylem conditions.

**INTRODUCTION**

*Xylella fastidiosa* (*Xf*) is mainly vectored by the glassy-winged sharpshooter (GWSS) in southern California (Raju et al., 1983; Sorensen and Gill, 1996). Previous studies in California have identified 94 plant species in more than 28 plant families as hosts of *Xf* (Costa et al., 2004). Most of them are symptomless but may serve as inoculum sources for vector acquisition of *Xf*. Studies in the Temecula Valley showed that the proximity of citrus groves to vineyards influenced the incidence and severity of Pierce's disease (PD) in grapevine (Perring et al., 2001). PD infection is most severe when the grapevines are adjacent to citrus, and the damage declines as one moves away from citrus. Although the GWSS feeds on and moves back and forth between citrus and grape plants, there is no *Xf*-caused disease symptom in citrus in the area. This implies that citrus plants are resistant or tolerant to *Xf* but may be a reservoir, harboring the pathogen for GWSS acquisition while grape plants are susceptible (Bi et al., 2007b). Transmission of PD by leafhoppers from citrus to grapevines has indeed been documented (Hopkins et al., 1978). Little is known about the biochemical mechanisms involved in host plant resistance/susceptibility to *Xf* in this citrus and grape system.

It was recently reported that certain amino acids are essential for *Xf* growth, and that glucose stimulates growth, while fructose and sucrose have an inhibitory effect (Leite et al., 2004). Our preliminary data indicated that there were large differences in xylem fluid amino acid and sugar contents between grapes and citrus currently growing in the Temecula Valley (Bi et al., 2007a). Xylem fluid of citrus significantly inhibits biofilm formation by PD strains of *Xf* compared to xylem fluid of grape (Bi et al., 2007b). However, the xylem fluid chemical components in citrus and grape, and their role in *Xf* gene expression and host plant resistance and susceptibility to *Xf*, are not well known. Further research is needed to determine the effect of host plant xylem fluid on expression of *Xf* virulence factors and to elucidate the mechanisms that are involved. Host plant resistance has been recognized as the most cost effective and environmentally safe method for controlling many major microbial pathogens of economic plants. Understanding the biochemical mechanisms involved may lead to the development of resistant varieties or anti-*Xf* chemicals for existing grapevines.

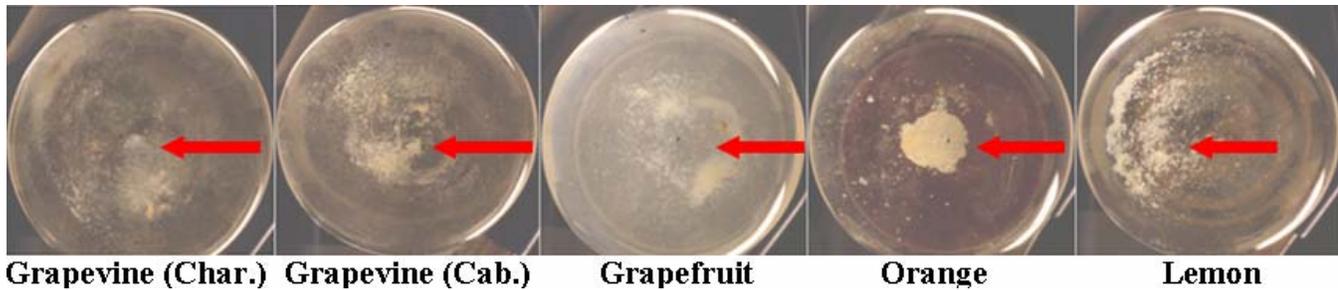
**OBJECTIVES**

1. Investigate the effect of host plant xylem fluid on *Xf* aggregation, biofilm formation, and gene expression *in vitro*.
2. Determine the role of specific chemical components in citrus xylem fluid in *Xf* resistance.

**RESULTS AND DISCUSSION**

**Bacteria cell aggregation in xylem fluid.** Aggregation and attachment of *Xf* was observed after culturing a PD strain in pure xylem fluid of grapefruit, orange, lemon, and grape. The bacterial cells aggregated to form large clumps in grapefruit, orange, and lemon fluid (**Figure 1**). This may cause the bacteria to remain in only a few xylem vessels after introduction to

the citrus by a sharpshooter, without much mobility within the xylem of citrus. In contrast, the bacterium formed less aggregation in grape fluid, consistent with the known ability of PD strains to move easily within the xylem of grapevines.



**Figure 1.** A fresh *Xf Temecula A05* culture (Costa et al., 2004) was inoculated into xylem fluid of grape, grapefruit, orange, and lemon at  $OD_{600}=0.02$  in borosilicate glass culture tubes on a rotary shaker at room temperature (around 24°C). All tubes were covered with a black box to prevent the xylem fluid from light during the shaking culture. Photographs were taken after 10-12 weeks. The red arrow indicates the aggregated cells in xylem fluid.

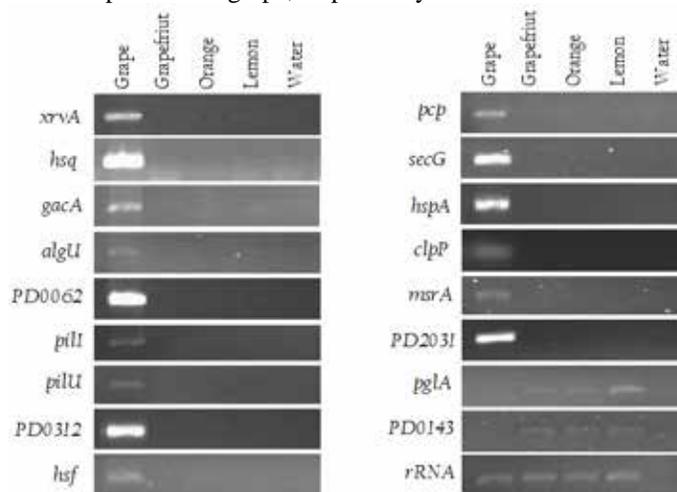
**DNA macroarray analysis of gene expression in citrus and grape xylem fluid.** DNA macroarray membranes were prepared with 111 selected genes with putative roles in virulence, as well as others involved in nucleic acid and protein metabolism, cellular transport and stress tolerance, based on the genome sequences of *Xf 9a5c* (a CVC strain) (Simpson, et al, 2000) and *Temecula1* (a PD strain) (Van Sluys, et al., 2003). Total RNAs were extracted from *Xf Temecula1* cultured in the pure xylem fluid of grapefruit, orange, lemon and grape using a Quiagen RNeasy mini kit (Quiagen, CA). The purified mRNA was separated from total RNA using an mRNA-ONLY™ Prokaryotic mRNA Isolation Kit according to the manufacturer's protocol (Epicentre, WI), and used for synthesizing cDNA probes for array hybridization by reverse transcription (RT). DNA macroarray nylon membranes were hybridized with DIG-labeled cDNA probes following the manufacturer instructions (Roche, Molecular Biochemical, Indianapolis, IN). The signal intensities of spots on the membranes were analyzed using the Quantity one® software (Biorad). Thirty genes were differentially expressed in grape fluid compared to citrus fluid (**Table 1**).

**Validation of macroarray data.** Several potential virulence-related genes were chosen to validate the differential expression levels of genes in xylem fluid of grape and citrus using RT-PCR (**Figure 2**). rRNA was detectable in all xylem fluid in this RT-PCR condition. No RNA was detectable in a water control. Expression of the genes *xrvA*, *hsq*, *gacA*, *algU*, PD0062, *pill*, *pilU*, PD0312, *hsf*, *pcp*, *secG*, *hspA*, *clpP*, *msrA* and *tapB* RNA was increased in grape fluid, and *pglA* and PD0143 RNA were increased in grapefruit, orange and lemon fluids (**Figure 2**). Genes predicted to be involved in virulence regulation, such as the virulence regulator *xrvA*, transcriptional regulators *algU*, *agmR*, *gcvR*, two-component regulators *gacA* and *colS*, and posttranscriptional regulator *hsq*, were expressed at higher levels in grape fluid. Inside the plant's xylem, *Xf* is exposed to a range of variable stress factors, such as changes in osmolarity, availability of nutrients, and agents generating reactive oxygen intermediates (Alves et al., 2004). To ensure survival, *Xf* may respond to these stress situations via regulatory mechanisms involving specific regulatory genes. The regulatory genes *algU* (Shi et al, 2007) and *gacA* (Cooksey, 2007) were previously shown to have roles in regulating many potential virulence factors in *Xf*. Hfq, an abundant RNA-binding protein, may indirectly affect biofilm formation in *Xf* through a complex *hfq/rsmB/rsmA*-mediated system (Shi et al, 2007). Genes involved in surface structures and attachment components, such as PD0312, *hsf*, and *xadA*, were expressed more highly in grape fluid than citrus. *hsf* (PD0744) has a high similarity to the *hsf* adhesin gene of *Haemophilus influenza* (St Geme et al., 1996), and *xadA* encodes a putative afimbrial outer membrane protein adhesion (Simpson et al., 2000). The expression of *hsf* and *XadA* was increased in grape fluid, likely contributing to an enhanced ability to adhere to xylem cell walls. It was reported that *hsq* and *hspA* were regulated by *algU* (Shi et al, 2007) and *xadA* and *hsf* were regulated by *gacA* (Cooksey, 2007). Genes involved in the biogenesis and twitching motility of type I pili and type IV pili in *Xf*, such as PD0062, *fimT*, *pill*, *pilT*, *pilU*, *pilY1* (Simpson et al., 2000), were shown to have higher expression in grape fluid. It is reported that type I pili function in cell-cell aggregation and biofilm formation, and type IV pili are involved in twitching motility within the xylem of host plants (Meng et al., 2005). The expression of genes encoding type I and type IV pili was increased in grape fluid, likely contributing to an enhanced ability to aggregate, form biofilm, and move within the xylem, contributing to PD symptoms in grapevines. Since the expression of *secD* and *secG* was increased in grape fluid, the secretion of proteins by the type II, *sec*-dependent secretion system may enhance bacterial survival in grape. Genes involved in physiological metabolism under stress, such as heat shock protein genes *hspA* and *cplP*, and sulfoxide reductase gene *msrA*, cation tolerance protein *cutA*, and hypothetical protein PD0008, PD1741 and PD2031, were also more highly expressed in grape fluid. In contrast, the polygalacturonase gene, *pglA*, and hemolysin, had increased expression in citrus fluid. The data indicate that the chemical compounds or elements in xylem fluid of different plants differentially affect the regulation of virulence and the survival of *Xf* within xylem.

**Table 1. Differential expressed genes of *Xf* in grape fluid comparing to citrus fluid**

Gene ID <sup>a</sup>	NAME	HYPOTHETICAL FUNCTION	Grape/Citrus <sup>b,c</sup>	P Value <sup>d</sup>	Expression in Grape
PD1905	<i>xrvA</i>	Virulence regulator	1.8	1.2E-03	Higher
PD2040	<i>acvB</i>	Virulence protein	1.9	3.4E-03	Higher
PD0066	<i>hsq</i>	RNA-binding protein	2.7	1.5E-03	Higher
PD2068	<i>gacA</i>	Two-component regulator	1.8	3.0E-04	Higher
PD1276	<i>algU</i>	Transcriptional regulator	1.6	1.5E-03	Higher
PD0268	<i>agmR</i>	Transcriptional regulator (luxr/uhpa family)	1.9	5.2E-03	Higher
PD1738	<i>gcvR</i>	Transcriptional regulator	1.6	2.4E-03	Higher
PD1920	<i>colS</i>	Two-component system, sensor protein	1.6	1.4E-03	Higher
PD0019	<i>fimT</i>	Pre-pilin like leader sequence	2.0	1.8E-03	Higher
PD0062	-	Fimbrial subunit precursor	2.6	1.1E-03	Higher
PD0846	<i>pilI</i>	Pilus biogenesis protein	1.8	4.5E-03	Higher
PD1147	<i>pilT</i>	Twitching motility protein	1.6	3.2E-03	Higher
PD1148	<i>pilU</i>	Twitching motility protein	1.8	1.5E-03	Higher
PD1611	<i>pilYI</i>	Fimbrial assembly protein	2.3	2.1E-04	Higher
PD0312	-	Outer membrane protein precursor	2.5	1.2E-03	Higher
PD0731	<i>xadA</i>	Outer membrane protein	2.3	4.5E-03	Higher
PD0744	<i>hsf</i>	Surface protein	1.7	3.4E-03	Higher
PD0757	<i>pcp</i>	Peptidoglycan-associated outer membrane lipoprotein precursor	1.6	1.7E-03	Higher
PD0182	<i>secD</i>	Protein-export membrane protein	2.1	1.4E-03	Higher
PD0246	<i>secG</i>	Protein-export membrane protein	2.4	1.2E-03	Higher
PD1280	<i>hspA</i>	Low molecular weight heat shock protein	2.3	1.8E-03	Higher
PD0472	<i>clpP</i>	ATP-dependent Clp protease proteolytic subunit	1.6	1.1E-03	Higher
PD0859	<i>msrA</i>	Peptide methionine sulfoxide reductase	1.6	2.1E-03	Higher
PD1536	<i>cutA</i>	Periplasmic divalent cation tolerance protein	1.8	1.6E-03	Higher
PD1475	<i>cmaA</i>	Heme ABC transporter ATP-binding protein	1.8	4.1E-03	Higher
PD2031	-	Hypothetical protein	2.5	1.2E-03	Higher
PD0008	-	Hypothetical protein	1.8	1.2E-03	Higher
PD1741	-	Hypothetical protein	2.0	1.7E-04	Higher
PD1485	<i>pglA</i>	Polygalacturonase precursor	-1.7	1.8E-03	Lower
PD0143	-	Hemolysin III protein	-1.6	1.7E-03	Lower

<sup>a</sup>Genes were determined on the basis of *Xf* Temecula1 genomic sequences at the NCBI website (Simpson et al. 2000). <sup>b</sup>The hybridization signal intensity (mean of three hybridization replicates) obtained with grape was divided by that obtained with citrus to obtain grape/citrus ratio. <sup>c</sup>The normalized hybridization signals for those genes between grape and citrus are significantly different as analyzed by Student's *t* test ( $P < 0.05$ ). <sup>d</sup>Genes having  $>1.5$  or  $<0.66$  final grape/citrus ratios were designated as having higher or lower expression in grape, respectively.



**Figure 2.** Reverse transcription polymerase chain reaction (RT-PCR) of differentially expressed genes of *Xf* in xylem fluid of citrus and grape. Water was the control.

## CONCLUSIONS

Aggregation and biofilm formation of *Xf* were differentially influenced by the xylem fluid of citrus vs. grape. Grape fluid stimulated the expression of genes predicted to be involved in virulence, attachment, biofilm formation, and twitching motility of *Xf* within xylem, likely contributing to PD in grapevine. Citrus may be resistant or tolerant to the PD strain of *Xf*, in part, because citrus xylem fluid does not support the induction of a number of virulence genes, or has substances that repress expression. Identification of specific chemical components of citrus xylem fluid which influence expression of virulence genes in *Xf* is being assessed.

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## ROLE OF TYPE I SECRETION IN PIERCE'S DISEASE

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### ABSTRACT

In previous work, we discovered that: 1) *tolC* was absolutely required not only for pathogenicity, but also for survival of *Xylella fastidiosa* (*Xf*) strain Temecula in *Vitis vinifera* grapevines; 2) that the loss of multi-drug resistance (MDR) efflux through the Type I secretion system was the primary reason that *tolC* Temecula could not survive in grapevines; and 3) that gene knockouts of Type I system components associated with offensive Type I effector secretion also resulted in significant loss of pathogenicity. Both hemolysins and colicin V effectors are found in the Temecula genome. Surprisingly, knockout mutations of both 1) the Type I components associated with colicin secretion and 2) the three colicin V effectors in Temecula resulted in a significant loss of pathogenicity of the mutants, raising the possibilities that colonization and pathogenicity of grapevines by *Xf* involves the exclusion of other bacteria from the xylem niche and/or that these colicins might directly affect plant cells.

### INTRODUCTION

In Gram-negative bacteria, multidrug resistance (MDR) efflux pumps are composed of three protein components, two of which are localized in the inner membrane, and one, TolC, that traverses both the periplasm and outer membrane (Koronakis et al. 2004). The process of MDR efflux is energy dependant and utilizes either ATP or the transmembrane electrochemical gradient. At least five characterized families of MDR efflux pumps exist in bacteria: the ATP-binding cassette (ABC) family (Davidson and Chen 2004), the major facilitator (MF) family (Pao et al. 1998), the small multidrug resistance (SMR) family (Paulsen et al 1997), the resistance-nodulation-cell division (RND) family (Tseng et al. 1999), and the multidrug and toxic compound extrusion (MATE) family (Brown et al. 1999). All utilize TolC as a common periplasm/outer membrane protein component (**Figure 1**).

In addition to (defensive) MDR efflux, TolC is also essential for type-I dependent secretion of a variety of degradative enzymes and offensive effectors, some of which are antibiotic and others involved in plant or animal pathogenicity. These include a variety of hydrolases (proteases, phosphatases, esterases, nucleases and glucanases) and protein toxins, including hemolysins and bacteriocins (Koronakis et al. 2004). Orthologs of *tolC* are highly conserved among diverse Gram-negative pathogenic bacteria, and strains typically carry multiple homologues per strain (Sharff et al. 2001), including all sequenced strains of *Xanthomonas*, *Pseudomonas* and *Ralstonia*.

*Xylella fastidiosa* (*Xf*) is a xylem-inhabiting Gram-negative bacterium that causes serious diseases in a wide range of plant species (Purcell and Hopkins, 1996). Two of the most serious of these are Pierce's disease (PD) of grape and Citrus Variegated Chlorosis (CVC). Analyses of the CVC and PD published genomes showed that there was no type III secretion (*hrp*) system, but there were at least two complete type I secretion systems present, together with multiple genes encoding type I effectors in the RTX (repeats in toxin) family of protein toxins, including bacteriocins and hemolysins.

There are two main purposes for Type I secretion (refer **Figure 1**): multi-drug resistance or MDR efflux (in this case, defense against presumably anti-microbial chemicals in the xylem sap of grape), and effector secretion (offensive, to either promote pathogenicity or secrete antimicrobial peptides). The outer membrane protein TolC has been shown to be essential for MDR efflux and pathogenicity in *Erwinia chrysanthemi* (Barabote et al., 2003) and in *Xf* (Reddy et al., 2007). Our general working hypothesis has been that *Xf* is a highly opportunistic species and that (at least) many of its strains have a very wide host range that is limited by at least two factors of unknown weight: the host range of its insect vectors, and its *intrinsic host range factors that may or may not elicit obvious pathogenic symptoms*. This working hypothesis was based on several observations. First, Freitag (1951) identified 75 asymptomatic host species for PD out of 100 plant species tested. In support of these older published test results, it is now clear that at least some PD, CVC and coffee leaf scorch strains of *Xf* can grow well in coffee, tobacco and periwinkle. These results all strongly indicate that some strains are capable of using the xylem sap of many plant species as growth medium, and may be restricted primarily by the lack of vectors to take them to other plant species.

Second, PD strains inoculated on grape both grow and elicit leaf scorch symptoms, but on tobacco cultivar Samsun, PD strains grow, but elicit no symptoms (Harakava Ph.D. thesis); on citrus, PD strains neither grow nor elicit symptoms (Hopkins, 1977). Similarly, the coffee strain does not grow or cause symptoms in citrus, but the CVC strain causes limited symptoms in coffee and mainly chlorosis in tobacco (Harakava, personal communication). These results indicate: 1) that

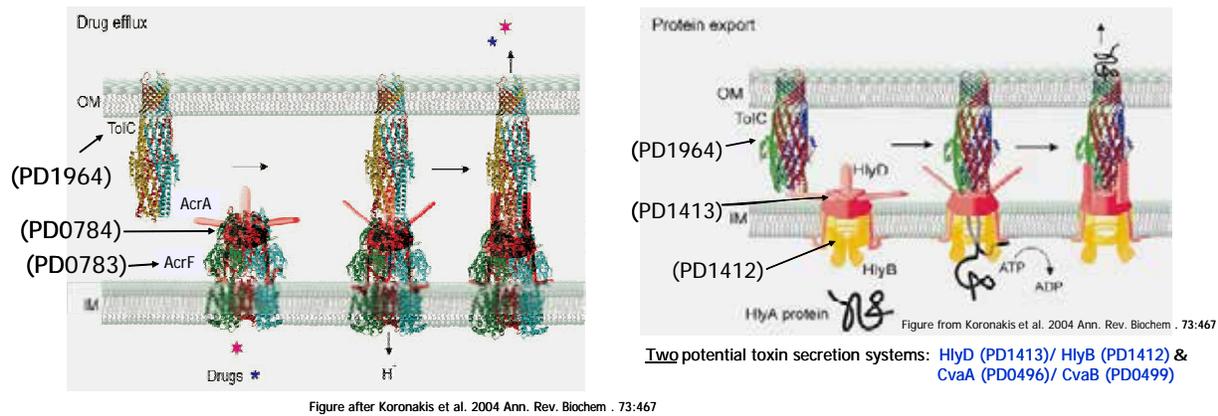
symptoms are host specific and induced only in a subset of host species; and 2) that the host specific symptom induction depends on the *Xf* strain as well as the plant species infected.

*PD strains produce a host-specific elicitor of PD involving programmed cell death (PCD).* Symptoms of leaf scorch in PD are not expected of a pathogen that merely blocks xylem vessels. Vascular wilts, such as periwinkle wilt, are more typical of xylem vessel blockage. Leaf scorch must be caused by another factor, long ago proposed to be a toxin (Raju and Wells, 1986). However, the limited evidence provided to support the toxin theory at the time was found to be an artifact caused by components of the culture medium (Goodwin et al., 1988) and the matter seemed settled (Hopkins, 1989) until very recently. At the 2007 Pierce's Disease Research Symposium, Gilchrist and colleagues reported evidence that *Xf* PD strains elicited PD and programmed cell death (PCD) or apoptosis in *V. vinifera*, but not *V. californica* grapes, and that anti-apoptotic genes cloned from grape variety Chardonnay and retransformed into plants using a strong (CaMV) promoter strongly suppressed symptoms of PD and PCD (Gilchrist & Lincoln, 2007 Pierce's Disease Research Symposium Proceedings, pp 252-5). In recent years, a large and growing number of bacterial protein toxins have been discovered that behave as virulence factors, and many of these bacterial toxins induce apoptosis (Schiavo, G.; van der Goot, F.G. 2001). One emerging theme from studies of these bacterial toxins is that they frequently interfere with host pathways, thereby eliciting programmed cell death (for a review see Weinrauch and Zychlinsky, 1999). Since symptoms of PD are suppressed by anti-apoptotic gene expression, it becomes likely that the pathogen is producing a PCD elicitor, or "toxin". This "toxin" or elicitor has yet to be identified.

*Elicitation of symptoms of PD and PCD enhances Xf growth in hosts.* A major question has always been whether or not the symptoms of leaf scorch on grape contribute to pathogen growth or spread on grape or are merely gratuitous. Gilchrist's lab discovered that the anti-apoptosis genes both strongly suppressed symptoms of PD and in addition, limited the bacterial titer (at six months post inoculation) to that which is usually seen on the asymptomatic host, *V. californica* (ie., to ca.  $10^4$  cfu/gram stem tissue instead of  $10^8$  cfu/gram stem tissue observed at point of death of *V. vinifera*; refer Table 2 of the PowerPoint presentation by Gilchrist, Lincoln, Ward and Cook, 2007 Pierce's Disease Research Symposium, available online; confirmed by Dave Gilchrist in personal communication). This data indicates that elicitation of programmed cell death (PCD) can contribute to additional *Xf* growth in hosts, but is not required for opportunistic (parasitic) growth of *Xf*, at least not in some hosts.

Of course, the early work of Freitag (1951) mentioned above demonstrated that PD symptom elicitation is not required for growth of PD strains in a variety of hosts. The converse is also true; several non-PD *Xf* strains are known to be capable of asymptomatic growth in *V. vinifera* (Hopkins, 2005). Indeed, *Xf* strain EB92-1, isolated from elderberry, has been found to be highly effective as a biological control agent against PD in the field for 12-18 months, and "only strains that were able to multiply and systemically colonize without producing significant symptoms were able to protect against virulent strains" (Hopkins, 2005). An important series of questions regarding host specific symptoms and host range now may be quantitative in nature: how much additional growth is provided by ability to elicit PCD and/or PD symptoms? Are multiple elicitors involved? How host-specific are these elicitors? Is some low level of PCD, below the level required to elicit symptoms, required for host range? The anti-apoptosis genes in Gilchrist's study suppressed, but presumably did not eliminate, programmed cell death (PCD) in the host, thereby resulting in suppression of symptoms and limiting bacterial growth. What if PCD were eliminated? Would all or most *Xf* growth also then be eliminated, and the plant be a nonhost?

A related question is whether or not ability to elicit PCD ultimately restricts ability to infect plants that might otherwise be hosts, such as PD strains inoculated on citrus. In other words, since PD strains do not grow in all plants, is (are) the PCD elicitors (all) host specific? Are there additional factors, aside from insect transmission and symptom elicitation that may limit host range? As described in some detail below, recent work from our lab indicates that the answer to the host range question indicates that there are likely additional factors aside from elicitors that may limit host range. These factors may involve colicins used for competitive exclusion of other bacteria that may colonize the same ecological niche. Our general working hypothesis regarding the very wide host range of the entire, highly opportunistic *Xf* species but more limited range of individual strains has been expanded to include three factors affecting host range: 1) the host range of its insect vectors (not examined by our methods); 2) the ability of *Xf* to elicit PCD with or without leaf scorch symptoms on *V. vinifera*; and 3) ability of *Xf* to competitively exclude other bacteria from its xylem vessel niche. If the primary factor(s) that determine host range can be identified, then additional targets for chemical, biological and/or transgenic controls would be made available.



**Figure 1:** Type I machine for MDR (“Drug) efflux in *X. fastidiosa* utilize *tolC* and *acrF/A* or *acrC/D* (left). Type I machine for protein export or secretion in *X. fastidiosa* utilize *tolC* and *cvaA/B* or *hlyB/D* (right). Figures from Koronakis et. al. (2004).

## OBJECTIVES

Two specific Type I toxin secretion systems are found in both CVC strain 9a5c and PD strain Temecula. These are an alpha-hemolysin-like system [in Temecula, it utilizes TolC (outer membrane, PD1964) and HlyB/D (inner membrane, PD1412/periplasm, PD1413)], and a colicin V-like system [in Temecula, it utilizes TolC (outer membrane, PD1964) and CvaA/B (inner membrane, PD0496/periplasm, PD0499)]. These Type I systems are also found together with multiple genes encoding type I effectors of the RTX (repeats in toxin) family of protein toxins (Lally et al., 1999).

Critically, all sequenced *Xf* strains carry only a single *tolC* gene; therefore, a knockout of this single gene in Temecula eliminated all Type I secretion, both offensive and defensive (Reddy et al. 2007). Unfortunately, the loss of MDR efflux in Temecula resulted in the strain being undetectable even a few minutes after inoculation of the *tolC* mutation; therefore, the loss of pathogenicity reflected the loss of the strain itself and any offensive role of Type I secretion in pathogenicity, due to loss of secretion of either hemolysin and/or colicins could not be tested.

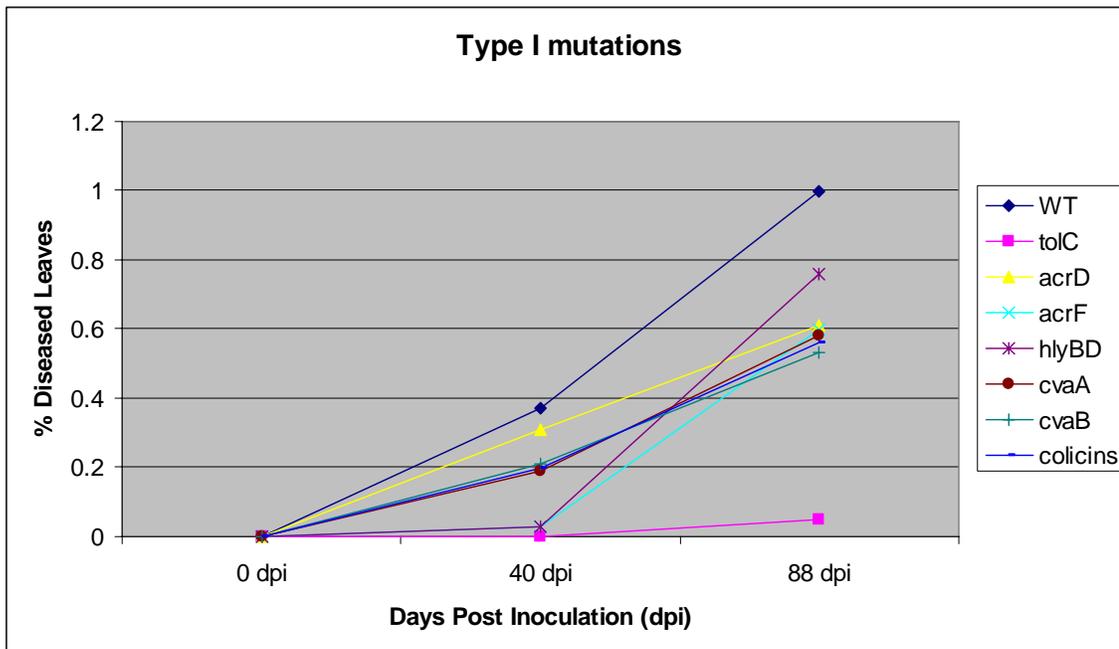
Type I secreted effectors found in the sequenced CVC and PD strains were a bacteriocin (XF2407 in CVC and ortholog PD1427 in PD) that resembles a *Rhizobium* host range factor (Oresnik et al., 1999), three hemolysins (XF0175, XF0984 and XF1280) in CVC and orthologs PD0413, PD0282 and PD0536, respectively, in PD), calcium binding hemolysin-type proteins (XF0668, XF1011, XF2759 in CVC and orthologs PD1506, PD0305, PD2094, respectively, plus an additional calcium binding protein PD2097), and three colicin V precursors (XF0262, XF0263 in CVC and orthologs PD0215, PD0216 and PD0217 in PD). The discovery of such a large group of RTX toxins is likely significant because both genomes carry representatives of both major RTX toxin types: the alpha-hemolysin group that are toxic to a very wide range of eukaryotic cell types (Lally et al., 1999), and the colicin V group, which is not known to us to affect eukaryotic cells. Among the symptoms elicited by CVC strains on citrus are brown, necrotic and slightly gummy lesions on the undersides of leaves that are suggestive of toxin activity. The earliest symptom caused by PD strains on grape is leaf scorch, which is also strongly suggestive of toxin activity.

## RESULTS

Rather than attempt knockouts of multiple and potentially redundant effectors, initial experiments focused on knockouts of three apparently separate components of Type I secretion: 1) MDR efflux only: *acrD* (PD1404) and *acrF* (PD0783); 2) Type I hemolysin secretion only: the periplasmic component *hlyB* (PD1412) and the inner membrane component *hlyD* (PD1413); and 3) colicin V secretion only: the inner membrane component *cvaA* (PD0496) and the periplasmic component *cvaB* (PD0499). Surprisingly, knockouts of any of the three Type I system strongly reduced pathogenicity (**Figure 2**).

The colicin V precursors (PD0215, PD0216 and PD0217) are clustered in the Temecula genome, allowing the simultaneous knockouts of all three colicins in a single recombination event by marker exchange, which was accomplished and documented as described (Reddy et al. 2006). Plant inoculation assays using the colicin V knockout mutant were performed in collaboration with Dr. Don Hopkins, at the Mid-Florida Research and Education Center, Apopka, Florida. Grape plants (var. Carnignae) were inoculated with the wild-type *Xf* Temecula strain and the mutant  $\Delta$  (PD0215, PD0216 and PD0217)::*nptII* strain (labeled “colicins” in **Figure 2**). The plants were maintained under green-house conditions and were evaluated for PD symptoms at 60 and 90 days after inoculation. All plants inoculated with the wild-type Temecula strain exhibited typical PD (not shown).

Again to our surprise, pathogenicity was strongly reduced by eliminating just the colicin effectors (**Figure 2**).



**Figure 2.** Grape var. Carignane inoculated with marker exchanged mutants of *acrF* (PD0783), *acrD* (PD1404), *hlyBD* (PD1412-1413), *cvaA* (PD0496) and *cvaB* (PD0499), and the three colicin V precursors PD0215, PD0216 and PD0217 (labeled “colicins”) and assessed for % diseased leaves at 40 and 88 days post inoculation.

Note that genes considered to be dedicated to both hemolysin (*hlyBD*) and colicin (*cvaAB*) secretion exhibited greatly reduced symptoms, and not just delayed symptoms. The *acrF* (MDR efflux) mutant, but not *hlyBD* (hemolysin secretion) was sensitive to berberine chloride (Gabriel, 2007 Pierce’s Disease Research Symposium Proceedings, p190-3), as expected. These results strongly support a role of both colicins and hemolysins in pathogenicity. In this regard, it is important to note that hemolysins are known to behave as apoptotic toxins in insects and animal pathogens (Vigneux et al., 2007). Therefore hemolysins may have a direct role in PD pathogenicity. Some colicins have a structural domain similar to Bcl-2 like proteins that are involved in apoptosis of animal cells (Boya et al. 2001), and can inhibit proliferation of cancer cells. Therefore the colicins may have a direct role in PD pathogenicity as well; alternatively or additionally, they may have a role in suppressing growth of bacteria that may compete for colonization of the xylem niche. These potential roles are currently under investigation.

## CONCLUSIONS

This work demonstrates that not only is multidrug efflux critical to survival of *Xf* in grape, but also that Type I secretion is needed for full pathogenicity, including the putative Type I effectors annotated as “colicin V precursors”. Both multidrug efflux and Type I secretion depend upon a single *tolC* gene present in the *Xf* genome. Since TolC is exposed to the outer surfaces of bacteria, these combined results make TolC a vulnerable and specific target for both chemical and transgenic approaches to control PD. Additionally, since colicin-like effectors appear to be important in conditioning pathogenicity, they represent additional targets for chemical, biological and/or transgenic disease control strategies.

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# UNDERSTANDING CONTROL OF *XYLELLA FASTIDIOSA* CELL AGGREGATION: IMPORTANCE IN COLONIZATION AND BIOFILM DEVELOPMENT IN GRAPEVINE AND SHARPSHOOTER FOREGUT

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

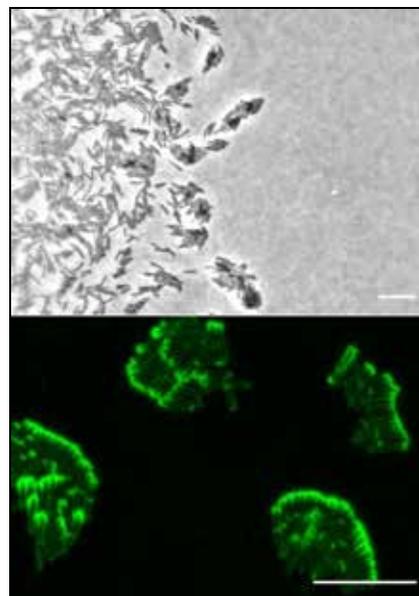
## ABSTRACT

Investigations reported herein focused on aggregation and ‘raft’ development by *Xylella fastidiosa* (*Xf*) cells. This study was particularly directed toward elucidating how *Xf* cells aggregate, to cause Pierce’s disease symptoms. It was shown that 100% grape xylem sap, for the first time, could be routinely used to culture *Xf*. Furthermore, the biofilm formed in sap was significantly different than biofilms formed in the more commonly used nutrient rich PD2 media. Additionally, we were able to visualize using immunocytochemical approaches, type I and type IV pili by light microscopy.

## INTRODUCTION

Colonization of grapevine xylem by *Xylella fastidiosa* (*Xf*) develops over an extended period of time before symptoms of Pierce’s disease are recognized. Such colonization, initially as individual cells, then as aggregates of a few cells, and finally as very large multicellular aggregates, coalesce to form a biofilm. From a disease standpoint such aggregates and biofilms are important for several reasons, including possible direct blockage of sap flow through xylem vessels or indirect blockage through initiation of tylose formation. Cell aggregates may also facilitate pathogen spread from vessel element to vessel element via enzyme digested pit membranes (Newman et al., 2004) — individual cells likely lack sufficient ‘enzymatic power’ to breach pit membranes, but a compact aggregate of cells would be much more effective in this regard. Furthermore, enzyme production may not be expressed in individual cells, but be regulated in aggregates associated with quorum sensing. From the standpoint of the pathogen, cell aggregates and biofilms likely facilitate nutrient adsorption, protection from environmental stresses, and phytochemicals.

Ascertaining how *Xf* inhabits the xylem environment and how it blocks the transpiration stream through the production of biofilms and bacterial cell masses is deemed informative toward facilitating development of novel control approaches. Furthermore, insight into the selective acquisition, retention, and transmission of *Xf* by leafhopper vectors represents a priority area of interest. Earlier, we demonstrated several unique and important features of *Xf* biology not previously recognized, including the observation that the bacteria possess functional type IV pili that allow the cells to migrate via twitching motility upstream against the transpirational flow in grape xylem elements (<http://www.nysaes.cornell.edu/pp/faculty/hoch/movies/>; Meng et al., 2005; **Figure 1**), that they possess type I pili that function in adhering the cells to xylem (De La Fuente et al., 2007a; 2007b; Li et al., 2007), and more recently that, at some as yet undefined time or condition, individual bacteria that are separated by relatively great distances



**Figure 1.** ‘Rafts’ of *Xf* cells actively migrate outward from colony margins (upper). Migration is through extension and retraction of type IV pili at one pole of the cell. Immunostaining of similar rafts with an antibody (Agdia) reveals the common polar site of both type I and type IV pili and the cell alignment that allows raft migration. Bars, 10µm.

'autoaggregate' into large masses (De La Fuente et al., 2008). In our *in vitro* studies, this occurred after six or more days of growth (initiated from only a few cells) in PD2 media. Aside from a slow population build up of cells in xylem vessels at or near sites of sap flow constrictions (pits, element end-wall openings) which we consider cell aggregates, it is possible that many individual cells normally distributed throughout xylem elements are able to quickly autoaggregate into large cell masses contributing to vessel blockage. This phenomenon may explain, in part, why Pierce's disease symptom development (reddening and drying of leaf margins) occasionally occurs within a short time span.

## OBJECTIVE

Understanding the relationship of *Xf* cells within the confines of the xylem environment is our long term goal. To that end, this project centers on the development and importance of bacterial cell aggregates and biofilms, and their involvement in expression of Pierce's disease. We previously reported that *Xf* cells 'autoaggregate' as the cell population matures in PD2 media within microfluidic devices (De La Fuente et al., 2008). That observation has led us to examine the biological and genetic mechanism associated with this phenomenon by generating aggregation and autoaggregation-defective mutants. Such mutants were examined for their activities within microfabricated 'artificial' xylem vessels (which provide superior observation opportunities) as well as in *bona fide* xylem vessels, for disease development, and for vector transmission. More recently we have directed our attention toward similar aggregation phenomena and biofilm development in these artificial xylem vessels using grape xylem sap in addition to the nutrient rich PD2 medium.

Specific objectives are to:

1. Identify genes associated with aggregation and autoaggregation of *Xf* cells.
2. Assess spatially and temporally aggregation and autoaggregation activities as they occur in planta and in microfluidic 'artificial' xylem vessels.
3. Assess selected aggregation and autoaggregation-defective mutants in planta for disease development and movement within the plant.
4. Assess aggregation mutants generated in the first objective, and related attachment mutants already in hand, for acquisition, retention, and transmission by sharpshooter vectors.

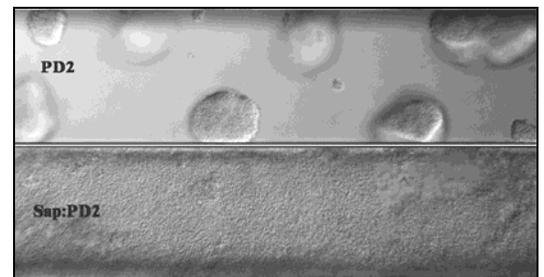
We reported previously many aspects of the results of our studies toward these objectives (in 2005, 2006, 2007 Pierce's Disease Research Symposium Proceedings, as well as in several publications—De La Fuente et al., 2007a; 2007b; 2008). This report summarizes our most recent observations pertaining to aspects of the objectives which remained to be investigated, as well as reporting coincidental observations made during the course of our studies.

## RESULTS

**Mutants, aggregation, rafts.** Numerous *Xf* mutants, including *pilB*, *pilO*, *fimA*, *pilY1*, *hecA*, *xadA*, double mutants *fimA-pilO*, etc. were generated and screened for deficiencies in the ability to form cell aggregates when suspended in a fluid environment. Thus far, only the *xadA* mutant from Lindow's group exhibited such a deficiency; all other mutants formed aggregates. Furthermore, mutants deficient in either type I or type IV pili, or both (e.g., *fimA-pilO*), aggregated from cell suspensions. This indicates that cell aggregation is not entirely dependent upon pilus-related adhesins, but more likely on cell surface adhesins. In addition, raft formation *in vitro* on firm surfaces (agar, cellophane, etc.), a phenomenon we relate to aggregation in liquid environments (xylem sap, PD2, etc.) occurred in *fimA* mutants, indicating that type I pili were not necessary for this phenotype and that the sole presence of type IV pili was sufficient for rafts to form. Rafts do not form in mutants deficient for type IV pili, e.g., *pilB*, because they cannot twitch-migrate (Meng et al., 2005). The side-to-side arrangement of *Xf* cells in rafts is likely reliant upon cell surface adhesins, in addition to the presence of type IV pili (for movement and alignment). We were not able to relate the surface adhesin of *xadA* to raft development since this mutant was either developed from a twitch-deficient parent or this characteristic was lost in course of the mutation. Our next goal will be to site direct a *xadA* mutation into an existing *fimA* mutant to test this possibility.

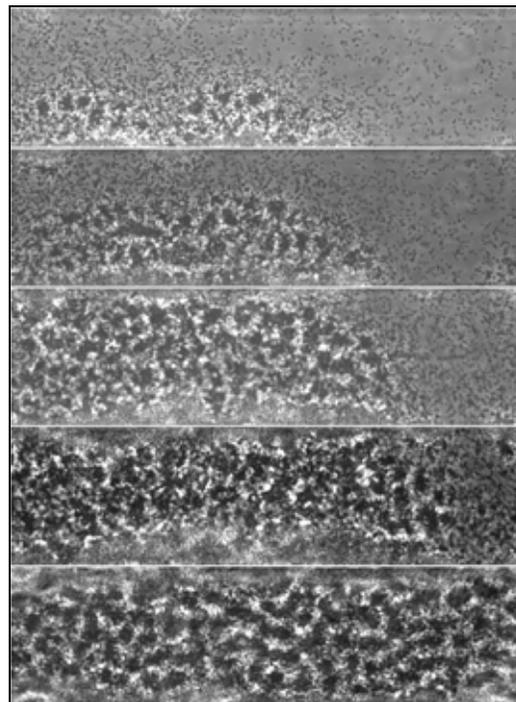
### ***Xf* aggregation and biofilm development in PD2 and xylem sap.**

Until now all our studies with cell motility, aggregation and biofilm development were in PD2 broth—in both microfluidic devices and in culture flasks (Meng et al., 2005; De La Fuente et al., 2008). This was because we were not able to culture *Xf* in grape xylem sap, even though that is where the organism lives *in planta*. Either collected sap was oxidized and became inhospitable to *Xf*, or possibly *Xf* lost its ability to grow in sap after being continually cultured in a nutrient rich media such as PD2. We were able to grow *Xf* in summer grape xylem sap (Chardonnay, provided by Hong Lin, USDA, Parlier, CA) following initial growth in PD2, and gradually increasing the percentage of sap over several days. Subsequently, we used spring sap obtained from Chardonnay as well as from *Vitis riparia* and *Vitis labrusca* grapes grown in Geneva, NY and noted no difference in *Xf* growth. *Xf* grown in side-by-side microfluidic channels, one with PD2 and one with a mixture of 50:50 (PD2:Chardonnay sap) exhibited different growth



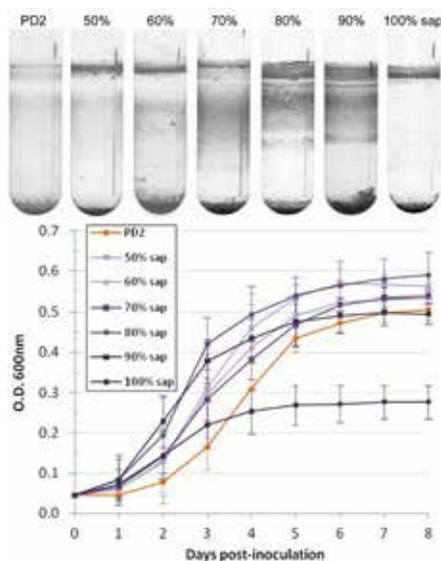
**Figure 2.** Aggregation of *Xf* WT cells on PD2 medium and in a 50:50 mixture of grape (Chardonnay) sap and PD2 after 7-8 days of growth.

habits; grown in PD2, *Xf* developed cell aggregates by seven days as observed previously (De La Fuente et al., 2008), while in the PD2:sap mixture *Xf* growth occurred as a 'lawn' of cells (biofilm) (**Figure 2**). Best growth occurred in mixtures of 20:80 (PD2:sap), although continual growth and biofilm formation also occurred in 90 and 100% sap in microfluidic devices (**Figure 3**). Also, while not quantitatively assessed, it appeared that *Xf* cell type IV pili motility was significantly greater in sap and sap mixtures than in PD2 alone. Being able to grow *Xf* in grape sap provides a more natural environment in the microfluidic devices to assess other aspects of *Xf* biology. Since culturing of *Xf* in sap in microfluidic devices, we have now been able to grow it in tubes and flasks containing sap, again by increasing sap concentrations gradually to 100%. Also, we are now able to store *Xf* at -80 C in 100% sap, and retrieve viable cells, shortening the time interval for sap media culture. Notable is the observation that not only is biofilm formation in 80-90% sap significantly more robust and greater than in PD2, but cell growth is also greater in higher percentage mixtures of sap and PD2 (**Figure 4**).



**Figure 3.** Sequential development of WT *Xf* biofilm over 6 days in a mixture of 10:90 PD2:Chardonnay sap in microfluidic channels.

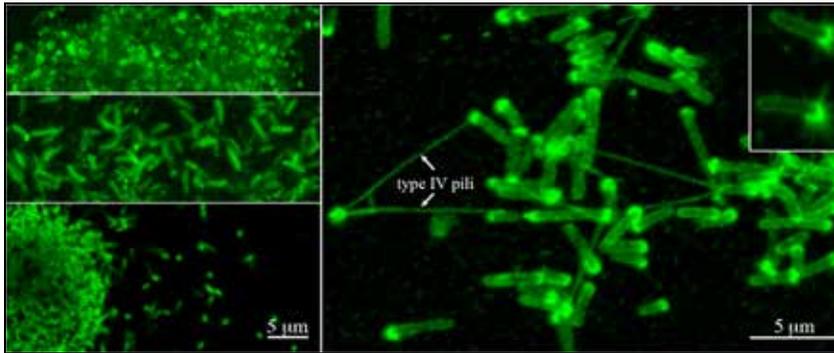
**Production of antibodies against *Xf*.** Characterization of *Xf* pili and other cell surface characteristics, e.g., adhesins, is important to understanding the biology of the pathogen. One means of approaching this is to use antibodies and specifically monoclonal antibodies (MAB's) to visualize localization of these properties. In addition, such antibodies may be useful in inhibition of migration and colonization of *Xf* *in vitro*, and possibly *in planta*. We have thus produced MAB's in mice toward various surface proteins of *Xf*. A number of cell surface localizations are visualized using these antibodies (**Figure 5**). In addition, blood serum (polyclonal antibodies) from immunized mice exhibited excellent recognition of *Xf* cell surface antigens including both type I and type IV pili (**Figure 5**).



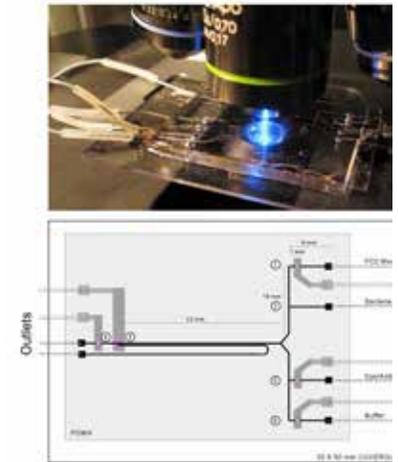
**Figure 4.** Biofilm formation and growth of *Xf* in mixtures of PD2 and grape xylem sap. *Xf* biofilms were most robust and greatest in 80-90% sap following 7 days growth (upper). Similarly, growth of *Xf* was greatest in sap:PD2 mixtures (lower).

#### **Presentation of antibodies, ligands, dispersion chemicals, etc. to *Xf* cells.**

One of our goals is to examine temporally, activities of *Xf* cells when presented with various chemicals that may affect cell motility, aggregation, and biofilm development. We wish to do this in a way that excess non-bound chemical can be removed from the cell environment, and at the same time observe cell behavior. To accomplish this, we developed microfluidic devices with valves that can be activated open or closed have been devised in which the environment around treated cells can be flushed, and the treated cells moved to an adjacent chamber for observation (**Figure 6**). We will next expose cells to fluorescent protein stains such as CY3 to observe activities of the type IV pili under different environmental conditions, as well as to treatments that may influence cell activities.



**Figure 5.** Immunocytochemical localization of cell surface components of WT *Xf* with MAb's reveals predominately one cell pole (left top), the site of type I pili, and outer surface proteins (left middle, left bottom). Polyclonal mouse serum localizes several cell surface features, including cell surface, type IV pili, and type I pili as a bright pole (right), and individual type I pili (right inset).



**Figure 6.** 'Valved' microfluidic device (upper) and schematic of components (lower). Five pneumatic independently operated valves (gray components) control flow and exposure of *Xf* cells to various treatments.

## CONCLUSIONS

Observations from this period demonstrate the pronounced role that pili have in *Xf* attachment, aggregation, and biofilm formation. We have demonstrated that microfluidic devices can effectively serve as 'artificial xylem vessels' to gain valuable information about the biology of *Xf*, and to infer roles for these phenomena *in planta*. To our knowledge, this is the first time that *Xf* has been reported to be cultured in 100% grape xylem sap, and advancement that should provide better insight into *Xf* cell colonization and biofilm development.

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# EXPLOITING A CHEMOSENSORY SIGNAL TRANSDUCTION SYSTEM THAT CONTROLS TWITCHING MOTILITY AND VIRULENCE IN *XYLELLA FASTIDIOSA*

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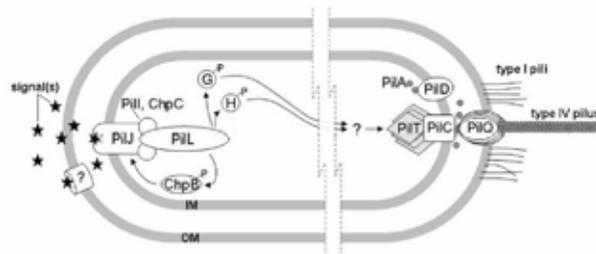
**Reporting Period:** The results reported here are from work conducted July 2008 to September 2008.

## ABSTRACT

Here we provide evidence that twitching motility (TM) in *Xylella fastidiosa* (*Xf*) is dependent on a cluster of signal transduction pathway proteins (*tonB*, *pilG*, *pilI*, *pilJ*, *pilL*, *chpB* and *chpC*), which is related to the system that controls flagella movement in *Escherichia coli*, and highly similar to chemosensory system controlling TM in *Pseudomonas aeruginosa*. The gene *pilL*, coding for a putative kinase, is shown to be essential for TM as an insertional mutation in this gene resulted in a twitching –minus phenotype in agar and inside microfluid chambers, and reduced biofilm formation. We constructed a new site-directed mutant on *pilL* (called *pilL<sub>2</sub>*), which confirms the minus phenotype observed for this gene. This second mutation on *pilL* affects biofilm formation as well. We demonstrate that *pilG-chpC* region is organized as an operon. In addition, we showed that *tonB* gene is also required for TM and complementation experiments restore the TM phenotype of *tonB* mutant. TEM revealed that type IV (and type I) pili are present on all mutants in the cluster, indicating that none of the chemosensory-related genes affects the pili production and instead are likely to be involved in the sensory regulation of TM. We also report our advances on the heterologous complementation of swarming motility phenotype in *E. coli* methyl-accepting chemotaxis protein (MCP) mutant using the *Xf* chemoreceptor, *pilJ*.

## INTRODUCTION

Bacteria sense and respond to changes in their environment, integrating the signals to produce a balanced response. *Xf* is non-flagellated xylem-restricted gram-negative bacterium that moves inside of grapevines via TM that employs type I and type IV pili (Meng et al. 2005). Movement controlled by a chemosensory system was first reported in *E. coli* where a group of *che* genes regulated the rotation movement of its flagella. These proteins work by means of a phosphorylation cascade to ultimately control the direction of flagella rotation (Blair, 1995). In *P. aeruginosa* the chemosensory regulation of type IV pili is controlled by genes in the clusters *pilGHIJK* and *chpABCDE* (Whitchurch, 2006). We previously described the new cluster of genes involved in TM likely to be responsible for the chemosensory regulation of type IV pili in *Xf*. (**Figure 1**). Herein, we further characterize this cluster reproducing mutations in *pilL* and describing a new gene *tonB* as part of this cluster.



**Figure 1.** Model for chemosensory regulation of twitching motility in *X. fastidiosa*. PilJ, the single polar methyl-accepting chemotaxis protein senses environmental signal(s). PilL phosphorylates, PilG, PilH and ChpB. ChpC and PilI couples PilL to PilJ. ChpB mediates adaptation to a constant chemical concentration by adjusting the methylation level of the receptor. Some aspects still unknown are, for example, the nature of the signal(s) and whether they diffuse or are actively transported across the outer membrane. For schematic purposes not all pili components are shown. (from Burr et al. 2007)

## OBJECTIVES

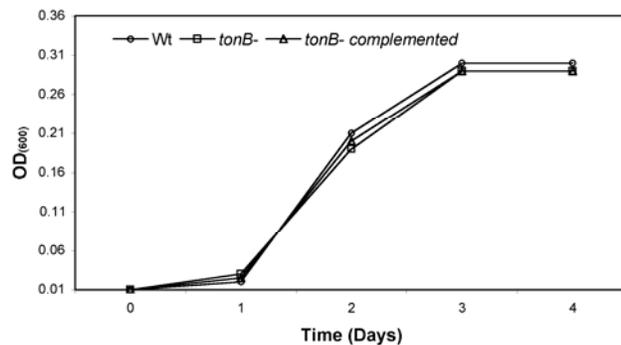
1. Complete characterization of the single chemosensory regulatory system of *Xf* and its function in Pierce's disease and, in particular, we will focus on its role in mediating bacterial movement and biofilm formation. Toward this we will:
  - a. Obtain *Xf* mutants in the *pilJ* gene that encodes the single methyl-accepting chemotaxis protein in *Xf*.
  - b. Assess virulence and motility of *pilJ* mutants in grapevines, as well as previously created mutants deficient in related chemosensory genes, *pilL*.
2. Identify environmental signals that bind PilJ and activate chemosensory regulation. Toward this we will:

- Express PilJ or a chimeric form of PilJ in a strain of *E. coli* previously deleted of all methyl-accepting chemotaxis protein genes.
- Subsequently, candidate signals will be screened using the above *E. coli* system for activation of motility.

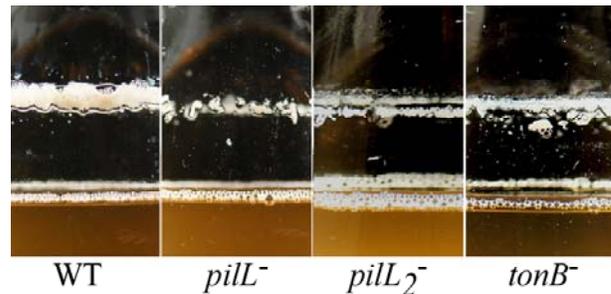
## RESULTS

**Construction of null mutants strains of *Xf* for the chemosensory cluster.** The construction of an allelic exchange mutant for *pilL* gene in *Xf* was performed according to Chatterjee et al. 2008 with slight modifications. The disruption of the *pilL* locus in marker-exchange mutants was confirmed by sequencing and PCR. The mutation occurred in the codon 968. The *pilL* mutant was designated as *pilL*<sub>2</sub>. The construction of a null mutant for the *pilJ* gene is underway.

**Growth, biofilm, and pilus formation.** We previously described a *tonB* mutant (Burr et al. 2007) and here we show that complementation analysis of the *tonB* mutant was accomplished by cloning the gene in pBBR1MCS-5 followed by transformation. No significant differences in growth rates between the mutant and complemented mutant were observed when compared to wild-type (**Figure 2**). Therefore, the lack of twitching observed in mutants was not correlated with growth. The development of biofilms by wild-type, *pilL*, *pilL*<sub>2</sub>, *tonB* mutant, and complemented *tonB* are shown in **Figure 3**. The *tonB* mutant formed significantly less biofilm than the wild-type strain, and biofilm formed by complemented *tonB* was similar to the wild-type (not shown). Similarly, the *pilL* and *pilL*<sub>2</sub> mutants formed less biofilm than the wild-type. Electron microscopy revealed that *pilL*, *pilL*<sub>2</sub>, and *tonB* mutants as well as the complemented *tonB* possess type I and type IV pili confirming that these genes are not involved in pili biogenesis. The twitching phenotype by *tonB* mutant therefore is due to the absence of a functional TonB protein, which is predicted to be accessory to the type IV pilus machinery contributing to the release of pili subunits. Similarly, we predict the abolishment of twitching in the *pilL* mutant is due to lack of histidine kinase binding to the chemoreceptor.



**Figure 2.** Growth curves of *Xf* wild-type, *tonB* mutant and complemented mutant from a 10-day period (4-day data shown). Experiments were repeated at least three times using five replicates each.

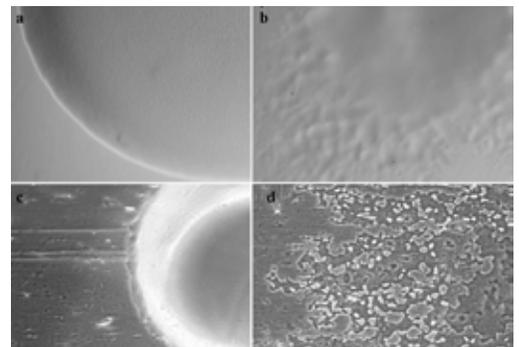


**Figure 3.** Biofilm formation by *Xf* grown for 10 days in culture flasks. The *tonB*, *pilL* and *pilL*<sub>2</sub> mutant biofilm layers were significantly smaller than *Xf* wild-type.

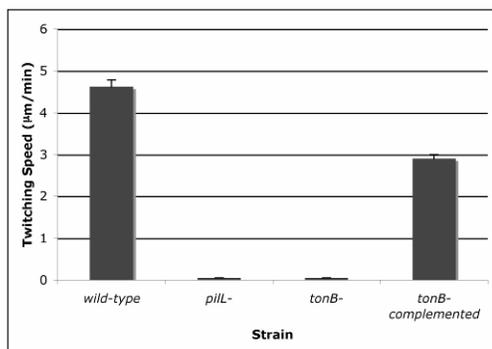
**Twitching motility.** Examination of *pilL*, *pilL*<sub>2</sub>, and *tonB* mutants on PW agar surfaces revealed colony morphologies with smooth margins consistent with loss of twitching function (**Figures 4a and 4c**). Complementation *tonB* (C) showed the restored fringe phenotype similarly observed in the wild-type (**Figures 4b and 4d**)

We measured the speed of movement of *Xf* wild-type, *pilL* and *tonB* mutant and *tonB*-complemented on PW agar surface. The complemented mutant shows a slight reduction in the speed of movement when compared to the wild-type, but TM phenotype was restored (**Figure 5**).

**Transcriptional analysis of the chemosensory cluster.** We also investigated the effect of the transposon insertion in *pilL* on the transcription of neighboring coding sequences by semi-quantitative RT-PCR. This analysis showed not only that wild-type expression levels are retained in the mutant (in this case *pilL*) but also that *pilG*-*chpC* comprise an operon (**Figure 6**). Transcriptional analysis of *tonB* and *tonB*-complemented are underway.



**Figure 4.** Colony morphology of *tonB* mutant (a, c) and *tonB*-complemented (b, d) grow on PW agar surface (a, b) and on cellophane overlaid on agar (c, d) for 4 days. TM fringe is pronounced in b and d.

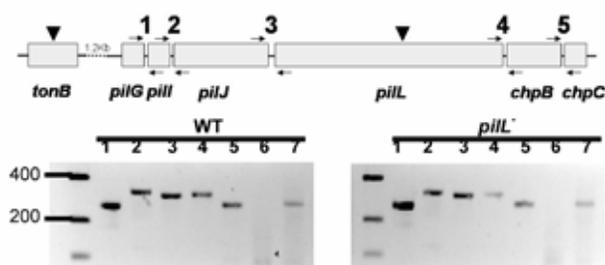


**Figure 5.** Speed of twitching movement of *X. fastidiosa* wild-type, *tonB* mutant, and complemented *tonB* mutant cells on PW agar. Values shown are means of at least 15 cells.

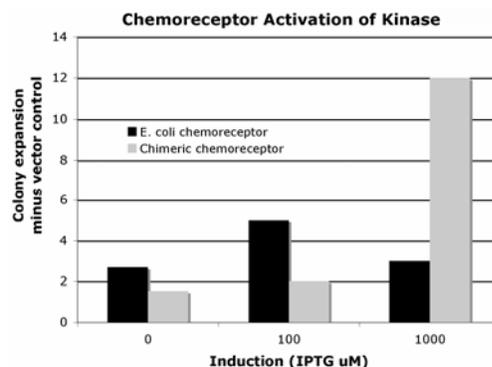
**Virulence and movement on grapevines.** The *pilL*, *pilL*<sub>2</sub>, and *tonB*, mutants were inoculated into grapevines in September 2008. The virulence and movement will be assessed in about 12 weeks. *pilJ* and *tonB*-complemented will be assayed in the Spring of 2009.

**Complementation of *Xf* chemoreceptor in *E. coli*.** The putative *Xf* chemoreceptor, *pilJ*, was cloned and expressed in an *E. coli* strain lacking all chemoreceptors and methylating proteins, UU1535 (Bibikov, et al. 2004). SDS-PAGE analysis of whole cell lysates suggested that limited PilJ protein was produced, presumably due to differences in codon usage between the two organisms (not shown). We are currently working towards eliminating this problem.

**Construction of a chimeric chemoreceptor.** A chimeric chemoreceptor was constructed that contains the periplasmic ligand binding domain of the *Xf* putative chemoreceptor PilJ fused to the cytoplasmic signaling domain of *E. coli* chemoreceptor Tsr. The construct was expressed in an *E. coli* strain UU1535 (Bibikov et al. 2004), and Western blot analysis, using antibodies to the *E. coli* chemoreceptor portion (Ames and Parkinson. 1994), suggested that the chimeric chemoreceptor was produced, although at lower levels than wild-type *E. coli* chemoreceptor. Again differences in codon usage were suspected to be affecting protein production. Over-expression of the chimera protein revealed its ability to activate the chemotaxis kinase as measured by pseudotaxis (Wolfe and Berg, 1989; Ames and Parkinson, 1996), an assay measuring expansion of the colony on a soft agar plate (Figure 7). We are currently examining if the chimera supports chemotaxis.



**Figure 6.** RT-PCR showing operon structure of *pil-chp* cluster. Total RNA treated with DNase was used to amplify fragments indicated by black arrows in the top diagram. (1) *pilG-pilI*; (2) *pilI-pilJ*; (3) *pilJ-pilL*; (4) *pilL-chpB*; (5) *chpB-chpC*; (6) *pilG-pilI* with no reverse transcriptase but with DNA polymerase; (7) *pilG-pilI* fragment amplified from genomic DNA.



**Figure 7.** Expansion of colonies (pseudotaxis) in mm on soft agar plates indicates activation of the chemotaxis kinase. *E. coli* chemoreceptor optimal induction at 100µM IPTG while chimeric protein required ten-fold more induction. Chemoreceptors were expressed in an *E. coli* strain lacking chemoreceptors and methylating proteins, and grown on tryptone soft agar plates incubated for 22.5 hours at 30 C.

## CONCLUSIONS

Our results with the complementation of *tonB* and a construction of a second mutation in *pilL*, *pilL*<sub>2</sub>, show these genes are required for twitching motility in *Xf*. They also play a role in biofilm formation i.e. the mutation reduces the amount of biofilm and may play a role in virulence. Initial studies with the chemoreceptor suggest that both the *Xf* chemoreceptor, *pilJ*, and chimeric protein express in *E. coli* and that the chimeric protein successfully interacts with the chemotaxis kinase. This project is in initial stages and over the next nine months we will finish investigating *pilL* and the roles of *pilJ*. We will also be able to begin exploring the signals that trigger the chemosensory regulation in *Xf*.

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## THE ROLE OF TYPE V SECRETION AUTOTRANSPORTERS IN THE VIRULENCE OF *XYLELLA FASTIDIOSA*

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**Reporting Period:** The results reported here are from work conducted October 1, 2007 to September 30, 2008.

### ABSTRACT

Autotransporters are multi-domain proteins that are responsible for secreting a single specific polypeptide (passenger domain) across the outer membrane of Gram-negative bacteria. Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xylella fastidiosa* (*Xf*) Temecula 1 (*Xf*-PD). Most of our work during the period under review has focused on PD0528 and PD1379, the AT-1 autotransporters whose passenger domains contain tandem repeats of a 50-60 amino acid motif that is only found in *Xf* species. These studies indicated that both PD0528 and PD1379 have a major impact on autoaggregation and biofilm formation *in vitro*. Furthermore, grapevines infected with a strain carrying a mutation in PD0528 do not develop Pierce's disease. We have also initiated experiments to characterize PD0218, PD0313, and PD0950, the three autotransporters predicted to have proteolytic activity. We have generated strains containing single mutations and a strain containing mutations in both PD0218 and PD0950. Experiments are currently underway to generate a strain carrying mutations in all three genes. Characterization of the triple mutant should provide insight into the role of the secreted serine proteases in the *Xf* infection cycle.

### INTRODUCTION

*Xylella fastidiosa* (*Xf*) is a Gram-negative, xylem-limited bacterium and is the causative agent of Pierce's disease (PD), a devastating disease of grapevines (for a recent review, see (Chatterjee et al. 2008)). The ability of *Xf* to colonize grapevines and to incite disease is dependent upon the capacity of this bacterium to produce a diverse set of virulence factors. Many of these virulence factors are proteins that must be secreted to the bacterial cell surface or released into the external environment before they can contribute to pathogenicity. In Gram-negative bacteria, this secretion occurs through one of seven major pathways, Types I to VI and the chaperone-usher pathways (Henderson et al. 2004, Hodak and Jacob-Dubuisson 2007, Cascales 2008). These pathways are highly conserved and exhibit functionally distinct mechanisms of protein secretion.

One of the simplest secretion mechanisms is exhibited by the AT-1 autotransporters, a subcategory of Type V secretion systems (Henderson et al. 2004, Hodak and Jacob-Dubuisson 2007). AT-1 systems are dedicated to the secretion of a single specific polypeptide called the passenger domain across the outer membrane. Virulence functions associated with passenger domains include proteolytic activity, adherence, biofilm formation, intracellular motility, cytotoxic activity, or maturation of another virulence determinant. Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xf*-PD. Functional sequence predictions of these genes indicate that three of these secreted proteins have proteolytic activity (PD218, PD0313, PD0950), one protein has lipase/esterase activity (PD1879), and two of the proteins encode tandem repeats of a 50-60 amino acid motif that is only found in *Xf* species (PD0528, PD1379). Establishing the role of these secreted proteins in *Xf* cell physiology and virulence will provide new targets for researchers to use in generating tactics that disrupt the ability of *Xf* to colonize plant tissue and to initiate the PD disease cycle in susceptible grapevines.

### OBJECTIVES

The primary goal of this project is to determine the role of the six *Xf*-PD autotransporter proteins and their passenger domains in *Xf* cellular physiology and virulence. Given the importance of AT-1 proteins in the virulence of other Gram-negative pathogens, it is highly likely that most of the *Xf*-PD AT-1 proteins will play an important role in *Xf* virulence.

1. Generate a mutation in each of the six AT-1 genes and determine their impact on *Xf* cell physiology and virulence. The construction of strains carrying double and triple mutations in the various autotransporters is also part of this objective.
2. Examine the biochemical properties and location of the six AT-1 passenger domains. Priority will be given to any gene identified in Specific Aim 1.

### RESULTS AND DISCUSSION

We have successfully generated mutations in five of the six AT-1 genes by the gene replacement method (Feil et al. 2003). This procedure involved generating plasmids, which contain an antibiotic resistance marker flanked on each side by ~750 base pairs of the appropriate chromosomal sequence. To facilitate the construction of double and triple mutants, one of four different antibiotic resistance markers was used: chloramphenicol (Cm<sup>R</sup>), erythromycin (Em<sup>R</sup>), gentamicin (Gm<sup>R</sup>), or kanamycin (Kn<sup>R</sup>). The resulting plasmids were then introduced into *Xf* by electroporation. *Xf* cells containing the desired mutations were identified by plating the cells onto antibiotic-containing PD3 plates. Polymerase chain reaction (PCR) was

then used to confirm that a double crossover event had occurred between the disrupted gene carried on the plasmid and the wild-type gene on the *Xf* chromosome.

To date, this series of experiments has resulted in the construction of five strains carrying a mutation in one of the AT-1 autotransporters. We have also generated a strain, which carries a mutation in PD0794. Although PD0794 is not predicted to encode an AT-1 autotransporter or to be localized to the outer member, it is classified as a paralog of PD0528 and PD1379 based on its similarity to their passenger domains. Since PD0794 might be secreted to the cell surface by a different type of secretion system, we decided to include it in our characterization of the PD0528 and PD1379 passenger domains.

Characterization of the AT-1 autotransporters with *Xf*-species specific passenger domains:

The passenger domains of PD0528 and PD1379 contain tandem repeats of a 50-60 amino acid motif. PD0528 contains six copies of this repeat, whereas PD1379 contains three copies. Their paralog PD0794 contains four copies. To investigate the role of this *Xf*-species specific motif in cell physiology and virulence, we first generated strains that carried a single mutation in each of the genes: TAM103 (PD0528::Cm<sup>R</sup>), TAM127 (PD1379::Gm<sup>R</sup>), and TAM145 (PD0794::Em<sup>R</sup>). We then began to construct strains containing mutations in multiple genes. To date, two of these strains have been generated: the double mutant TAM128 (PD0528::Cm<sup>R</sup>, PD1379::Gm<sup>R</sup>) and the triple mutant TAM140 (PD0528::Cm<sup>R</sup>, PD1379::Gm<sup>R</sup>, PD0794::Em<sup>R</sup>).

**Table 1.** Properties of strains carrying mutations in genes having the *Xf*-species specific motif.

Strain	Mutations	Doubling Time (hr)	Clumping in Liquid	Biofilm formation
Temecula	Wild-type	13.8	++	0.55 ± 0.13
TAM103	PD0528::Cm <sup>R</sup>	13.5	+	0.34 ± 0.14
TAM127	PD1379::Gm <sup>R</sup>	13.9	+	0.42 ± 0.13
TAM145	PD0794::Em <sup>R</sup>	13.4	+	0.29 ± 0.04
TAM128	PD0528::Cm <sup>R</sup> PD1379::Gm <sup>R</sup>	13.7	+	0.23 ± 0.07
TAM140	PD0528::Cm <sup>R</sup> PD1379::Gm <sup>R</sup> PD0794::Em <sup>R</sup>	13.5	+	0.23 ± 0.11

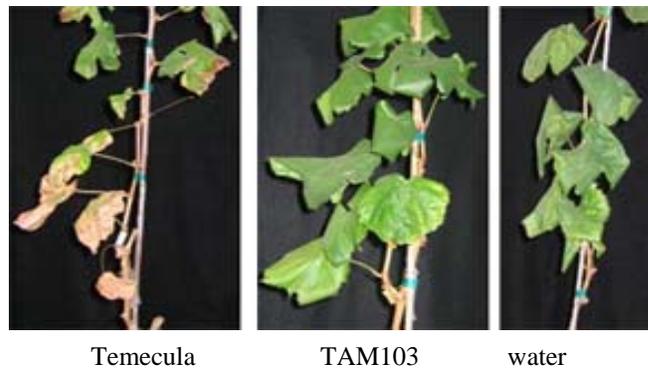
The next step was to investigate the impact of these mutations on *Xf* cell physiology. As shown in **Table 1**, the growth rate of *Xf* is not affected by the elimination of genes carrying the *Xf*-species specific passenger domain. However, even single mutations had an impact on the ability of *Xf* to form clumps in liquid and a biofilm on a glass surface. This would suggest that all three genes contribute to the ability of *Xf* to autoaggregate and to form a biofilm on a solid surface.

Another method for determining the contribution of the individual *Xf*-species specific passenger domains to cell physiology and virulence is to express the protein in a heterologous system. This strategy has been used to generate *E. coli* strains that display the passenger domain of heterologous autotransporter proteins on their cell surface. These recombinant strains have been employed for binding assays, for developing antibody specificity tests, and for exposing antigenic determinants for vaccine development (Yang et al. 2004). In last year's Symposium Report (Igo 2007), we described our successful use of this strategy for analyzing the PD0528 passenger domain. Specifically, we introduced the plasmid pAM61, which carries the gene encoding PD0528 into the *E. coli* strain UT5600. UT5600, which has been used to express other autotransporter proteins, is deficient in the outer membrane proteases OmpT and OmpP. The presence of the PD0528 gene in UT5600 (UT5600/pAM61) results in autoaggregation and the formation of a biofilm, properties not normally demonstrated by the parental *E. coli* strain. Thus, expression of PD0528 in *E. coli* establishes that PD0528 plays a direct role in autoregulation and biofilm formation in this heterologous system and by inference, in *Xf*.

During the period under review, we performed a more detailed analysis of the *E. coli* strain containing the PD0528 gene. To facilitate this analysis, we generated an antibody to the PD0528 passenger domain and then used it to perform Western analysis on UT5600 and UT5600/pAM61. As expected, the antibody did not hybridize to any proteins in UT5600 and recognized a single band in UT5600/pAM61 that corresponds to the predicted size of the PD0528 protein. We next examined the location of PD0528 in *E. coli*. Using a protease accessibility assay (Yen et al. 2007), we established that PD0528 is present on the *E. coli* cell surface. The PD0528 antibody was also used to perform immunofluorescence microscopy. Together, these results indicated that PD0528 is localized to the *E. coli* surface and suggest that the components necessary for secreting autotransporters like PD0258 to the cell surface are conserved between *E. coli* and *Xf*.

We also performed Western analysis on a number of *Xf* strains. In one study, we examined the specificity of the PD0528 antibody by comparing the hybridization pattern obtained for three *Xf* strains: Temecula, TAM103 (PD0528::Cm<sup>R</sup>), and TAM127 (PD1379::Gm<sup>R</sup>). This study revealed that antibody made with the PD0528 passenger domain does not hybridize to the PD1379 protein. Given the fact that there is only ~50% identity between the two proteins, this is not surprising. Based on this result, we plan to use the PD0528 antibody to examine the localization of PD0528 to the *Xf* cell surface using the protease accessibility assay and immunofluorescence microscopy.

Finally, we examined the impact of the absence of PD0528 on *Xf* virulence *in planta*. In this experiment, grapevines were infected by pinprick using the protocol provided by our cooperator Dr. Bruce Kirkpatrick (Guilhabert and Kirkpatrick 2005). Briefly, *Xf*-Temecula and TAM103 were grown at 28°C on PD3 plates. The cells were harvested after 7-10 days and the suspension was adjusted to a concentration of 10<sup>9</sup> cells/ml. Then, 20 µl of the adjusted suspension was used to inoculate three Thompson seedless grapevines by the standard pinprick method. The parental Temecula wild strain served as a positive control, whereas a water inoculation served as a negative control. The vines were then monitored for symptom development.



**Figure 1.** PD0528 impacts *Xf* virulence *in planta*. An *Xf* suspension of Temecula or TAM103 was used to inoculate Thompson seedless grapevines using the standard pinprick method. These photographs show a representative vine 16 weeks after infection.

As shown in **Figure 1**, the vine inoculated with TAM103 does not exhibit the symptoms associated with Pierce's disease, suggesting that PD0528 plays an important role in *Xf* virulence. As expected, symptoms were observed in the grapevines inoculated with *Xf*-PD Temecula and were not observed in the grapevines inoculated with water. To confirm the presence of *Xf* in both the Temecula-infected and TAM103-infected vines, petiole tissues from each vine were harvested one inch and six inches above the inoculation sites. The samples were then processed using published protocols (Guilhabert and Kirkpatrick 2005). Serial dilutions of the samples were made and plated onto PD3. The plates were then incubated at 28°C for 7-10 days and the numbers of *Xf* colonies were compared. *Xf* colonies were observed on both sets of PD3 plates, confirming the presence of *Xf* in the Temecula-infected and TAM103-infected vines. Interestingly, the TAM103-infected vines appeared to have approximately 10-fold fewer *Xf* cells. However, further experiments are needed to determine whether or not this 10-fold difference is significant.

#### Characterization of the AT-1 autotransporters with subtilisin-like serine protease passenger domains:

The passenger domains of three AT-1 autotransporter proteins (PD0218, PD0313, and PD0950) are predicted to encode subtilisin-like serine proteases (Bateman et al. 2004). Extracellular subtilisin-like serine proteases have been implicated in defense, growth on proteinaceous compounds, and the proteolytic maturation of virulence factors (Henderson et al. 2004). In order to investigate the role of these genes in *Xf* pathogenicity, we first generated null mutations in each of these genes and then examined their impact on *Xf* cell physiology. As summarized in **Table 2**, the mutation in PD0218 affects clumping in liquid and biofilm formation, whereas the mutation in PD0950 only affects clumping in liquid. In contrast, the mutation in PD0313 does not appear to affect clumping in liquid, but may result in increased biofilm formation. Although still preliminary, these results suggest that the proteolytic activities of the PD0218, PD0313, and PD0950 passenger domains may make different contributions to *Xf*-PD physiology and virulence.

**Table 2.** Properties of strains carrying mutations in genes having a serine protease passenger domain.

Strain	Mutations	Appearance of Single Colonies	Clumping In Liquid	Biofilm Formation
Temecula	Wild-type	6-8 days	++	0.55 ± 0.13
TAM148	PD0218::Cm <sup>R</sup>	6-8 days	+	0.34 ± 0.09
TAM100	ΔPD0313::Km <sup>R</sup>	6-8 days	++	0.79 ± 0.11
TAM147	PD0950::Em <sup>R</sup>	6-8 days	+	0.50 ± 0.03
TAM149	PD0218::Cm <sup>R</sup> PD0950::Em <sup>R</sup>	6-8 days	+	0.44 ± 0.07

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# EFFECTS OF GRAPE XYLEM SAP AND CELL WALL CONSTITUENTS ON *IN VITRO* GROWTH AND CELL WALL DEGRADING GENE EXPRESSION OF *XYLELLA FASTIDIOSA*

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## ABSTRACT

Purified cell-wall constituents or grape xylem sap added to media affected *Xylella fastidiosa* (*Xf*) *in vitro* growth, biofilm formation, cell aggregation and gene expression. Media containing xylem sap from Pierce's disease (PD)-susceptible plants provided better support for bacterial growth and biofilm formation than media supplemented with xylem sap from PD-resistant plants. Culturing *Xf* on media containing various purified cell-wall constituents demonstrated that CM-cellulose, xylan,  $\beta$ -D-glucan, k-carrageenan, cello-oligosaccharide and laminarin promoted bacterial growth whereas lichenan strongly suppressed growth. However, only laminarin, xylan, and k-carrageenan promoted biofilm formation *in vitro*. Lichenan, oligosaccharide, k-carrageenan, laminarin, xylan and  $\beta$ -D-glucan all significantly decreased bacterial cell aggregation *in vitro*. Quantitative real-time PCR assays revealed that expression of genes encoding extracellular endoglucanase, endo-1,4-beta-glucanase, and periplasm protease were differentially regulated in response to amendment of media with xylem sap from PD-resistant and PD-susceptible grapevines. This study indicates that composition of xylem sap and cell walls may influence the interaction of *Xf* with grape hosts *in planta* and may account for differences in pathogenesis of *Xf* on PD-resistant and -susceptible grapevines.

## INTRODUCTION

*Xylella fastidiosa* (*Xf*) is a Gram negative, xylem-limited bacterium causing Pierce's disease (PD) of grapevine (1). *Xf* is transmitted by xylem-feeding insects, including the polyphagous and invasive glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (2). However, the mechanisms of *Xf* pathogenicity in host plants are not fully understood. The currently accepted explanation for development of PD in grapevine is water stress resulting from occlusion of xylem vessels by bacterial biofilm and/or accumulation of extracellular polysaccharides and subsequent blockage of xylem vessels with pectins, tyloses and gums produced by the plant host in response to *Xf* infection (2). There also is a functional relationship between xylem chemistry and *Xf* planktonic growth, aggregation and biofilm formation within *Vitis* germplasm (3, 4). We hypothesize that cell wall degradation products may affect *Xf* cell growth, aggregation, biofilm formation, and movement within xylem vessels either directly as a source of nutrients and/or indirectly by induction or repression of *Xf* genes.

## OBJECTIVES

1. Determine the comparative effects of xylem sap from PD-resistant and PD-susceptible grapes on growth, biofilm formation, and cell aggregation *in vitro*;
2. Determine the effects of a variety of cell-wall constituents on *Xf* growth, biofilm formation, and cell aggregation *in vitro*; and
3. Analyze the effects of xylem saps from PD-resistant and PD-susceptible grapes pre-inoculated with or without *Xf* on the cell-wall degrading enzyme-related gene expression *in vitro*.

## RESULTS

*Effects of xylem sap from PD-resistant and PD-susceptible grapevines and other cell-wall constituents on bacterial planktonic growth:* After one day of culture in liquid PW (-BSA) medium amended with 1X PW medium (minus BSA) – containing xylem sap from PD-resistant or -susceptible plants, no significant differences ( $p < 0.4873$ ) in *Xf* growth was observed. However, after three or seven days, growth of *Xf* in liquid PW (-BSA) medium amended with xylem sap from PD-susceptible plants was significantly greater than growth in the same medium amended with xylem sap from PD-resistant plants ( $p < 0.0282$  and  $p < 0.0177$ , respectively) (**Figure 1A**). Inclusion of BSA in the growth medium at half the normal concentration (1/2X BSA) resulted in more rapid growth of *Xf* (**Figure 1B**) than in PW (-BSA) medium (**Figure 1A**). Nonetheless, *Xf* growth after three days was significantly greater ( $p < 0.0071$ ) when cultured in PW (1/2X BSA) medium amended with xylem sap from susceptible plants relative to growth in the same medium amended with xylem sap from PD-resistant plants (**Figure 1B**). To confirm this result, *Xf* cells grown in liquid PW (-BSA) medium amended with xylem sap

for seven days were plated onto complete solid PW medium (**Figure 1C**). The number of viable cells recovered from growth medium amended with xylem sap from PD-susceptible plants averaged 2.24-fold more than the number of viable cells recovered from medium supplemented with xylem sap from PD-resistant plants (number of *Xf* colonies: 431±7 for xylem sap from PD-susceptible plants versus 192±89 for xylem sap from PD-resistant plants, with  $n = 3$ ,  $p < 0.01$ ). In addition, we determined the effects of cell-wall components on *Xf* growth. Most cell-wall components had positive effects on bacterial growth *in vitro*, among these, cellulose had the most promoting effect for all time points examined ( $p < 0.0001$  at Day 1,  $p < 0.0001$  at Day 3 and  $p < 0.0014$  at Day 7), followed by laminarin ( $p < 0.0550$  at Day 1,  $p < 0.0111$  at Day 3 and  $p < 0.0014$  at Day 7), xylan ( $p < 0.1163$  at Day 1,  $p < 0.0228$  at Day 3 and  $p < 0.0182$  at Day 7), glucan ( $p < 0.8569$  at Day 1,  $p < 0.0126$  at Day 3 and  $p < 0.0244$  at Day 7), and carragerran ( $p < 0.0476$  at Day 1,  $p < 0.0009$  at Day 3 and  $p < 0.9496$  at Day 7); oligosaccharides promoted *Xf* growth only at one day ( $p < 0.0120$  at Day 1,  $p < 0.8030$  at Day 3 and  $p < 0.1636$  at Day 7). In contrast, lichenan inhibited bacterial growth on the third and seventh days ( $p < 0.2126$  at Day 1,  $p < 0.0004$  at Day 3 and  $p < 0.0001$  at Day 7) of culture (**Figure 1D**).

*Effects of in vitro growth medium amendment with xylem sap from resistant or susceptible grapevines and cell wall components on bacterial biofilm formation and aggregation:* As shown in **Figure 2A**, xylem sap from PD-susceptible grapevine significantly increased *Xf* biofilm formation *in vitro* (1.57 times higher than in unamended control,  $p < 0.0162$ ; 1.60 times higher than resistant xylem sap,  $p < 0.0082$ ). In contrast, xylem sap from both PD-susceptible and -resistant grapevines significantly decreased *Xf* cellular aggregation *in vitro* by factors of 3.28 ( $p < 0.0074$ ) and 2.20 ( $p < 0.0333$ ) times lower than the unamended control (**Figure 2B**). Laminarin and k-carrageenan significantly enhanced *Xf* biofilm formation (2.48 fold greater than control,  $p < 0.0006$  for laminarin; 1.59 fold greater than unamended control,  $p < 0.0055$  for k-carrageenan). Laminarin and k-carrageenan also significantly decreased *Xf* cellular aggregation 5.25-fold ( $p < 0.0397$ ) and 4.2 fold ( $p < 0.0178$ ), respectively, compared to that in unamended control medium (**Figure 2B**). Lichenan and cello-oligosaccharide decreased the *Xf* cellular aggregation very significantly (3.00 fold less than control,  $p < 0.0090$ ) and significant (4.67 fold less than unamended control,  $p < 0.0137$ ) levels, respectively, but did not affect *Xf* biofilm formation (**Figures 2A and 2B**). In contrast, *Xf* biofilm formation and cellular aggregation were not significantly different in medium supplemented with xylan and  $\beta$ -D-glucan from that in unamended medium controls.

*Effects of xylem sap from resistant and susceptible grapevines on bacterial cell-wall degrading-related gene expression:* Expression of endo-1,4- $\beta$ -glucanase gene was significantly increased (2.83 fold greater,  $p < 0.05$ ) in PW medium amended with plant xylem sap from PD-resistant grapevine pre-infected with *Xf*, but increased only slightly in medium amended with susceptible plant xylem sap from PD-susceptible grapevine pre-infected with *Xf* (**Figure 3A**). The *Xf* periplasm protease gene was significantly down-regulated (1.67 fold less,  $p < 0.01$ ) in medium amended with xylem sap from PD-resistant grapevines pre-infected with *Xf*. In contrast, the periplasm protease gene expression was significantly up-regulated (2.21 fold greater,  $p < 0.01$ ) in medium amended with xylem sap from PD-susceptible grapevines pre-infected with *Xf* (**Figure 3B**).

*Total protein content and composition changes in PD-resistant and PD-susceptible grape plant xylem sap in response to Xf infection:* As shown in **Figure 4**, the majority of *Xf*-induced host proteins in PD-resistant plants are of low molecular weight (15 – 35 Kd) with high pI values (pI 7 – 10), although a small group of proteins with lower pI values (pI 3.5 - 4) were also induced (**Figures 4A and 4B**). In contrast, only a few host proteins with the similar range of molecular weights and pI values were induced by *Xf* in PD-susceptible plants (**Figures 4C and 4D**). Some *Xf*-induced xylem sap proteins were genotype specific, whereas others were specific to *Xf* infection.

## DISCUSSION

### *Genetic differentiation of xylem sap from PD-resistant and PD-susceptible grapevines*

Highly PD-resistant and -susceptible *Vitis* species were used in this study. Differential host responses to *Xf* infection between the two lines are controlled by a single major locus (the dominant resistance allele is *PdRI*) (7). Host plant response to *Xf* infection differs between resistant and susceptible genotypes at both molecular and physiological levels and also varies with plant organ, as stem and leaf tissues of the same plant respond differently (5,10). Given that *Xf* is limited to xylem vessels, we hypothesized that xylem cell wall properties and chemical composition of xylem fluid may significantly affect *Xf* pathogenesis. Although the biochemical properties of xylem sap from these two grapevines have not been determined in detail, our bioassay and protein analysis indicated that xylem sap from PD-resistant and PD-susceptible grapevines differed in protein composition, especially following *Xf* infection (**Figure 4**).

### *Roles of cell-wall constituents in bacterial growth, biofilm formation and cellular aggregation of Xf*

Within xylem, *Xf* is confined to a poor nutritive environment (8, 9). Upon degradation of xylem cell-walls, xylem fluid in PD-resistant and -susceptible grapevines likely differ both qualitatively and quantitatively with respect to the chemical composition of cell wall degradation products. Our results suggest that different cell-wall constituents have different effects on growth, biofilm formation and cellular aggregation of *Xf* at least *in vitro* (**Figures 1 and 2**). Several cell-wall constituents (cellulose, xylan, glucan, laminarin, carragerran and oligosaccharides) enhanced *Xf* growth *in vitro*. Some cell-wall constituents (laminarin, k-carrageenan, cellulose, lichenan and oligosaccharides) inhibited *Xf* cellular aggregation. Only laminarin and k-carrageenan significantly enhanced biofilm formation, and only cellulose significantly enhanced cellular aggregation *in vitro*. It seems clear that different cell-wall constituents are required and actively involved in the different

processes of bacterial growth, biofilm formation and cell aggregation, of which algae and seaweed laminarin- and k-carrageenan-related cell wall components significantly enhanced both bacterial growth and biofilm formation *in vitro*, whereas only cellulose significantly enhanced bacterial growth and aggregation, and most other cell-wall ingredients tested inhibited cell aggregation *in vitro*. Aggregation may result from clumping of cells facilitated by extracellular polysaccharides and may be the initial step of biofilm formation (11, 12). Cellular aggregation of *Xf* in response to xylem sap from PD-resistant and -susceptible grapevine plants, and to different cell-wall constituents, did not mirror responses in planktonic growth and biofilm formation to the same treatment. It is not clear whether cellular aggregation process *in vitro* is different from that *in planta*.

#### *Cell-wall degrading enzymes potentially involved in the early stage of pathogenicity through interaction of Xf with xylem sap of host plants*

Pathogenicity of *Xf* likely requires biofilm formation leading to xylem vessel blockage and subsequent water stress (12, 13). Regulatory pathways are responsible for the transition from planktonic growth to biofilm formation (14, 15). Gene expression during the early stage of biofilm formation with planktonic bacteria exposed to plant xylem sap resulted in expression of endo- $\beta$ -1,4-glucanase and periplasm protease genes in the xylem sap from PD-susceptible grapevines pre-infected was elevated. Increased expression of these genes by *Xf* in PD-susceptible grapevines presumably would result in more efficient degradation of cell-walls and release more free cell-wall constituents available to support bacterial growth (Figures 3A and 3B). This conclusion is supported by recent studies showing plant pathogenic bacteria are able to lyse and grow on viable host cells by producing a variety of cell-wall degrading enzymes, including endo- $\beta$ -1,3-glucanases, proteases,  $\beta$ -1,6-glucanases, mannanases, and chitinases (16).

## CONCLUSIONS

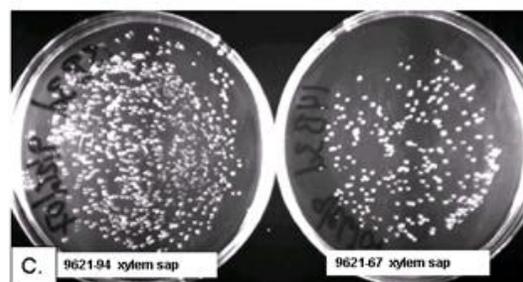
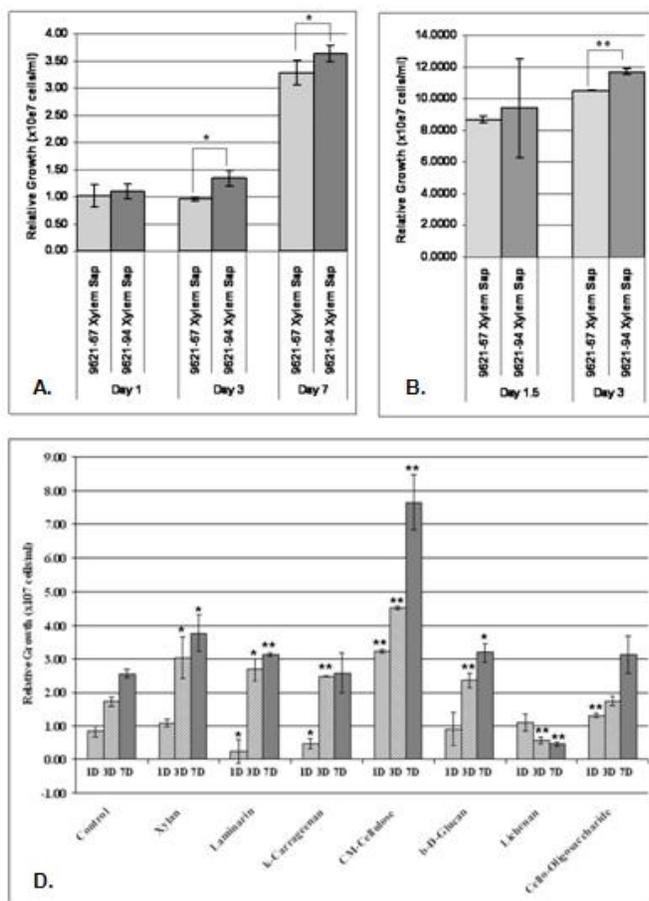
Our observations support the hypothesis that *Xf*-grapevine host-pathogen interactions are mediated by xylem sap constituents, as opposed to a direct connection between bacteria and metabolically active host cells. Therefore, xylem vessels may serve as a unique niche for host plants to recognize and interact with *Xf* in xylem sap *in planta*. If this is the case, identification of components of xylem sap that differ among PD-resistant and -susceptible grapevines may facilitate elucidation of mechanisms through which *Xf*-host plant interactions result in resistance or susceptibility. Xylem sap could be used to screen grapevines for PD resistance breeding.

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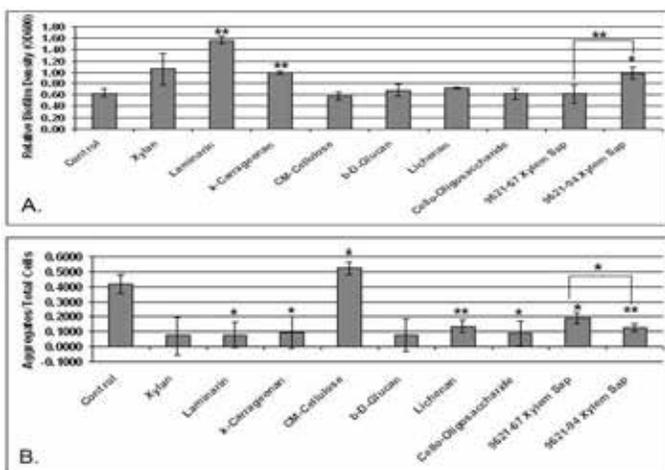
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**FUNDING AGENCIES**

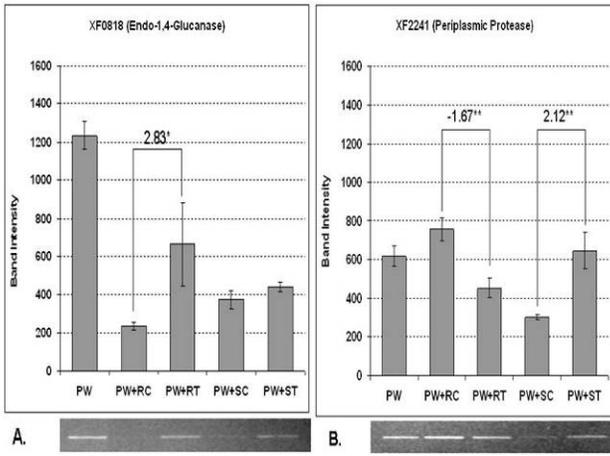
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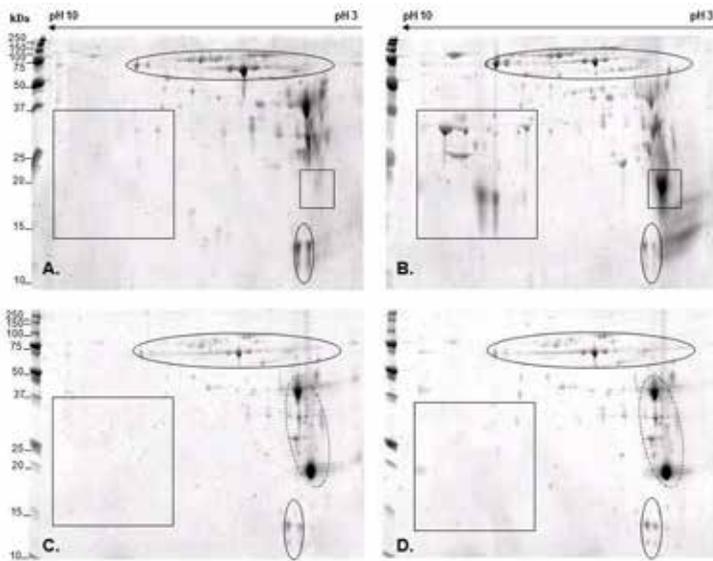
**Figure 1.** Effects of amendment of liquid PW medium with xylem sap from PD-resistant (9821-67) and PD-susceptible (9621-94) grape plants (A-C) or cell wall components (D) on *Xylella fastidiosa* (*Xf*) growth *in vitro*. Asterisk (\*) indicates ANOVA-test results of significance at  $P < 0.05$ ; double asterisk (\*\*) indicates significance at  $P < 0.01$ . A. *Xf* growth in PW medium containing 1/10 X BSA and amended with xylem sap. Bacterial growth was measured after 1, 3 and 7 days of culture in amended media. B. *Xf* growth in PW medium containing 1/2 X BSA and amended with xylem sap. Bacterial growth was measured after 1.5 and 3 days of culture. C. *Xf* colony formation on agar-solidified PW medium (1X BSA) following culture for 7 days in liquid PW (1/10 X BSA) amended with xylem sap from PD-resistant or PD-susceptible plants. D. Effects of cell-wall components on *Xf* growth. Bacterial growth was measured after 1 (1D), 3 (3D), and 7 days (7D) of culture in liquid PW medium (1/10 X BSA) amended with purified cell-wall polymers. Control cultures were grown under the same conditions without amendment with cell-wall polymers.



**Figure 2.** Effects of amendment of liquid PW medium with xylem sap from PD-resistant (9821-67) and PD-susceptible (9621-94) grape plants or cell wall components on *Xf* biofilm formation (A) or cell aggregation (B). Asterisk (\*) indicates ANOVA-test results of significance at  $P < 0.05$ ; double asterisk (\*\*) indicates significance at  $P < 0.01$ .



**Figure 3.** Effect of grape xylem saps on *Xf* gene expression *in vitro*. PW represents PW liquid medium, PW+RC indicates PW liquid medium plus xylem sap from uninoculated PD-resistant plants, PW+RT indicates PW liquid medium plus xylem sap from PD-resistant plants pre-infected with *Xf*, PW+SC indicates PW liquid medium plus xylem sap from uninoculated PD-susceptible plants, and PW+ST indicates PW liquid medium plus xylem sap from PD-susceptible plants pre-infected with *Xf*. Bacterial cultures were subsequently collected for RNA isolation and gene expression analyses.



**Figure 4.** 2D-PAGE analyses of proteins in xylem sap from PD-resistant (9621-67) and PD-susceptible (9621-94) grape plants in response to *Xf* infection. A. 9621-67 uninoculated control; B. 9621-67 *Xf*-infected; C. 9621-94 uninoculated control; D. 9621-94 *Xf*-infected. The pI range is shown on the top, molecular weight standards and sizes are shown on the left. The *Xf*-induced protein zones are boxed, constitutively expressed proteins are circled with solid lines or dashed lines to highlight the different display patterns of protein spots on the gels. Only qualitative analysis was performed to show the presence and variation of major visible protein spots between different xylem sap samples.

## ASSESSMENT OF THE PROCESS OF MOVEMENT OF *XYLELLA FASTIDIOSA* WITHIN SUSCEPTIBLE AND RESISTANT GRAPE VARIETIES

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**Reporting Period:** The results reported here are from work conducted November 2006 to September 2008.

### ABSTRACT

We have followed the movement and population size of a green fluorescent protein (gfp)-marked strain of *Xylella fastidiosa* (*Xf*), simultaneously in both the stems and petioles of Cabernet Sauvignon, Chenin Blanc, Roucaneuf and Tampa grape varieties which differ in susceptibility to Pierce's disease (PD). Very low populations of *Xf* and less frequent occurrence in xylem vessels in the stem were observed in the resistant varieties compared to more susceptible varieties. There was no simple relationship between the population size of *Xf* in the stem and the proportion of vessels colonized when considered over the several varieties; a much higher population size of *Xf* was observed than expected, even after accounting for the higher number of infected vessels, in susceptible varieties. To better understand the distribution of the *Xf* population, particularly in the stem vascular system, we distinguished between high moderate and low levels of cell numbers in a given infested vessel. The higher populations in susceptible genotype stems are achieved because of both higher numbers of infected vessels and particularly due to the much higher extent of colonization of those vessels that become infested with *Xf*. Lower populations in resistant genotype stems are achieved because of both lower numbers of infected vessels and also because of a lower number of cells in the vessels that are colonized. This suggests that in resistant genotypes the movement and multiplication of *Xf* in the stem are both impaired and are co-dependent phenomena. In contrast, similarly high percentages of vessels in petioles of susceptible and resistant plants were colonized, and similar population sizes were attained, suggesting that *Xf* is unrestricted in movement and growth within the petiole. These results indicate that resistance to PD is not due to inhibitory compounds that circulate through the xylem or to host defenses since they might be expected to operate similarly in all tissues. Also, large-scale cell agglomeration in a single vessel is not required for *Xf* to move laterally in the stem to adjacent vessels as the majority of vessels were categorized as having few cells in the vessels in all the genotypes. These results are consistent with earlier work done on Cabernet petioles. In the resistant genotype Roucaneuf we found only low numbers of cells in any vessel, although *Xf* was able to move a distance greater than the average vessel length from the point of inoculation.

Work is continuing using mixtures of isogenic strains of *Xf* to examine the apparent bottlenecks that occur when cells move from one infected vessel into other adjacent uninfected vessel. The efficiency with which cells move from one vessel to another is expected to be related directly to overall susceptibility to PD and should be manifest as a rate of spatial segregation in the plant of the two strains that is inversely related to susceptibility to disease.

### INTRODUCTION

Nearly all studies of *Xf* colonization of grapes have focused on the petioles, with little examination of *Xf* movement and distribution in the stems has been made. Importantly, the work from the Walker lab has noted that the mechanism of resistance to *Xf* is localized within the stem xylem and not fully functional or absent in the xylem of petioles and leaf blades. This was based on the observation that there was little difference in the colonization of the petioles and leaf blades, as opposed to the stems. They speculate that a more constitutive resistance mechanism is present in the stem xylem based on nutritional or structural differences between resistant and susceptible types. Our study was designed to examine differences in the colonization process of the stem of different grape genotypes to identify resistance mechanisms.

In an effort to better understand the process of colonization of grapevines by *Xf*, and develop a method of screening for resistant plant genotypes, we are investigating the spatial segregation of *Xf* cells within the xylem vessel systems of different grape varieties. Single *Xf* strains or an equal mixture of two different isogenic *Xf* strains, are being co-inoculated in different varieties and their movement is being followed closely by culturing and epifluorescence microscopy, with time and distance from the point of inoculation to determine how rapidly spatial segregation of the cells might occur, presumably due to stochastic processes occurring by transfer of only a few cells from one infected vessel to other uninfected vessels. Before initiating studies of the segregation of differentially marked strains of *Xf* in various grape varieties, we explored the process of colonization of *Xf* in stems of Cabernet Sauvignon to establish control data and optimize sampling schemes for the *Xf* strain mixtures. We set out to determine how quickly *Xf* moves within stems throughout the plant, the fraction of the xylem vessels colonized as a function of time and distance from the point of inoculation, and the relative likelihood of finding *Xf* in xylem vessels as compared to tracheal elements. We specifically considered the longitudinal movement of *Xf* in the xylem vessels in the internodal stem locations and the rate at which segregation of the two strains occurs.

## OBJECTIVES

1. Study the process of movement of *Xf* cells between xylem vessels and through plant by determining the changes in proportion of genetically distinct strains of the pathogen initially inoculated into plants at an equal proportion with distance and time from point of inoculation
2. Determine if bottlenecks in movement of cells of *Xf* from xylem vessel to xylem vessel is more extreme in resistant plants than in susceptible plants and whether this phenomenon can be exploited as a tool to screen germplasm for resistance to *Xf*.

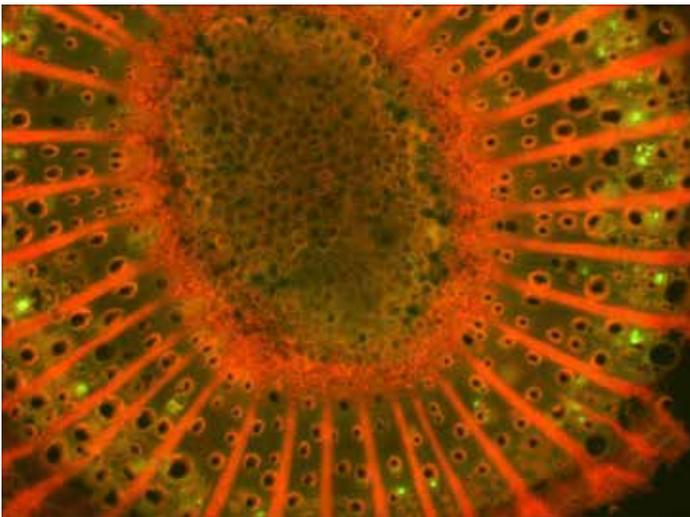
## RESULTS

### Objective 1:

We initiated our investigation by co-inoculating Cabernet Sauvignon stems with a mixture containing an equal amount of wild-type and gfp-marked (KLN59.3) *Xf* strains. This was designed specifically so that the segregation of the two strains could be tracked and correlated to resistance characteristics of the plant variety. The population size of the gfp-marked strain of *Xf* was somewhat smaller at a given location and time after inoculation than the wild-type strain. It was known that this strain caused disease symptoms slightly slower than the wild-type strain, and this difference thus appears to be due to a slower growth in the plant. Given that future experiments will emphasize the spatial segregation of this gfp-marked strain and a similar cyan fluorescent protein (cfp)-marked strain which is expected to have a similar growth rate as the gfp-marked strain we do not expect that this lower growth compared to the wild-type strain will complicate our measurements of ratios of these two strains in up-coming experiments. To best test our model of stochastic processes influencing spatial segregation, it is important that two isogenic bacterial strains used in such studies have nearly identical behavior in the plant. We thus have tested other such strain pairs for suitability for this study. It was found that an *rpfB* mutant of *Xf* was more virulent to grape and moved and multiplied somewhat better in Cabernet than the wild type *Xf*. This was unexpected given that when inoculated singly they each had yielded similar disease severity and progression in the plant. Studies are underway with other isogenic strain pairs of *Xf*. These strain pairs include *Xf* harboring different marker genes introduced into the same intergenic region in *Xf* by the Igo lab, as well as random Tn5 mutants of *Xf* generated by the Kirkpatrick lab that exhibited similar virulence as the wild type strains.

### Objective 2:

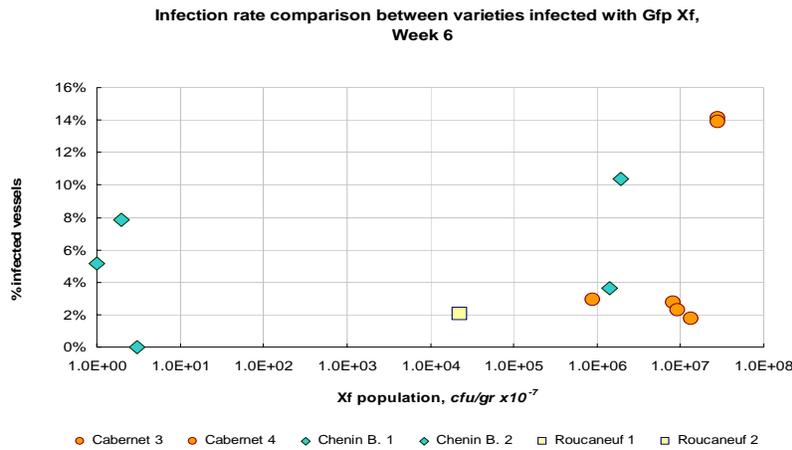
Colonization of susceptible Cabernet Sauvignon and resistant genotypes like Tampa and Roucaneuf by *Xf* was examined by sequential culturing and epifluorescence microscopy. Roucaneuf is a complex hybrid that includes *Vitis cinerea* and *V. berlandieri* and has been described as “fully-resistant” in field conditions to PD. Tampa also is a PD resistant genotype. Microscopy did not reveal any obvious differences in anatomy of the stem and petiole tissues of the resistant and susceptible varieties. Cabernet Sauvignon, Roucaneuf and Tampa plants were inoculated with a gfp-marked *Xf* strain. We followed population growth by culturing and also visually by microscopy (**Figure 1**). Culture sampling was done at weeks two, three, four, six, and 11 following inoculation. A total of six plants at each time point, two from each resistant genotype and two from the susceptible genotype were evaluated. Each plant was sampled at the petiole near the point of inoculation and at six internodal locations 10, 20, 30, 60, 80, and 120 cm away. The sample sites were examined the same day by epifluorescence microscopy. Petioles and portions of the stems were sectioned and prepared for microscopy. An average of nine sections was prepared for each stem location and photos were taken from each sample.



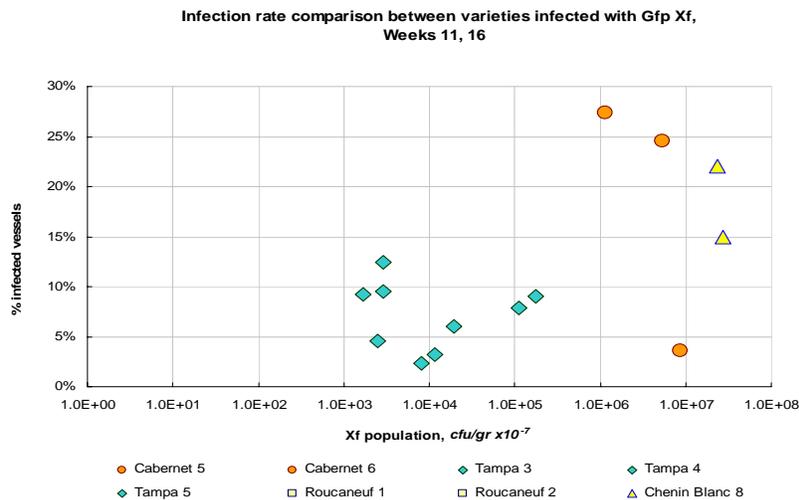
**Figure 1:** Visualization of colonization of Cabernet Sauvignon stems with a gfp-marked strain of *X. fastidiosa*. The plant was sectioned 11 weeks after inoculation and this section was taken from the stem at 30 cm from the point of inoculation. This image is typical of stem tissue from susceptible grape varieties in that a relatively high proportion of vessels harbor at least some cells of *Xf* while most vessels harbor relatively few cells of the pathogen.

It was clear from our observations that a very low proportion of the stem vessels at sites away from the point of inoculation of Roucaneuf and Tampa were colonized by any cells of *Xf* compared to that of Cabernet. There was also a higher viable population sizes of *Xf* in Cabernet in the stem tissue compared to that of Roucaneuf and Tampa. However, there was no simple relationship between the population size of *Xf* in the stem and the proportion of vessels colonized when considered

over the several varieties; a much higher population size of *Xf* was observed than expected, even after accounting for the higher number of infected vessels, in susceptible varieties (**Figure 2**). This raised the question as to whether cells in the resistant varieties may die as they age, or whether there was a large difference in the extent of colonization of those vessels that become infested with *Xf*.



**Figure 2:** Relationship between incidence of colonization of stem vessels of different grape varieties by *Xf* as determined by a microscopic detection of gfp-tagged *Xf* strain (Y-axis) and the population size of *Xf* determined by culturing of small samples of tissue near the site of examination (X-axis).

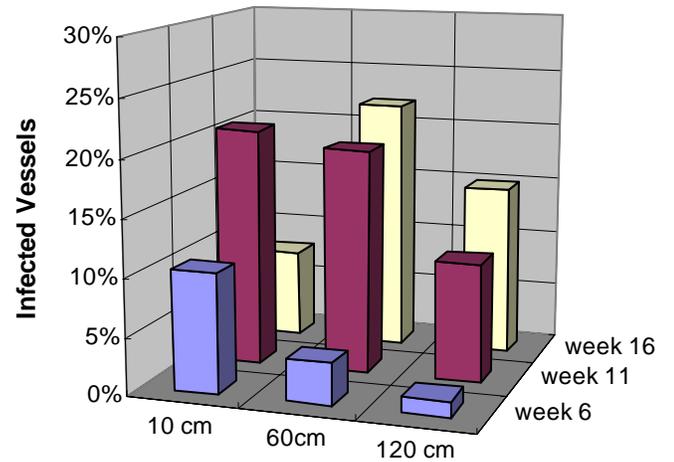
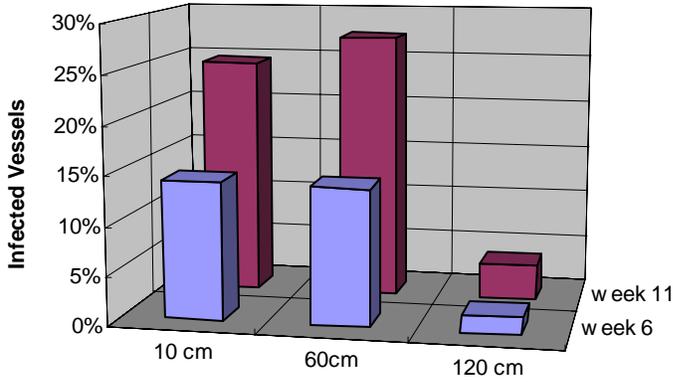


In contrast to the stem tissue, visualization of cells of *Xf* in petioles of Cabernet, Roucaneuf and Tampa reveal that petioles of these plants were both equally well colonized by the gfp-tagged cells of *Xf*. This is in contrast with the stems of these two varieties where very few vessels of Roucaneuf were colonized but a large percentage of vessels of Cabernet were colonized. It was evident that there was no significant difference in bacteria population between the resistant and susceptible genotypes in the petioles (**Table 1**) which is consistent with the work of the Walker lab. In addition, The proportion of the total stem xylem vessels that are colonized by *Xf* appears to be much less than that of the xylem vessels in the petiole for a given variety. Thus the petiole seems to offer little resistant to movement and or multiplication of *Xf* compared to stem tissue.

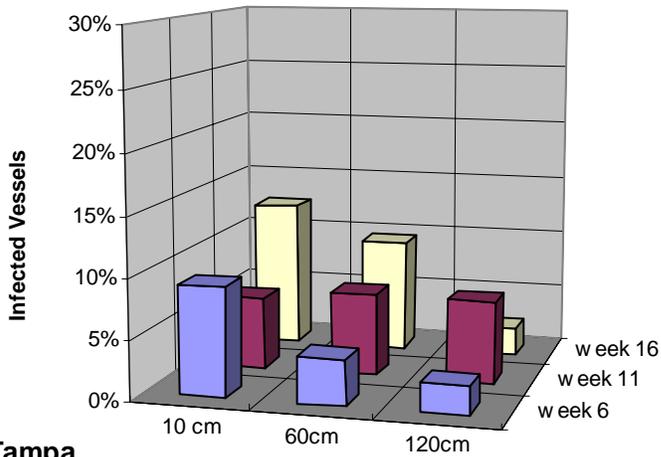
**Table 1.** *Xf* populations in petioles of different grape varieties determined by dilution plating at a given time after inoculation.

week	Xf Concentration, log[(cfu/g+1)]		
	Petiole		
	<i>Roucaneuf</i>	<i>Tampa</i>	<i>Cabernet Sauvignon</i>
3	7.77	4.86	7.60
4	7.71	5.55	7.43
6	7.18	5.22	6.26
11		6.12	8.46

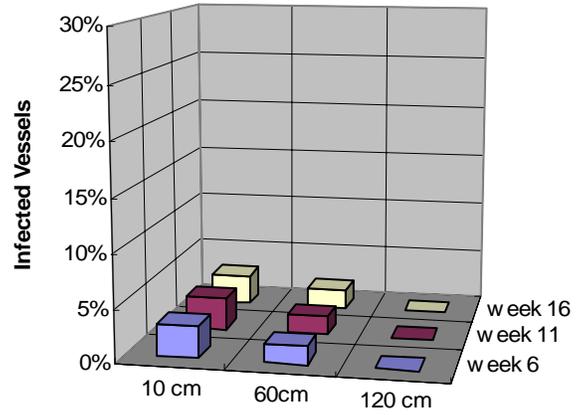
To investigate the model that not only does *Xf* move into more vessels of susceptible varieties than resistant varieties, but it also multiplies more extensively in those vessels into which it moves we performed a more robust examination of colonization of the varieties Tampa, Roucaneuf, Cabernet, as well as Chenin Blanc, a susceptible variety with a slightly more resistance to PD than Cabernet. In addition, to counting number of stem vessels that were colonized by any number of *Xf* cells, we distinguished between those having high levels of colonization (which we estimated to be about 100,000 cells/vessel (labeled “full” in the figures), those having moderate levels of colonization (about 1000 cells/vessel) (labeled “medium” in the figures) or those having minor colonization (less than 10 cells/vessel) (labeled “few” in the figures). The colonization was assessed in the stem for each variety at several different times and distances from the point of inoculation. At each sampling location and time, 12 stem sections were examined under the fluorescence microscope to obtain robust estimates of both incidence and intensity of colonization of vessels. More than 10,000 plant vessels were observed at each sampling. It was clear that the incidence of infestation of stem xylem vessels by *Xf* was related directly to the resistance of these varieties to PD; The highest incidence of colonization of vessels was observed in the highly susceptible Cabernet sauvignon with the lowest in the most resistant variety, Roucaneuf (**Figure 3**). The varieties with intermediate susceptibilities exhibited intermediate levels of colonization incidence. It is evident that near the point of inoculation the proportion of vessels that harbor any number of cells of *Xf* are higher than at more distal sites. With increasing time, the number of vessels colonized also increase. The reduced number of colonized vessels, particularly at distal sites suggests that in resistant genotypes the lateral movement to adjacent vessels is what it is impaired. More importantly, a large difference in the extent of colonization of vessels was observed between varieties. In all varieties, the large majority of vessels harbored relatively few cells of *Xf* (**Figures 4 and 5**). Vessels that harbored very large numbers of *Xf* were only observed in the most susceptible variety Cabernet sauvignon (**Figures 4 and 5**). Likewise, the more susceptible varieties Cabernet Sauvignon and Chenin Blanc both had higher numbers of vessels that harbored intermediate extents of colonization by *Xf* (**Figures 4 and 5**). These differences in extent of colonization were highly statistically different between varieties in most cases (**Table 4**). At increasing distances from the point of inoculation, the resistant genotypes respond more like each other and become more statistical divergent from Cabernet and Chenin Blanc varieties, having lower colonized vessels.



**Cabernet sauvignon**



**Chenin blanc**



**Tampa**

**Roucaneuf**

**Figure 3.** Percentages of infected vessels determined by microscopy (12 stem cross sections each of 28  $\mu$ m thickness examined per location) sampled at different times and distances from the point of inoculation for four grape varieties.

**Table 2:** Differences in extent of colonization of stem xylem vessels in different grape varieties determined by microscopic detection of a gfp-marked strains of *Xf* at different distances from the point of inoculation. The results of an LSD test performed on the mean number of colonized vessels 11 weeks post-infection are shown. Means followed by the same letter within a column do not differ ( $P < 0.05$ ). Vessels having large numbers 100,000 cells/vessel (full), moderate numbers (1000) of cells/vessel (medium) or few (<10) cells/vessel were differentiated.

**Table 2: LSD test for mean number of colonized vessels**

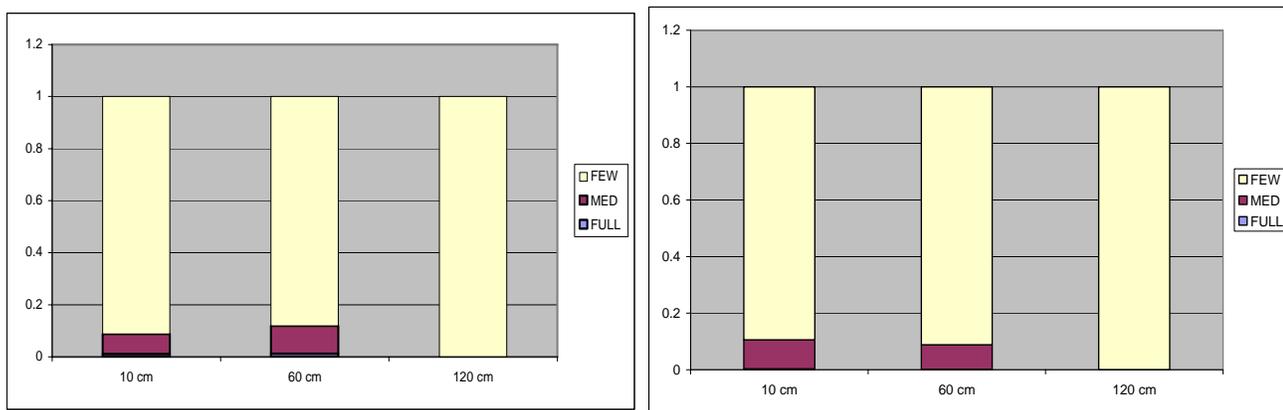
<b>Few cells colonization</b>			
	<b>10 cm</b>	<b>60 cm</b>	<b>120 cm</b>
Cabernet Sauvignon	68.2a	75.7b	8.1a
Tampa	25.5b	13.3a	38c
Chenin Blanc	60.9c	46.9c	4.1ab
Roucaueuf	8d	4.7a	0b

<b>Medium vessels colonization</b>			
	<b>10 cm</b>	<b>60 cm</b>	<b>120 cm</b>
Cabernet Sauvignon	5.7b	8.3b	0a
Tampa	3.4a	0.41a	0a
Chenin Blanc	3.2a	1.41a	0a
Roucaueuf	0c	0a	0a

<b>Full vessel colonization</b>			
	<b>10 cm</b>	<b>60 cm</b>	<b>120 cm</b>
Cabernet Sauvignon	0.9b	0a	0a
Tampa	0a	0a	0a
Chenin Blanc	0a	0a	0a
Roucaueuf	0a	0a	0a



**Figure 4.** Proportion of colonized vessels having different extents of colonization by *Xf* in Cabernet (left) and Chenin Blanc (right).

**Table 3:** Differences in extent of colonization of stem xylem vessels in different grape varieties determined by microscopic detection of a gfp-marked strain of *Xf* at different distances from the point of inoculation. The results of an LSD test performed on the mean number of colonized vessels 11 weeks post-infection are shown. Means followed by the same letter within a column do not differ ( $P < 0.05$ ). Vessels having large numbers 100,000 cells/vessel (full), moderate numbers (1000) of cells/vessel (medium) or few (<10) cells/vessel were differentiated.

**Table 3 : LSD test for mean number of colonized vessels**

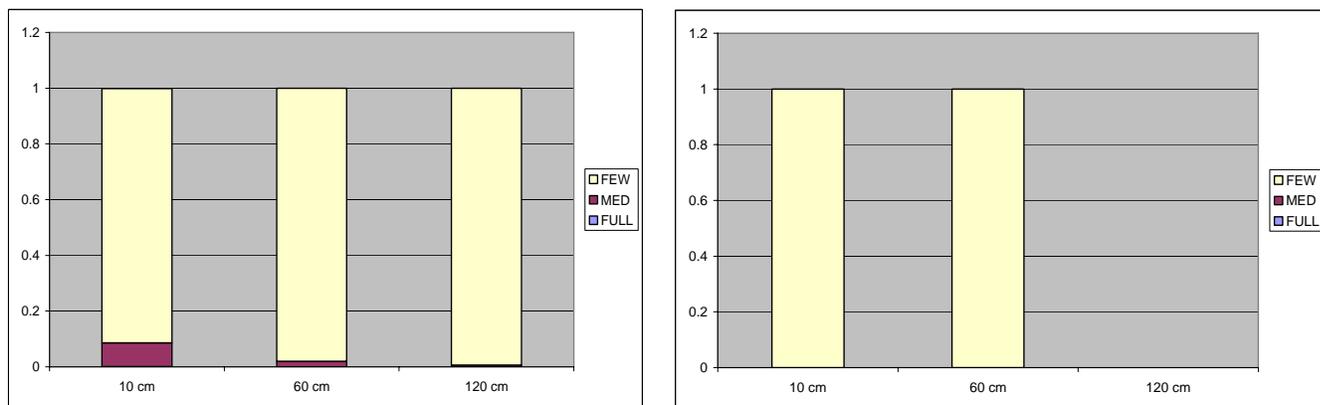
<b>Few cells colonization</b>			
	<b>10 cm</b>	<b>60 cm</b>	<b>120 cm</b>
Cabernet Sauvignon	68.2a	75.7b	8.1a
Tampa	25.5b	13.3a	38c
Chenin Blanc	60.9c	46.9c	4.1ab
Roucaueuf	8d	4.7a	0b

<b>Medium vessels colonization</b>			
	<b>10 cm</b>	<b>60 cm</b>	<b>120 cm</b>
Cabernet Sauvignon	5.7b	8.3b	0a
Tampa	3.4a	0.41a	0a
Chenin Blanc	3.2a	1.41a	0a
Roucaueuf	0c	0a	0a

<b>Full vessel colonization</b>			
	<b>10 cm</b>	<b>60 cm</b>	<b>120 cm</b>
Cabernet Sauvignon	0.9b	0a	0a
Tampa	0a	0a	0a
Chenin Blanc	0a	0a	0a
Roucaueuf	0a	0a	0a



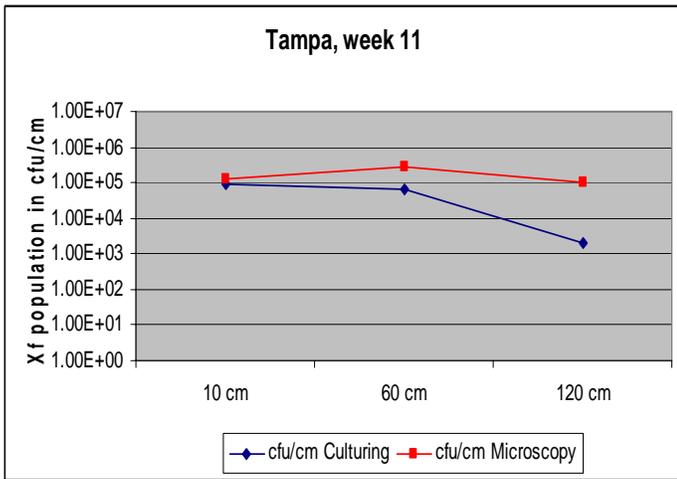
**Figure 5.** Proportion of colonized vessels having different extents of colonization in Tampa (left) and Roucaneuf (right).

**Table 4.** Differences in the proportion of vessels from different grape varieties that had been colonized by any cells of *Xf* that exhibited varying extents of colonization. Microscopic detection of a *gfp*-marked strain of *Xf* at different distances from the point of inoculation was determined. The results of an LSD test performed on the mean number of colonized vessels 11 weeks post-infection are shown. Means followed by the same letter within a column do not differ ( $P < 0.05$ ). Vessels having large numbers 100,000 cells/vessel (full), moderate numbers (1000) of cells/vessel (medium) or few (<10) cells/vessel were differentiated. (Data of **Table 3** expressed as a proportion of the total colonized vessels).

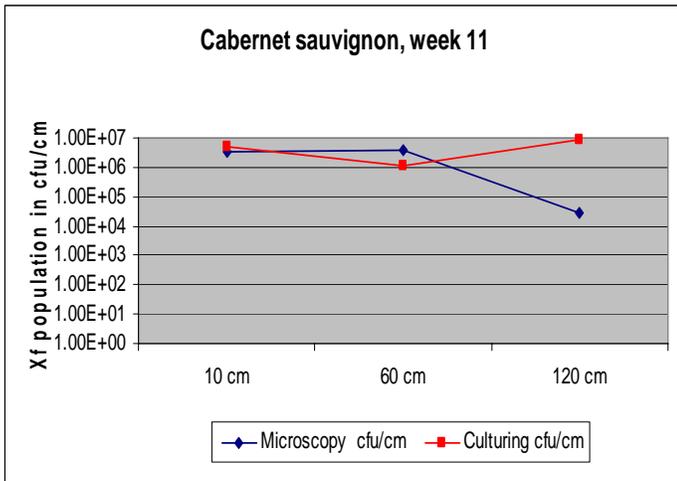
**Table 4: LSD test for proportion mean of vessels colonization**

<b>Few cells colonization</b>			
	10 cm	60 cm	120 cm
Cabernet Sauvignon	0.91a	0.88b	1a
Tampa	0.93a	0.97a	0.99a
Chenin	0.9a	0.92c	1a
Roucaneuf	0.96a	0.98a	0b
<b>Medium vessels colonization</b>			
	10 cm	60 cm	120 cm
Cabernet Sauvignon	0.07a	0.07a	0a
Tampa	0.06a	0.16b	0a
Chenin	0.09a	0.07a	0a
Roucaneuf	0b	0c	0a
<b>Full vessel colonization</b>			
	10 cm	60 cm	120 cm
Cabernet Sauvignon	0.01b	0.01b	0a
Tampa	0a	0.01a	0a
Chenin	0.03c	0.07c	0a
Roucaneuf	0a	0a	0a

Since we had made independent measures of both the incidence and extent of colonization of stem xylem vessels by *Xf* by microscopy as well as direct measures of viable population sizes of *Xf* by culturing of the adjacent tissue, we tested the model that cells of *Xf* had similar frequencies of viability in different grape varieties. We estimated population sizes from microscopy measurements by multiplying the number of infected vessels by the number of cells enclosed in a given vessel and with knowledge of the amount of plant material that had been examined (28  $\mu\text{m}$ /section examined). In locations more proximal to point of infection (POI), the total populations estimated by microscopy were very similar to that of the culturable population, suggesting that most of the cells were viable, irrespective of grape variety (**Figure 6**). At the most distal sites from the point of inoculation, the numbers of *Xf* estimated by microscopy were somewhat lower than the culturable populations; we believe this is due to sampling issues since the relatively few vessels that were colonized by *Xf* at such distances (**Table 3**) made accurate estimates of incidence and extent of colonization difficult and subject to underestimation. Given that the numbers of *Xf* in stems of resistant varieties such as Roucaneuf are low and apparently spatially variable, at a given sampling time, not all visualized stem segments (28  $\mu\text{m}$ /section  $\times$  12 sections/sample) include detectable cells of *Xf*.



**Figure 6.** Comparison of population sizes of *Xf* in in Tampa and Cabernet sauvignon stem segments at different distances from the point of inoculation estimated by culturing (blue) and by microscopy (red) at week 11 post-infection.



## CONCLUSIONS

Resistance to movement of *Xf* in different grape varieties appears to be restricted to the stem tissue and is due to structural differences in the vessels of the resistant varieties and is associated with a limitation of the number of vessels into which *Xf* can spread and thus in which they can grow. It is apparent that the relatively high populations in susceptible genotype stems are achieved because of higher numbers of infected vessels and also due to more extensive colonization of the vessels into which it moves. Since *Xf* was frequently detected in petioles, even in resistant varieties and at some distance from the point of inoculation, it appears that *Xf* follows a sinuous path up the vessels in the stem, never colonizing a large number of vessels, but when it enters the petiole it can multiply to high numbers. The similar populations, estimated by microscopy or plating suggest that most cells in the stem appear to be alive. This suggests that in resistant genotypes in-stem tissue movement and multiplication are impaired as separate or co-dependent phenomena, which doesn't seem to be the case in petioles. Presumably the process of movement of *Xf* from one infected vessel to other adjacent vessels involves the degradation of pit membrane. This degraded plant material is apparently a source of considerable nutrition to *Xf*. That is, those grape varieties that are most easily digested by *Xf* will be both more easily invaded and support more extensive multiplication by *Xf*.

## FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

## GENOTYPING GRAPE *XYLELLA FASTIDIOSA* ISOLATES IN TEXAS

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**ABSTRACT**

Pierce's disease (PD) pressure has always been intense along the Texas Gulf Coast, but the disease has been steadily moving north and west. Additionally, PD has been discovered in counties thought to be beyond the ecological range of either the *Xylella fastidiosa* (*Xf*) bacterium or the insect. As part of our genetic analysis we are analyzing conserved genes (such as *gyrB*) to distinguish new isolates as either 'grape,' 'ragweed' or 'oleander.' However, base pair changes within conserved genes are usually too limited to track genetically relatedness within short time periods. To improve discrimination power, we are using multi-locus simple sequence repeat markers for genotyping each individual isolate. Eighteen grape isolates have been fingerprinted so far. The most similar isolates are found in the same county, but some counties have multiple, genetically distinct isolates. Isolates from new infection areas will be run to determine the relationships with other sources. Additionally, *Xf* SSR genotyping profile in Texas will be analyzed and compared with the profile from California isolates.

**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.



***Section 4:  
Pathogen  
and  
Disease Management***





## RESPONSES OF GROUND COVER PLANT SPECIES TO MECHANICAL INOCULATION WITH DIVERSE *XYLELLA FASTIDIOSA* ISOLATES

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

### ABSTRACT

Ten plant species with potential benefits as groundcovers in and near vineyards at risk for Pierce's disease were grown in containers in greenhouse and screenhouse for mechanical inoculation with *Xylella fastidiosa* isolated from weed, tree, or grape in Texas. Isolates were from *Ambrosia trifida* var. *texana*, *Helianthus annuus*, *Iva angustifolia*, *Nerium oleander*, *Platanus occidentalis*, and *Vitis vinifera*. No symptoms developed and evaluations were with ELISA. Based on mechanical inoculations, lower risk (susceptible to fewer isolates and no grape isolates, low test mean ELISA OD, low test mean Rx [proportion OD $\geq$ 0.300]) species were *Coreopsis tinctoria*, *Verbena rigida*, and *Lolium multiflorum*. Higher risk species were *Phlox drummondii*, *Lupinus texensis*, *Medicago polymorpha*, *M. polymorpha*, *Trifolium incarnatum*, *T. repens*, and *Gaillardia pulchella*. Data from plant species interactions with vector species and senescence dates (vs. vector peak populations) under field conditions may alter our interpretation of risk status.

### INTRODUCTION

Pierce's disease (PD) management suggestions include vegetation management in and near vineyards (Black et al., 2005, 2008), including competition from plants that are poor hosts of *Xylella fastidiosa* (*Xf*) and not commonly used by insect vectors. Traits of *Xf*-safe plants may include low cost to establish, re-seed or re-grow from roots/crowns, require minimal maintenance once established, senesce without mowing or herbicide, provide temporary standing mulch, minimal competition with grapevines for water and nutrients, fix nitrogen, affect other pests in neutral or beneficial manner, enhance vector biological control as insectary plants, and/or seasonal tourist appeal.

Our long term approach to ranking species for risk as ground cover includes ELISA testing (positive was OD $\geq$  0.300, reaction [Rx] was proportion positive) of a) environmental samples from the vicinity of PD vineyards, b) mechanically inoculated greenhouse and/or screenhouse container-grown plants (Black et al., 2006), and c) seeded or transplanted small plots near a PD vineyard. This report addresses only mechanical inoculations with selected isolates (Table 11) in the absence of *Xf* vectors.

### OBJECTIVES

Mechanically inoculate diverse Texas *Xf* isolates into potential ground cover plants and compare PD risk in and near vineyards.

### RESULTS

Candidate species for vineyard and vicinity use represented Polemoniaceae (Table 1), Fabaceae (Tables 2-6), Asteraceae (Tables 7, 8), Verbeneaceae (Table 9) and Poaceae (Table 10). Every potential ground cover species was infected with one or more Texas *Xf* isolates (Tables 1-10). Species were ranked using arbitrary criteria (number of grape isolate treatment means OD $\geq$ 0.3, test mean OD $\geq$ 0.7, test mean Rx $\geq$ 0.6). Based on mechanical inoculations of container-grown plants, lower risk species were *Coreopsis tinctoria*, *Verbena rigida*, and *Lolium multiflorum*. Higher risk species (susceptible to more isolates and grape isolates, high mean OD, high mean Rx) were *Phlox drummondii*, *Lupinus texensis*, *Medicago polymorpha*, *M. polymorpha*, *Trifolium incarnatum*, *T. repens*, and *Gaillardia pulchella*. Data from plant species interactions with vector species and senescence dates vs. vector peak populations under field conditions may alter our interpretation of risk status.

### CONCLUSIONS

No species was immune to grape and non-grape *Xf* isolates. With the criterion of mechanical inoculation in the absence of insect vectore, three plant species may pose low PD risk if used in and near vineyards. Seven plant species may pose significant PD risk if planted or allowed to grow in and near vineyards. Data from field plants exposed to *Xf* vectors are needed to confirm risk status.

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## FUNDING AGENCIES

Funding for this project was provided by the USDA Animal and Plant Health Inspection Service.

**Table 1.** Responses of Drummond phlox (*Phlox drummondii*) to diverse *Xf*. Test A inoculated 17-25Mar08; evaluated 21May08. Test B inoculated 7-22Apr08; evaluated 16Jun08.

Test	Isolate	OD	Rx	OD	Rx	
A	GILGRA288 Ha	2.53	a	1.00	a	
	GILGRA286 At	2.04	ab	1.00	a	
	TRAFILA377 Vv	1.58	bc	1.00	a	
	GIL007 Po	1.47	bc	0.75	abc	
	GILBEC625 Vv	1.18	cd	0.83	ab	
	UVA119 Po	0.68	de	0.58	bc	
	MEDPRI025 No	0.53	ef	0.50	c	
	SCP Buffer	0.08	f	0.00	d	
				<b>Mean</b>	<b>1.26</b>	<b>0.71</b>
				<b>C.V., %</b>	<b>57</b>	<b>48</b>
B	GILGRA286 At	2.45	a	1.00	a	
	TRAFILA377 Vv	1.32	b	1.00	a	
	GILBEC625 Vv	1.30	b	0.83	ab	
	GILGRA288 Ha	1.04	b	0.71	bc	
	UVA119 Po	0.50	c	0.50	cd	
	GIL007 Po	0.49	c	0.25	de	
	MEDPRI025 No	0.13	cd	0.08	e	
	SCP Buffer	-0.01	d	0.00	e	
				<b>Mean</b>	<b>0.90</b>	<b>0.54</b>
				<b>C.V., %</b>	<b>54</b>	<b>60</b>

**Table 2.** Responses of Texas bluebonnet (*Lupinus texensis*) to diverse *Xf*. Test A inoculated 13Apr07; evaluated 2May07. Test B inoculated 3-6Dec07, 22-25Jan08; evaluated 14Jan-11Mar08, 8Apr08. Test C inoculated 22-25Jan08; evaluated 8Apr08.

Test	Isolate	OD		Rx		Site	OD		Rx	
A	GILGRA286 At	0.37	a	0.53	a	Inoculation	0.38	a	0.50	a
	TRAFILA302 Vv	0.37	a	0.33	ab	Above	0.10	b	-0.03	b
	GILBEC625 Vv	0.27	ab	0.33	ab					
	GILBEC532 Vv	0.18	b	0.19	ab					
	TRAFILA377 Vv	0.16	b	0.08	b	<b>Mean</b>	<b>0.27</b>		<b>0.29</b>	
	SCP Buffer	0.09	b	-0.06	b	<b>C.V., %</b>	<b>52</b>		<b>107</b>	
B	UVA119 Po	1.74	a	0.85	ab					
	GIL007 Po	1.74	a	0.95	a					
	GILGRA286 At	1.19	b	0.70	b					
	GILGRA288 Ha	1.16	b	0.90	a					
	GILBEC625 Vv	1.15	b	0.68	b					
	TRAFILA377 Vv	1.03	b	0.90	a	<b>Mean</b>	<b>1.13</b>		<b>0.72</b>	
	SCP	-0.13	c	0.01	c	<b>C.V., %</b>	<b>40</b>		<b>40</b>	
C	GILGRA286 At	1.99	a	1.56	a	Inoculation	0.89	a	0.79	a
	GILGRA288 Ha	1.67	b	1.51	a	Above	0.77	b	0.70	b
	GIL007 Po	0.67	c	0.49	b					
	UVA119 Po	0.53	c	0.51	b					
	TRAFILA377 Vv	0.45	c	0.51	b					
	GILBEC625 Vv	0.43	d	0.66	b	<b>Mean</b>	<b>0.83</b>		<b>0.74</b>	
	SCP	0.07	e	-0.03	c	<b>C.V., %</b>	<b>32</b>		<b>41</b>	

**Table 3.** Responses of burr medic (*Medicago polymorpha* 'Armadillo') to diverse *Xf*. Test A inoculated 17-25Mar08; evaluated 23Apr08.

Test	Isolate	OD		Rx		OD	Rx
A	GILGRA288 Ha	1.58	a	1.00	a		
	GIL007 Po	1.36	ab	1.00	a		
	GILGRA286 At	1.30	ab	0.92	ab		
	TRAFILA377 Vv	1.23	ab	1.00	a		
	UVA119 Po	1.18	ab	0.83	ab		
	GILBEC625 Vv	1.02	bc	1.00	a		
	MEDPRI025 No	0.68	c	0.75	b	<b>Mean</b>	<b>1.06</b>
	SCP Buffer	0.11	d	0.00	c	<b>C.V., %</b>	<b>49</b>

**Table 4.** Responses of small burr medic (*Medicago polymorpha* 'Devine') to diverse *Xf*. Test A inoculated 17-25Mar08; evaluated 28Apr08. Test B inoculated 7-22Apr08; evaluated 28May08.

Test	Isolate	OD		Rx		Site	OD		Rx	
A	UVA119 Po	2.71	a	1.00	a	Inoculation	1.89	a	0.88	a
	GILGRA286 At	2.45	ab	1.00	a	Above	1.72	b	0.77	b
	GIL007 Po	2.34	b	1.00	a					
	GILBEC625 Vv	2.22	bc	1.00	a					
	GILGRA288 Ha	2.17	bc	1.00	a					
	TRAFILA377 Vv	1.94	c	1.00	a					
	MEDPRI025 No	0.51	d	0.58	b	<b>Mean</b>	<b>1.81</b>		<b>0.82</b>	
	SCP Buffer	0.11	e	0.00	c	<b>C.V., %</b>	<b>23</b>		<b>22</b>	
B	GILGRA286 At	3.16	a	1.02	a					
	UVA119 Po	2.92	ab	1.00	a					
	GILGRA288 Ha	2.90	ab	1.02	a					
	GIL007 Po	2.47	ab	1.02	a					
	MEDPRI025 No	1.74	c	1.00	a					
	GILBEC625 Vv	1.50	cd	0.75	b	<b>Mean</b>	<b>1.86</b>		<b>0.79</b>	
	TRAFILA377 Vv	1.10	cd	0.75	b	<b>C.V., %</b>	<b>34</b>		<b>31</b>	
	SCP Buffer	0.15	e	0.00	c					

**Table 5.** Responses of crimson clover (*Trifolium incarnatum*) to diverse *Xf*. Test A inoculated 7-22Apr08; evaluated 14May08. Test B inoculated 29Apr-5May08; evaluated 3Jun08.

Test	Isolate	OD		Rx		OD	Rx	
A	GILGRA286 At	1.37	a	1.00	a			
	GILBEC625 Vv	1.33	a	1.00	a			
	UVA119 Po	1.22	ab	0.83	a			
	GIL007 Po	1.12	ab	0.92	a			
	GILGRA288 Ha	0.97	abc	1.00	a			
	MEDPRI025 No	0.83	bc	0.83	a	<b>Mean</b>	<b>0.96</b>	<b>0.83</b>
	TRAFILA377 Vv	0.58	cd	0.92	a	<b>C.V., %</b>	<b>56</b>	<b>34</b>
	SCP Buffer	0.26	d	0.17	b			
B	GILGRA288 Ha	1.86	a	0.92	ab			
	GILBEC625 Vv	1.77	a	0.92	ab			
	GILGRA286 At	1.76	a	0.75	ab			
	TRAFILA377 Vv	1.65	a	1.00	a			
	MEDPRI025 No	1.53	a	0.83	ab	<b>Mean</b>	<b>1.45</b>	<b>0.73</b>
	UVA119 Po	1.49	a	0.67	b	<b>C.V., %</b>	<b>58</b>	<b>46</b>
	GIL007 Po	1.47	a	0.75	ab			
	SCP Buffer	0.07	b	0.00	c			

**Table 6.** Responses of white clover (*Trifolium repens* 'Durana') to diverse *Xf*. Test A inoculated 1-8Apr08; evaluated 9Jun08. Test B inoculated 16-23Apr08; evaluated 10Jun08.

Test	Isolate	OD		Rx			OD	Rx
A	TRAFILA377 Vv	1.62	a	1.00	a			
	MEDPRI025 No	1.52	ab	0.83	ab			
	GILGRA286 At	1.16	abc	0.67	b			
	GILBEC625 Vv	1.15	abc	0.83	ab			
	GILGRA288 Ha	1.09	bc	0.83	ab			
	GIL007 Po	1.05	bc	0.83	ab			
	UVA119 Po	0.94	c	0.75	ab	<b>Mean</b>	<b>1.07</b>	<b>0.72</b>
	SCP Buffer	0.04	d	0.00	c	<b>C.V., %</b>	<b>57</b>	<b>47</b>
B	MEDPRI025 No	1.59	a	1.00	a			
	GILGRA286 At	1.19	b	1.00	a			
	GIL007 Po	1.09	b	0.92	a			
	GILBEC625 Vv	0.86	bc	0.75	ab			
	GILGRA288 Ha	0.74	c	0.83	a			
	UVA119 Po	0.67	cd	0.50	b			
	TRAFILA377 Vv	0.37	de	0.50	b	<b>Mean</b>	<b>0.82</b>	<b>0.69</b>
	SCP Buffer	0.03	e	0.00	c	<b>C.V., %</b>	<b>52</b>	<b>49</b>

**Table 7.** Responses of plains coreopsis (*Coreopsis tinctoria* var. *tinctoria*) to diverse *Xf*. Test A inoculated 7-22Apr08; evaluated 18Jun08. Test B inoculated 29Apr-5May08; evaluated 24Jun08.

Test	Isolate	OD		Rx		Site	OD	Rx
a	GILGRA288 Ha	1.19	a	0.92	a			
	GILGRA286 At	0.96	b	1.00	a			
	GIL007 Po	0.22	c	0.20	b			
	UVA119 Po	0.05	c	0.00	c			
	TRAFILA377 Vv	0.05	c	0.00	c			
	GILBEC625 Vv	0.03	c	0.00	c			
	SCP Buffer	0.03	c	0.00	c	<b>Mean</b>	<b>0.32</b>	<b>0.27</b>
	MEDPRI025 No	0.02	c	0.00	c	<b>C.V., %</b>	<b>78</b>	<b>63</b>
B	GILGRA286 At	1.14	a	0.83	a	Inoculation	0.264	b
	GILGRA288 Ha	0.80	b	0.83	a	Above	0.418	a
	UVA119 Po	0.39	c	0.42	b			
	GIL007 Po	0.20	cd	0.17	bc			
	MEDPRI025 No	0.06	d	0.08	c			
	GILBEC625 Vv	0.05	d	0.00	c			
	TRAFILA377 Vv	0.05	d	0.00	c	<b>Mean</b>	<b>0.34</b>	<b>0.29</b>
	SCP Buffer	0.04	d	0.00	c	<b>C.V., %</b>	<b>98</b>	<b>109</b>

**Table 8.** Responses of Indian blanket (*Gaillardia pulchella* var. *pulchella*) to diverse *Xf*. Test A inoculated 1-8Apr08; evaluated 18Jun08. Test B inoculated 16-23Apr08; evaluated 23Jun08.

Test	Isolate	OD		Rx			OD		Rx	
A	GILGRA286 At	2.53	a	1.00	a					
	GIL007 Po	2.42	a	1.00	a					
	UVA119 Po	2.38	a	1.00	a					
	GILGRA288 Ha	2.34	a	1.00	a					
	GILBEC625 Vv	2.29	a	1.00	a					
	TRAFILA377 Vv	1.25	b	0.67	b					
	MEDPRI025 No	0.19	c	0.17	c	<b>Mean</b>	<b>1.68</b>		<b>0.73</b>	
	SCP Buffer	0.02	c	0.00	c	<b>C.V., %</b>	<b>23</b>		<b>31</b>	
B	GILGRA286 At	2.76	a	1.00	a					
	GILGRA288 Ha	2.74	a	1.00	a					
	GIL007 Po	2.24	ab	1.00	a					
	UVA119 Po	1.91	bc	0.92	ab					
	TRAFILA377 Vv	1.46	c	0.67	b					
	GILBEC625 Vv	1.35	c	0.75	ab					
	MEDPRI025 No	0.26	d	0.33	c	<b>Mean</b>	<b>1.60</b>		<b>0.72</b>	
	SCP Buffer	0.08	d	0.08	c	<b>C.V., %</b>	<b>45</b>		<b>44</b>	

**Table 9.** Responses of tuber vervain (*Verbena rigida*) to diverse *Xf*. Test A inoculated 17-25Mar08; evaluated 14Jul08. Test B inoculated 7-22Apr08; evaluated 15Jul08. Test C inoculated 9-17Jun08; evaluated 13Oct08.

Test	Isolate	OD		Rx		Site	OD		Rx	
A	GILGRA286 At	1.08	a	0.79	a	Inoculation	0.462	a	0.40	ab
	GILGRA288 Ha	0.70	b	0.67	a	Above	0.588	a	0.48	a
	UVA119 Po	0.44	c	0.46	b	Below	0.258	b	0.27	b
	GILBEC625 Vv	0.23	d	0.33	b	Other stem	0.087	c	0.06	c
	TRAFILA377 Vv	0.11	d	0.04	c					
	GIL007 Po	0.11	d	0.08	c					
	SCP Buffer	0.08	d	0.04	c	<b>Mean</b>	<b>0.35</b>		<b>0.30</b>	
	MEDPRI025 No	0.05	d	0.00	c	<b>C.V., %</b>	<b>102</b>		<b>108</b>	
B	GILGRA286 At	1.13	a	0.79	a	Inoculation	0.53	a	0.46	a
	GILGRA288 Ha	0.70	b	0.54	b	Above	0.471	ab	0.44	a
	UVA119 Po	0.39	c	0.42	b	Below	0.324	b	0.25	b
	TRAFILA377 Vv	0.16	d	0.21	c	Other stem	0.078	c	0.06	c
	GILBEC625 Vv	0.15	d	0.21	c					
	MEDPRI025 No	0.10	d	0.13	c					
	SCP Buffer	0.09	d	0.08	c	<b>Mean</b>	<b>0.35</b>		<b>0.30</b>	
	GIL007 Po	0.07	d	0.04	c	<b>C.V., %</b>	<b>112</b>		<b>119</b>	
C	GILGRA286 At	0.51	a	0.38	ab	Inoculation	0.238	a	0.20	a
	GILGRA281 At	0.47	a	0.50	a	Above	0.23	a	0.20	a
	LLAFAL650 Ha	0.24	b	0.29	b	Below	0.144	ab	0.09	b
	TRAFILA345 Vv	0.11	bc	0.08	c	Other stem	0.107	b	0.07	b
	LLAFAL718 Ig	0.07	c	0.00	c					
	GILBEC530 Vv	0.07	c	0.00	c					
	GILBEC625 Vv	0.07	c	0.04	c					
	TRAFILA344 Vv	0.04	c	0.00	c	<b>Mean</b>	<b>0.18</b>		<b>0.14</b>	
SCP	0.04	c	0.00	c	<b>C.V., %</b>	<b>145</b>		<b>201</b>		

**Table 10.** Responses of annual ryegrass (*Lolium multiflorum* 'Jumbo') to diverse *Xf*. Test A inoculated 17-25Mar08; evaluated 5Jun08. Test B inoculated 7-22Apr08; evaluated 5Jun08.

Test	Isolate	OD		Rx			OD	Rx
A	GILGRA288 Ha	1.48	a	1.48	a			
	GILGRA286 At	1.31	a	1.31	a			
	MEDPRI025 No	1.08	ab	1.08	ab			
	GIL007 Po	0.65	bc	0.65	bc			
	TRAFILA377 Vv	0.30	cd	0.30	cd			
	GILBEC625 Vv	0.19	d	0.19	d			
	UVA119 Po	0.16	d	0.16	d	<b>Mean</b>	<b>0.65</b>	<b>0.52</b>
	SCP Buffer	0.01	d	0.01	d	<b>C.V., %</b>	<b>83</b>	<b>66</b>
B	GILGRA286 At	0.67	a	0.50	a			
	GILGRA288 Ha	0.44	ab	0.50	a			
	MEDPRI025 No	0.20	bc	0.17	b			
	TRAFILA377 Vv	0.16	c	0.25	ab			
	GIL007 Po	0.15	c	0.25	ab			
	GILBEC625 Vv	0.14	c	0.17	b			
	UVA119 Po	0.05	c	0.00	b	<b>Mean</b>	<b>0.23</b>	<b>0.23</b>
	SCP Buffer	0.00	c	0.00	b	<b>C.V., %</b>	<b>133</b>	<b>170</b>

**Table 11.** Host plant origins of *Xf* isolates.

Acronym	Family	Species	Common name
At	Asteraceae	<i>Ambrosia trifida</i> var. <i>texana</i>	Giant ragweed
Ha	Asteraceae	<i>Helianthus annuus</i>	Common sunflower
Ig	Asteraceae	<i>Iva angustifolia</i>	Narrowleaf sumpweed
No	Asclepiadaceae	<i>Nerium oleander</i>	Oleander
Po	Platanaceae	<i>Platanus occidentalis</i>	American sycamore
Vv	Vitaceae	<i>Vitis vinifera</i>	European grape

## EXPLOITING *XYLELLA FASTIDIOSA* PROTEINS FOR PIERCE'S DISEASE CONTROL

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### ABSTRACT

The aim of this project is to construct and express in grapevine, a protein or protein chimera capable of inactivating or otherwise interfering with the infectivity of, or disease-induction by, *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease (PD) of grapevine. A single-chain, monoclonal (scFv) antibody was selected for its ability to bind to *Xf* cells recovered by centrifugation of *Xf* liquid culture. Immunoblot analysis of total protein extracts from *Xf* cells revealed a single band corresponding to a ~47K protein target of this antibody. The identity of the target is unknown but is not likely to be major outer membrane protein MopB or the protein synthesis elongation factor EF-Tu, both of which have gel electrophoretic mobilities similar to the scFv antibody target. Previously we demonstrated that *Xf* EF-Tu induces chlorosis when pressure infiltrated into leaves of *Chenopodium quinoa*, suggesting that EF-Tu may be a protein recognized by plants as a signal of *Xf* infection. Although the primary function of EF-Tu in eubacteria is in protein synthesis, specific bacterial species have evolved to use EF-Tu for other applications, including binding the bacterium to host cells. We found *Xf* EF-Tu to be associated with an insoluble fraction which remains after treating *Xf* cells with lysozyme. Expression of *Xf* EF-Tu in *Escherichia coli* altered the cell morphology. The transformed *E. coli*, when introduced into the petioles of grapevine transformed with reporter constructions driven by a *Xf*-infection-specific promoter, activated synthesis of the reporter. These results suggest that EF-Tu may be a signal in grapevine of *Xf* infection, in addition to its role in protein synthesis.

### INTRODUCTION

Grapevine cultivars resistant to or tolerant of *Xf* presumably present the best approach to long term, effective, economical and sustainable control of PD. The mechanisms by which *Xf* induces symptoms in infected grapevine have not been established. However, interference with symptom development (i.e., creation of tolerance) is conceivable, and *Xf* virulence factors are potential targets for interfering with *Xf* infection and symptom induction. A strategy is to create transgenic rootstock(s) that will secrete a protein or proteins into the xylem for transport to scion xylem to provide protection against insect vector-delivered *Xf* or interfere with symptom development. *Xf* surface proteins are candidate targets in this strategy. Examples of *Xf* surface proteins are a major outer membrane protein MopB, the hemagglutinin-like minor outer membrane proteins HXfA and HXfB (Guilhabert and Kirkpatrick 2005), a protein that is recognized by a single chain, monoclonal antibody (described below), and possibly a form of the protein synthesis elongation factor "temperature-unstable" (EF-Tu). We report here on the single-chain, antibody and then, more extensively, on the properties of *Xf* EF-Tu.

We reported last year that EF-Tu was the major component of a minor trailing band observed after electrophoresis of partially purified MopB through sodium dodecyl sulfate- (SDS-) permeated polyacrylamide gel. *Xf* EF-Tu was recovered by elution from excised gel pieces from the trailing band and was shown to induce chlorosis in *Chenopodium quinoa* (Bruening et al. 2007), whereas *Xf* MopB produced in transformed *E. coli* failed to induce chlorosis in *C. quinoa*. These observations suggest that the chlorosis-inducing factor in our MopB preparations may be *Xf* EF-Tu and not MopB, formerly the candidate chlorosis-inducing factor. EF-Tu is one of a small number of highly conserved eubacterial macromolecules ("microbe-associated molecular patterns" = MAMPs) that have been discovered to induce defense responses in a variety of plants (Jones and Dangl 2006). Flagellin, chitin, certain lipopolysaccharides, and a few other molecules are other MAMPs. EF-Tu is the most abundant soluble protein of rapidly growing *E. coli* cells, so it is reasonable for it to be a signal for the presence of bacteria. That is, at least some EF-Tu proteins act as elicitors. The MAMP activity of *E. coli* EF-Tu is illustrated by alkalization of the medium of cultured *Arabidopsis thaliana* cells on exposure to subnanomolar concentrations of EF-Tu. EF-Tu, when introduced at 1  $\mu$ M by pressure-infiltration into *Arabidopsis* leaves, induced resistance to *Pseudomonas syringae* and accumulated defense gene mRNAs (Kunze et al. 2004). *E. coli* EF-Tu and *Xf* EF-Tu gene sequences show 77% identical and 88% similar in amino acid sequence. The regions of identity between the *E. coli* and *Xf* EF-Tu gene sequences also revealed >90% identity with >100 eubacterial EF-Tu sequences (Kunze et al. 2004). Some bacteria have evolved an EF-Tu protein with an additional function, beyond participating in polypeptide chain elongation or acting as an elicitor.

*Mycoplasma pneumoniae* and *Lactobacillus johnsonii* appear to use EF-Tu as an adhesin that is responsible for the binding of these bacteria to human cells, and, in the case of *M. pneumoniae*, antibody to EF-Tu was demonstrated to interfere with attachment to human cells (Dallo et al. 2002, Granato et al. 2004).

## OBJECTIVES

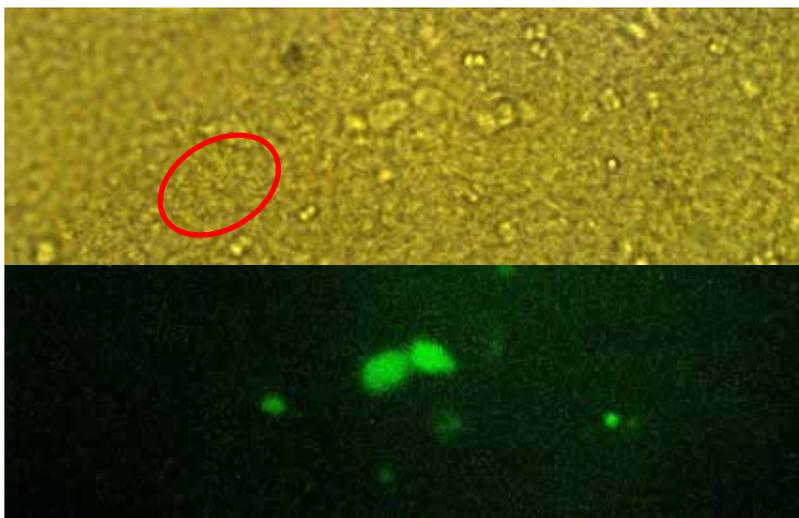
1. Discover or develop peptides and proteins with high affinity for the *Xylella fastidiosa* (*Xf*) cell exterior.
2. Test surface-binding proteins for their ability to coat *Xf* cells, for possible bactericidal activity or for interference with disease initiation following inoculation of grapevine or model plant with *Xf*.
3. In collaboration with the Gupta laboratory, develop gene constructions for chimeric proteins designed to bind tightly to and inactivate *Xf* cells; express and test the chimeric proteins against *Xf* cells in culture and in plants.
4. In collaboration with the Dandekar laboratory, prepare transgenic tobacco and grapevine expressing and xylem-targeting the candidate anti-*Xf* proteins; test the transgenic plants for resistance to infection by *Xf*.

## RESULTS AND DISCUSSION

### *A single chain antibody selected for affinity to Xf cell suspension*

We obtained a library of single chain (scFv) antibodies, expressed on bacteriophage M13 particles, from the University of Cambridge, UK. Cultures of the rapidly growing and minimally aggregating *Xf* strain HXfA-, which has a transposon insertion in the HXfA gene (Guilhabert and Kirkpatrick 2005), were centrifuged to collect the cells. This cell preparation was used to “pan” the M13-scFv library through three cycles. Ten of the 24 selected M13-scFv clones were sequenced, and all had the same deduced amino acid sequence. Therefore, all subsequent analyses were applied to just one selected scFv antibody, designated A2scFv. To identify the epitope bound by the A2scFv, *Xf* proteins were resolved by electrophoresis and were subjected to immunoblot detection using the A2scFv-p3 fusion protein-carrying bacteriophage particle as the “primary antibody” and anti-M13 major coat protein as the target of the secondary antibody conjugate. A single band was observed with mobility corresponding to slightly less than an apparent molecular weight of approximately 47K (results not shown). This is comparable in electrophoretic mobility to two other *Xf* proteins which we have investigated: *Xf* EF-Tu (formula weight 42.9K) and *Xf* mopB (mobility corresponding to molecular weight 45K). To test whether the A2scFv is recognizing either of these *Xf* proteins, we attempted to interfere with A2scFv binding by pre-incubating two immunoblots with peptide polyclonal antibody against *Xf* EF-Tu or polyclonal antibody against *Xf* MopB. No apparent interference with the binding of the A2scFv-carrying bacteriophage M13 particles was observed (data not shown), but the anti-EF-Tu peptide antibody might not block scFv binding to other parts of EF-Tu. However, results from experiments involving digestion of *Xf* cells with lysozyme, as described below, are not consistent with recognition of either MopB or EF-Tu as possessing the epitope of A2scFv (data not shown).

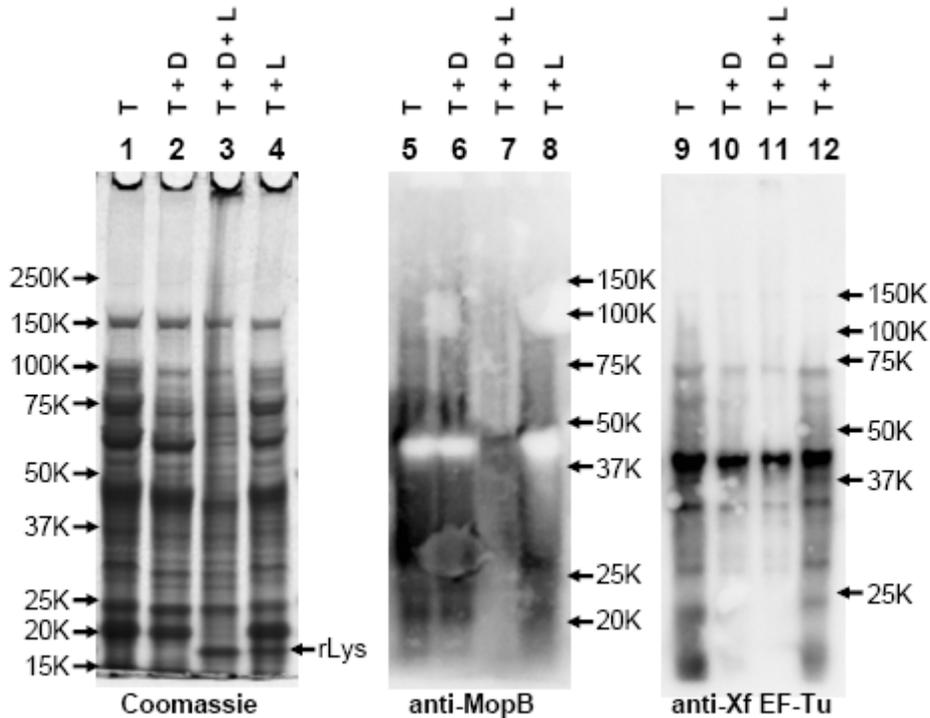
The A2scFv was purified under non-denaturing conditions from solution using its encoded hexahistidine sequence and was labeled with Alexa-488 (fluorescein). The Alexa-488 labeled A2 scFv was incubated with *Xf* cell preparations in phosphate-buffered saline-Triton X100 (PBS-T) (**Figure 1**), and the cell preparations were washed with PBS-T before observing fluorescence. The fluorescence was observed not in association with the cells, as might be expected, but with somewhat larger, amorphous structures that apparently had been collected with the cells during centrifugation or formed during centrifugation. Similar results were observed with the Temecula *Xf* strains bearing mutations in hemagglutinin-like protein genes, Hx fA- and Hx fB- (Guilhabert and Kirkpatrick 2005). These experiments do not eliminate the possibility of weak binding of the scFv to intact *Xf* cells, since weak binding would not have been detected after the washing procedures followed here. At this point, both the protein target and the larger-than-cells target of the A2 scFv monoclonal antibody remain unknown.



**Figure 1.** A Temecula strain *Xf* cell suspension, prepared by centrifugation, was observed under white light (upper panel) and 488nm light using an epifluorescence microscope. Alexa-488 (fluorescein) labeled A2scFv monoclonal antibody was added to the cell suspension, which subsequently was washed 3 times with phosphate-buffered saline containing 0.1% Triton X-100 (PBS-T). The red oval identifies an in-focus field of *Xf* cells.

***Xf* protein synthesis elongation factor “temperature unstable” (EF-Tu) in an insoluble fraction of a *Xf* extract**

Since EF-Tu was found in MopB preparations, and the MopB purification procedure (Bruening and Civerolo 2004) includes extraction from insoluble material, it is likely that at least some EF-Tu of *Xf* is in an insoluble form. Results presented in **Figure 2** suggest that EF-Tu is associated with an insoluble fraction from which some other proteins, including MopB, had been released. HXfA- cells were exposed to a proprietary detergent solution, “BugBuster®,” or they were exposed to lysozyme, or to both. Prior exposure of HXfA- cells to BugBuster® solution reduced the intensity of, or eliminated, a few protein bands (**Figure 2**, lanes 1 and 2), whereas treatment with lysozyme had no apparent effect on the pattern of protein bands (lane 4 compared to lane 1). Incubating the cells with BugBuster® and lysozyme together resulted in diminution or elimination of several protein bands (lane 3 compared to lane 1), including a prominent band with a mobility corresponding to an apparent molecular weight of about 43K, i.e., to the mobilities of *Xf* EF-Tu and *Xf* MopB.

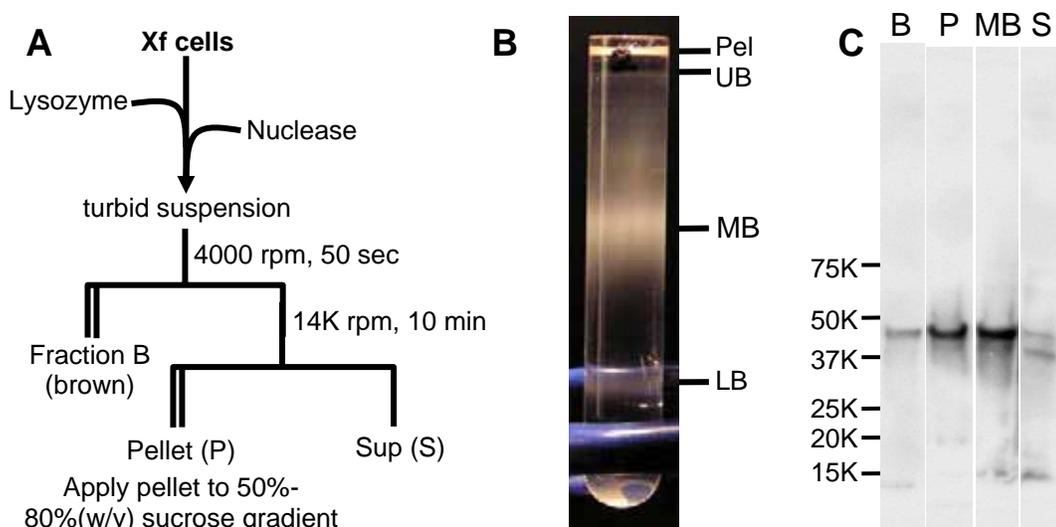


**Figure 2.** Effects of lysozyme treatment on the recovery of MopB and EF-Tu from an insoluble fraction of *Xf* cells. Each of the three images, lanes 1-4, 5-8 and 9-12, was derived from a different 12% polyacrylamide gel after electrophoresis of extracts of the relatively rapidly growing *Xf* strain HXfA-. Cells were harvested from liquid culture in late logarithmic phase. Cells were washed with water and cell pellets were quick-frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ . Rapidly thawed cells were suspended to approximately  $0.3 \mu\text{g}/\mu\text{L}$  total protein in 20 mM Tris-HCl, pH 8, alone (T, lanes 1, 5 and 9), in Tris-buffered “BugBuster®” (EMD Biosciences, proprietary detergent solution, T+D, lanes 2, 6 and 10), in Tris-buffered BugBuster® containing 45 U/ $\mu\text{L}$  recombinant lysozyme (EMD Biosciences, T+D+L, lanes 3, 7 and 11), and in Tris-buffered 45 U/ $\mu\text{L}$  recombinant lysozyme (T+L, lanes 4, 8 and 12). Samples were incubated at room temperature for 15 min with mixing, and insoluble material was collected by centrifugation at 14K rpm for 10 min. Precipitates were suspended in one-eighth the original volume of water, were mixed with SDS-mercaptoethanol-dithiothreitol disruption solution and were heated before loading the equivalent of 27  $\mu\text{g}$  (lanes 1-4) or 8.5  $\mu\text{g}$  (lanes 5-12) of starting protein on the gels. Detection was by staining with Coomassie Brilliant Blue (lanes 1-4), by immunoblotting using anti-MopB (lanes 5-6) or anti-EF-Tu peptide polyclonal antibody. Horse radish peroxidase-conjugated goat-anti-rabbit secondary antibody was located using the DuraSignal (Pierce) chemiluminescence system. The locations of bands for molecular markers and a presumed band for recombinant lysozyme (rLys) are indicated by arrows.

Immunoblot analysis using chemiluminescence detection is subject to a characteristic bleaching reaction when the target protein is present above a threshold amount. The apparent MopB signal of Fig. 2, lanes 5, 6 and 8, shows bleaching, as expected for the 8.5  $\mu\text{g}$  of *Xf* cell total protein and the known abundance of MopB. In contrast, the anti-MopB antibody provided only a weak signal from the insoluble fraction left after treatment with BugBuster® and lysozyme (lane 7). The anti-MopB antibody was raised against an immunogen MopB preparation that likely contained traces of EF-Tu, so it is possible that the lane 7 signal at about 43K apparent molecular weight reflects EF-Tu rather than MopB. Most of the EF-Tu remained in the insoluble fraction after treatment with BugBuster® and lysozyme (Fig. 2, compare lanes 11 and 9). Anti-*Xf*

HXfA polyclonal antibody (gift from Tanja Voegel and Bruce Kirkpatrick), which cross-reacts with *Xf* HXfB, and the A2 scFv antibody, gave a greatly diminished signal for cells treated with BugBuster® and lysozyme (data not shown). Thus, it appears that the treatment with the two reagents results in solubilizing two outer-membrane proteins, MopB, HXfB, and the scFv target, but not EF-Tu.

The insoluble product of the BugBuster® and lysozyme incubation (**Figure 3A**) was applied to a 50%-to-80% (w/v) sucrose gradient, which was centrifuged under conditions which should result in the various components of the digest moving through the gradient and reaching their own density. The main band of material (**Figure 3B**) was found to retain EF-Tu according to immunoblot results (**Figure 3C**). The centrifugation process, in effect, performs multiple washing steps as the main band material moves from the top of the tube to its isopycnic position, suggesting that the association of EF-Tu with the insoluble material is strong. Presumably this EF-Tu is not participating in protein synthesis but, as has been found for other systems (Dallo et al. 2002, Granato et al. 2004), *Xf* EF-Tu may have more than one function.



**Figure 3.** *Xf* EF-Tu is tightly associated with an insoluble fraction released from *Xf* cells by treatment with lysozyme **A**. *Xf* cells were suspended in a mild detergent solution (“BugBuster®,” EMD Biosciences), and the suspension was incubated at room temperature with lysozyme and then with a general nuclease (Benzonase, EMD Biosciences), which greatly reduced the viscosity of the suspension. Differential centrifugation produced a small brown precipitate (B), a white, fluffy precipitate (P), and a supernatant fraction (S). **B**. The P fraction was applied to a 50%-to-80%(w/v) sucrose gradient in 20 mM Tris-HCl, pH 8. Centrifugation was for 4 hr at 35,000 rpm in a Beckman SW-41Ti rotor at 4°C. The tube was mounted in a blue clamp, visible near the bottom of the image. In addition to the main band (MB) of white, turbid material, material also accumulated at the bottom of the tube, in a pellicle (Pel) floating on the gradient, and in two other small bands (UB and LB). **C**. Fractions from centrifugation and sucrose gradient centrifugation were heated in SDS-urea solution and were analyzed by SDS-PAGE on an 8-16% gradient gel. The bulk of the material reacting with peptide antibody to *Xf* EF-Tu in an immunoblot was associated with the MB fraction, which had a buoyant density of about 1.24 g/mL.

#### ***Phenotype of Xf EF-Tu expression in E. coli cells***

An *E. coli* strain was designed and constructed to express a fusion of the M13-like single-stranded DNA bacteriophage fd outer membrane protein P3 with *Xf* EF-Tu, *Xf* EF-Tu forming the amino end of the fusion. P3 is an adhesin protein responsible for initiating attachment of the bacteriophage M13 particle to the bacterial F-pilus. Prior to extrusion of the bacteriophage particle from the infected cell, or when P3 is expressed in transformed but uninfected cells, P3 resides in the cell outer membrane. Therefore, our expectation is that the fusion protein EF-Tu-P3 will be targeted to the outer membrane. When cells from the EF-Tu-P3-expressing and control P3-expressing strains were collected and treated with lysozyme (**Figure 4**), an insoluble residue remained. The insoluble material was subject to analysis by sucrose gradient centrifugation and electrophoresis in a fashion similar to the analysis of *Xf* cells presented in **Figure 3**. **Figure 4A** reveals a difference between the results obtained for P3-generating and EF-Tu-generating *E. coli*, in both amount and buoyant density of the product. Wildtype *E. coli* cells, incubated under the conditions reported in the **Figure 2 or Figure 3** legends, in BugBuster® and lysozyme solution, were completely liquefied, leaving no insoluble residue (data not shown). These results suggest that targeting a protein to the outer membrane may induce the accumulation of a new, lysozyme-resistant substance in the *E. coli* cell. Presumably proteases of *E. coli* prevent the accumulation of more than a trace of intact EF-Tu-P3 (Fig. 4C).

Comparison of **Figure 3B** and **Figure 4A** reveals that accumulation of EF-Tu-P3 in *E. coli* cells and EF-Tu in *Xf* cells do not generate lysozyme-resistant, insoluble materials of similar densities.

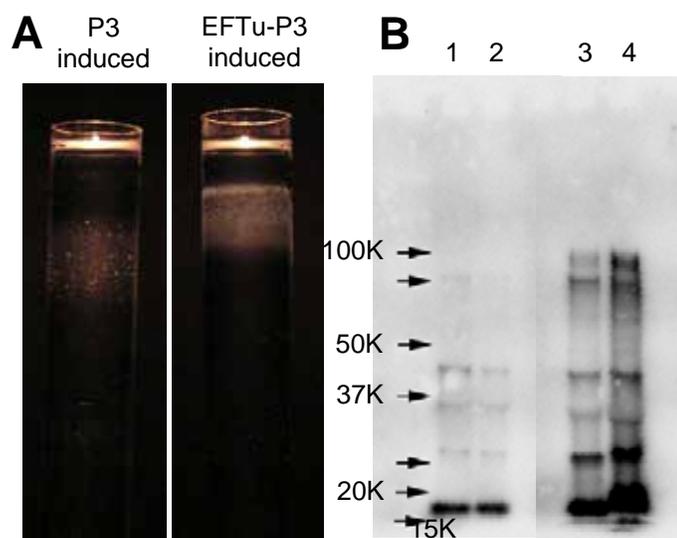
*E. coli* transformed and induced, by IPTG, for P3 expression increased in culture at a rate that was comparable to that for the corresponding untransformed strain. However, the *E. coli* strain bearing the EF-Tu-P3 construction grew slowly and very slowly after induction by IPTG. Cells from the two cultures, as viewed by light microscopy, had very different appearances. The presumed EF-Tu-P3-expressing cells were larger, in both length and diameter, than the P3-expressing or wildtype cells (data not shown). These results are consistent with incorporation of *Xf* EF-Tu, but not *E. coli* EF-Tu, into the *E. coli* cell wall.

***Does EF-Tu of Xf act as a MAMP or contribute to the symptoms seen on Xf-infected grapevines?***

The above results suggest that *Xf* EF-Tu is present in *Xf* in an immobilized or insoluble form unlikely to be active in protein synthesis. What function might this altered form of *Xf* EF-Tu have? The chlorotic response of *C. quinoa* leaves to pressure-infiltrated *Xf* EF-Tu (Bruening et al. 2007), although no defense response has been documented, suggests that *Xf* EF-Tu may be a MAMP in some plants. It is well established that local concentrations of *Xf* cells and symptom intensities on leaves do not correlate (Gambetta et al. 2007). Therefore, symptom development could be the result of factor(s) secreted or otherwise released by *Xf* cells. Such factors could accumulate at the leaf margin, for example, where scorch symptoms are first observed. Whether a *Xf* factor such as EF-Tu is involved in symptom induction or in defense response in grapevine, the relevant events must include recognition of the factor by grapevine as may be indicated, for example, by altered transcription.

Previous work from the laboratory of Prof. Douglas Cook identified grapevine promoters whose transcription was increased specifically in *Xf*-infected plants (Cook et al. 2005, da Silva et al. 2005). Transgenic Thompson Seedless grapes containing constructs with these promoters coupled to a green fluorescent protein (GFP) sequence were prepared in the laboratory of Prof. David Gilchrist (Gilchrist et al. 2007). Plants of the transformed line have been demonstrated to accumulate GFP in the pith region of petioles after inoculation of *Xf* but not after inoculation of another xylem-invading bacterium, *Xanthomonas campestris* (Gilchrist et al. 2008).

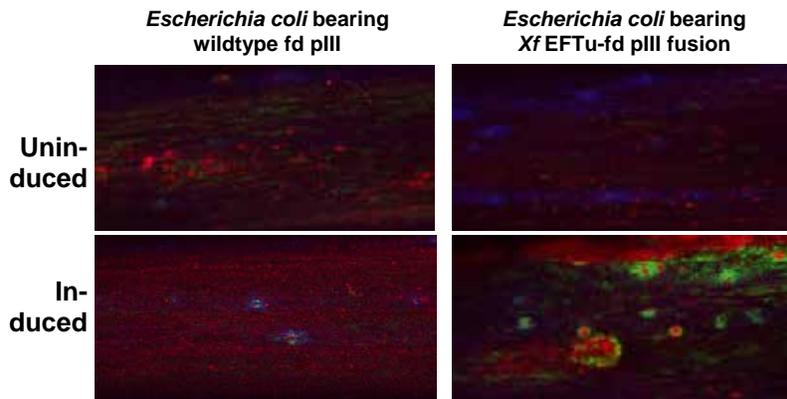
**Figure 4.** Expression of *Xf* EF-Tu in *E. coli* alters the insoluble residual found after digestion with lysozyme **A**. A 40 mg/mL (wet weight) suspension of *E. coli* cells that had been induced with IPTG was exposed to 30 U/μL recombinant lysozyme in BugBuster® detergent for 15 min and then to 0.05U/μL nuclease (EMD benzonase) for 10 min at room temperature. The resulting white precipitate was applied to the top of preformed 45%w/v – 75%w/v sucrose gradients. Gradients were centrifuged at 35,000 rpm for 4 hr at 4°C. **B**. An immunoblot was prepared using anti-*Xf* EF-Tu antibody. Lanes 1 and 3 received the flocculent lysozyme digestion product whereas lanes 2 and 4 were loaded with material recovered from the sucrose gradients. The uppermost band in lanes 3 and 4 has a mobility expected for a *Xf* EF-Tu-P3 fusion protein. Arrows indicate the locations of reference proteins by their molecular weight.



Significant accumulation of GFP was observed by confocal microscopy in the transgenic grapevine petioles after inoculation of the petiole with *E. coli* cells of the strain transformed for expression (**Figure 4B, lanes 3 and 4**) of the EF-Tu-P3 fusion protein, provided the cells were induced by exposure to IPTG (**lower right panel, Figure 5**). This image does not show GFP accumulation in pith cells, which is unlike the pith-cell accumulation seen when *Xf* cells were inoculated (Gilchrist et al. 2008). Significant GFP signals were not observed for the *E. coli* cells not exposed to IPTG or to cells transformed for P3 protein expression (**Figure 5**). In the **Figure 5** experiment, *E. coli* cells had been stored at 4°C overnight before infiltration. Cold storage may lead to substantial bacterial death (data not shown). Therefore, based on these results, we are not able to connect the observed induction of GFP synthesis to live cells actively accumulating EF-Tu-P3 or even to intact, EF-Tu-P3-containing cells. In a subsequent experiment, the transformed *E. coli* cells were held at room temperature, and GFP accumulation in pith cells was observed (not shown). The results presented here suggest that *Xf* EF-Tu protein may act as an elicitor that is recognized by grapevine. Whether this recognition has a role in symptom development or defense against *Xf* infection remains to be determined.

## CONCLUSIONS

A single-chain monoclonal antibody was isolated that reacts with a *Xf* protein that is most readily accessible in unusual structures found in liquid cultures of *Xf* cells but is also present in *Xf* cells. This protein is a potential target for interfering with *Xf* growth or colonization of grapevine. The protein synthesis elongation factor of *Xf* was demonstrated to have a bound form that may be recognized by grapevine in symptom development or defense reactions.



**Figure 5.** A *Xf*-specific grapevine promoter is activated by *E. coli* expressing EF-Tu-P3. The transgenic Thompson seedless grape plants bear constructions for green fluorescent protein (GFP) expression under the control of the *Xf*-infection inducible 9353 promoter (da Silva et al. 2005, Gilchrist et al. 2007). Transgenic grape petioles were inoculated with *E. coli* cells bearing either P3 (left panels) or EFTu-P3 (right panels), all at  $2 \times 10^8$  cells per ml. An aliquot of cells was supplied with the gratuitous inducer IPTG (lower two panels) for two hr before storing the cells overnight at 4°C and inoculation. At 13 days post inoculation, transverse sections of petiole taken a few mm from the inoculation site.

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## **IN PLANTA TESTING OF SIGNAL PEPTIDES AND ANTI-MICROBIAL PROTEINS FOR RAPID CLEARANCE OF XYLELLA**

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**Reporting Period:** The results reported here are from work conducted June 2008 to September 2008.

### **ABSTRACT**

*Xylella fastidiosa* (*Xf*), a Gram-negative bacterium, is the causative agent of Pierce's disease. Because *Xf* is xylem-limited, any potential anti-*Xf* gene product must be present in xylem at an effective concentration to provide disease control. Understanding how existing proteins are transported to xylem is necessary to target delivery of therapeutic proteins to this organ. We collected xylem exudate from *Vitis vinifera* cv. Chardonnay and analyzed its protein composition by two-dimensional gel electrophoresis, then purified and sequenced some of the abundant proteins to identify corresponding genes in the grapevine EST database. We identified the signal sequences present in these gene sequences and made vectors where these signals were fused to mature polygalacturonase inhibiting protein (mPGIP), which is secretion competent. Five different vectors were successfully constructed to test four signal sequences. These vectors were incorporated into *Agrobacterium* and used to transform grapevine. Callus and embryos were successfully selected and regenerated to give transformed grapevine lines for each construct. Plants have been obtained for all five constructs and protein inhibiting activity for two constructs has been tested. Next we will validate that our signal sequences are essential and sufficient to mobilize proteins into grapevine xylem. Such transgenic proteins, if synthesized in a rootstock, could confer resistance to xylem-specific infections such as Pierce's disease and assist in control of *Xf*.

As an alternative to signal peptide fused to PGIP, we designed a chimeric anti-microbial protein with two functional domains. One domain (the surface recognition domain, SRD) specifically binds to the bacterium outer-membrane and the other domain lyses the membrane and kills *Xf*. In this chimera, human neutrophil elastase (HNE) is the SRD that recognizes MopB, the major outer membrane protein of *Xf*. The second domain is cecropin B (CECB), a lytic peptide that targets and lyses gram-negative bacterial membranes. We have combined HNE and CECB using a flexible linker such that both components can simultaneously bind to their respective targets. This chimeric gene was synthesized in two versions, one with a mammalian signal peptide sequence designated HNE-CECB and the other with a signal peptide sequence from a plant *pgip* gene designated *pgip*-HNE-CECB, incorporated into binary vectors, and transformed into grapevine (*Vitis vinifera* var 'Thompson Seedless') and SR1 tobacco using *Agrobacterium*. Plant transformation experiments with both HNE-CECB and *pgip*-HNE-CECB were successful and plants have been obtained. Using PCR, the presence of HNE-CECB or *pgip*-HNE-CECB was confirmed in 37 and 7 plants, respectively. Methylation assay is in progress to confirm the presence of functional expression of HNE.

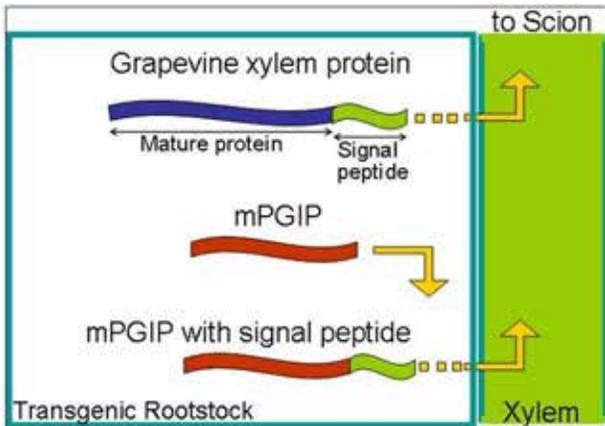
Preliminary testing of transgenic tobacco plants looks promising. To evaluate the signal sequences and to test the efficacy of the antimicrobial protein in grapevine, individual transgenic lines will be validated for transgene expression using RT-PCR. Plants will be tested as such and as rootstocks for wild type scions. Resistance will be validated in the greenhouse by challenging them with infected insects and by needle inoculations of *Xf*.

### **INTRODUCTION**

Pierce's disease (PD) in grapevines is caused by the Gram-negative bacterium *Xylella fastidiosa* (*Xf*). This bacterium infects xylem and kills grapevines by occluding the water-conducting vessels. The University of California reported that the disease destroyed over 1,000 acres of northern California grapevines between 1994 and 2000, causing \$30 million in damages. Globally, one-fifth of potential crop yields are lost to plant diseases primarily of bacterial origin. Xylem, the target tissue for

this organism, is composed of nonliving cells (tracheids and vessel elements) which join end to end to form water-conducting “pipes” from roots to the leaves and fruits. An oversimplified definition of xylem is a tissue involved in transporting water and dissolved mineral nutrients from soil to all other parts of the plant. However, xylem sap also contains significant organic material such as amino acids, proteins, sugars, and organic acids (Satoh 2006). Proteins have been reported in xylem sap from many species (Alvarez et al. 2006, Biles and Abeles 1991, Kehr et al. 2005, Young et al. 1995) and the number of identified proteins has recently increased considerably through multiparallel protein analysis (Aguero et al. 2006, Almeida and Purcell 2003a, Almeida and Purcell 2003b). In grapevine, proteins have been isolated from xylem tissue and separated through capillary electrophoresis to compare *Muscadinia* and *V. vinifera* profiles (Jain and Basha 2003).

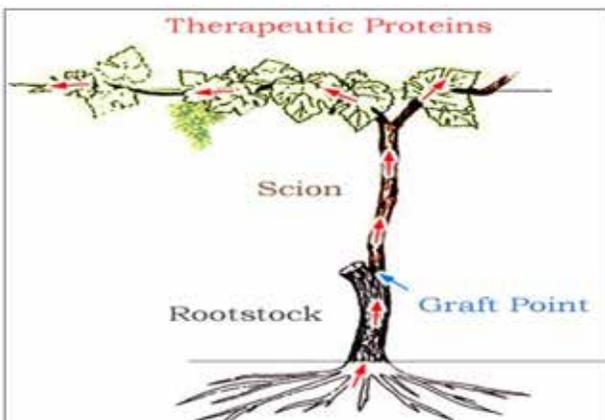
The purpose of the present study was to identify xylem sap proteins from grapevine and determine the signal sequences necessary for their secretion to the apoplast and xylem (Figure 1). Because *Xf* is xylem-limited, xylem-targeted expression of transgenic therapeutic proteins, such as PGIP and the antimicrobial chimera HNE-*cecropinB*, may be used to prevent and control PD infestations. The product of the pear PGIP gene, when expressed in transgenic grapevines, is present in xylem exudates and moves through the graft union (Aguero et al. 2006). This movement to the scion implies that a few transgenic



**Figure 1:** Theory: Identification of signal peptides of grapevine xylem proteins, modification of mPGIP with these peptides to test their secretion competence.

rootstocks could be used with many different scions, provided that the anti-*Xf* therapeutic protein is synthesized in effective concentrations in roots and targeted to the xylem for transport to the scion with the bulk flow of water through the root system (Figure 2).

Signal peptides control entry of virtually all proteins to the secretory pathway in both eukaryotes and prokaryotes. The N-terminal part of the amino acid chain is cleaved off when the protein is translocated through the endoplasmic reticulum membrane (Nielsen et al. 1997). Signal peptides are generally interchangeable, so proteins that are not usually secreted can become secretion-competent through attachment of a signal peptide to the N-terminus of the mature protein, allowing its entry into the vesicular transport system (Figure 1: Vitale and Denecke 1999). While many reports show successful recombinant protein targeting by signal peptides in transgenic plants, the signal sequence of recombinant proteins can affect the amount of protein produced. For example, the secretion efficiency of heterologous proteins in transgenic tobacco was improved by replacing the heterologous signal peptide with one from tobacco (Yoshida et al. 2004). Our final goal is to use signal sequences from grapevine xylem proteins to deliver therapeutic proteins into the xylem of transgenic rootstocks, thus conferring resistance to PD in the entire plant without modifying the scion or affecting the fruit (Figure 2).

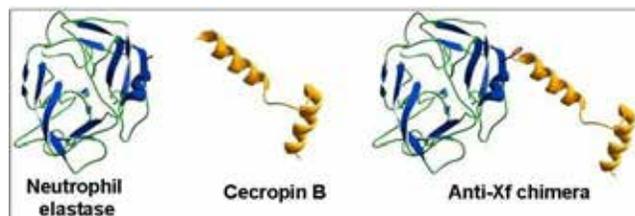


**Figure 2:** Application: Rootstock delivery of therapeutic proteins to enhance disease resistance in the scion.

In this project, we have taken a structure-based approach to develop chimeric anti-microbial proteins for rapid destruction of *Xf*. The strategy is based upon the fundamental principle of innate immunity: that plants recognize and clear pathogens rapidly (Pieters 2001, Baquero and Blazquez 1997). Pathogen clearance by innate immunity occurs in three sequential steps: pathogen recognition, activation of anti-microbial processes, and finally pathogen destruction. Different plant factors are involved in different steps of innate immunity. Our strategy of combining a pathogen recognition element and a pathogen killing element in

the chimeric molecule is a novel concept and has several immediate and long term impacts. During very early stages of *Xf* infection, specific carbohydrates/lipids/proteins on the outer membrane of *Xf* interact with plant cells and are important for virulence (Pieters 2001). A protein inhibitor that interrupts this step of plant-*Xf* interaction will be useful in anti-microbial therapy and controlling PD. In this project, we developed a novel, protein-based therapy that circumvents the shortcomings of traditional antibiotics. We designed a chimeric, anti-microbial protein with two functional domains (Figure 3). One domain (the surface recognition domain, SRD) specifically targets the bacterium’s outer membrane and the other will lyse the membrane and thus kills *Xf*. In this chimera, human neutrophil elastase (HNE) is the SRD that recognizes MopB, the major outer membrane protein of *Xf* (Bruening et al. 2002). The second domain is cecropin B, a lytic peptide that targets and lyses Gram-negative bacteria. We have combined HNE and cecropin B using a flexible linker so both components can bind simultaneously to their respective targets. This chimeric gene was synthesized and cloned into *Agrobacterium* vectors for

plant transformation. Transformation experiments are completed and we have plants of *Nicotiana tabacum* var SR1 and plants of *Vitis vinifera* ‘Thompson Seedless’ transformed with this gene. Preliminary results with some tobacco lines were very encouraging, as they showed tolerance/resistance to *Xf* infection of tobacco leaf tissues. This proposal will focus on evaluating transgenic grapevines expressing the chimeric antimicrobial protein for resistance to *Xf*.



**Figure 3:** Structures of the components of the chimeric anti-microbial proteins for the rapid clearance of *Xylella*.

The work described in this proposal corresponds to research priorities developed by the National Academies in their publication, “California Agriculture Research Priorities: Pierce’s Disease” as outlined in Chapter 4, Recommendations 4.3, 4.4, and 4.5 and Chapter 3, Recommendation 3.3. Additionally, the objectives of this research project are relevant to the research recommendations from the August 2006 PD/GWSS Scientific Summit: specifically, enhancing host resistance via transgenics, biological control of *Xf*, understanding the transmission of the disease, and perhaps biological control of GWISS through plant-incorporated proteins delivered through the xylem into the scion from the rootstock.

## OBJECTIVES

1. Evaluate the efficiency of different signal sequences in targeting PGIP to grapevine xylem tissue, through the graft union, and inhibiting infection with X.
2. Validate expression of chimeric antimicrobial proteins in transgenic grapevines, test for anti-*Xf* activity *in planta*, and test for graft transmissibility.

## RESULTS AND DISCUSSION

1. Evaluate the efficiency of different signal sequences in targeting PGIP to grapevine xylem tissue, through the graft union, and inhibiting infection with *Xf*:

In our previous research. Peptide spectrum and BLAST analysis showed that the proteins found in grape xylem exudates are secreted and share function similarities with proteins found in xylem exudates of other species (Buhtz et al. 2004). cDNA sequences matching two of them found in the TGI *Vitis vinifera* gene index (<http://compbio.dfci.harvard.edu/tgi/plant.html>) were used to design primers that were used to amplify the predicted fragments from genomic DNA of ‘Chardonnay’ and ‘Cabernet Sauvignon’ (Aguero et al. 2008). These fragments were annotated as Chi1b and NtPRp27 (**Table 1**). These fragments were then fused to DNA sequences that contained the mature polygalacturonase inhibiting protein (mPGIP) gene through gene splicing using a PCR-based overlap extension method (SOE) (Horton et al. 1990) and cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA). These two chimeric genes were then ligated into a plant expression vector containing the 35S cauliflower mosaic virus promoter and the octopine synthase terminator and the resultant expression cassettes were then ligated into the binary vector pDU99.2215 (Escobar, et al. 2001) which contains an *nptII*-selectable marker gene and a *uidA* ( $\beta$ -glucuronidase, GUS) scorable marker gene. The mature PGIP sequences without any signal peptide sequences was also incorporated into pDU99.2215 to serve as a control and this vector is designated pDU05.1002 (**Table 1**). We also incorporated signal peptides from the xylem sap protein XSP30 and the rice amylase protein Ramy3D that we have described in earlier reports. These binary vectors are designated pDA05.XSP and pDU05.0401, respectively (**Table 1**).

<b>Table 1.</b> Construction of vectors for the expression of mature PGIP with various signal peptide sequences.						
No	Signal Peptide	Binary Plasmid Map	Reporter Gene	Promoter	Marker Genes	Vector
1	None		Mature PGIP	CaMV35S	GUS and Kan	pDU05.1002
2	Rice amylase-Ramy3Dsp		Mature PGIP	CaMV35S	GUS and Kan	pDU05.0401
3	Xylem sap protein 30-XSP30sp		Mature PGIP	CaMV35S	GUS and Kan	pDA05.XSP
4	Chi1b signal peptide		Mature PGIP	CaMV35S	GUS and Kan	pDU06.0201
5	NtPRp27 signal peptide		Mature PGIP	CaMV35S	GUS and Kan	pDU05.1910

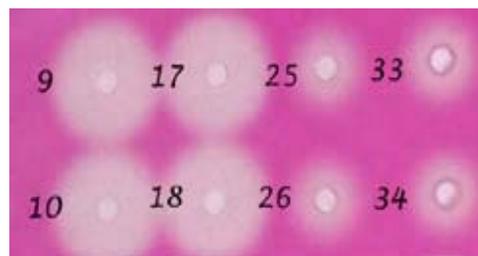
Binary vector # 1 is the control and should be immobile although PGIP with its native signal peptide is secretion competent in grape. In binary vector #2, mature PGIP has been fused to the signal sequence of rice amylase 3 (Ramy3D), which has been very effective in secretion of human  $\alpha_1$ -antitrypsin in rice cell cultures (Trexler et al. 2002). In binary vector # 3 mature PGIP has been fused to the signal sequence of cucumber XSP30, which is a xylem-specific protein. Constructs 4 and 5 have been described above. All five binary vectors were transformed into the disarmed *A. tumefaciens* strain EHA 105 pCH32 (Hamilton, 1997) by electroporation (Wen-jun and Forde 1989) and the stable transformation of *Vitis vinifera* 'Thompson Seedless' (TS) has been completed in all five vectors. The methods for Agrobacterium-mediated transformation have been reported earlier by us (Aguero et al. 2006).

**Table 2.** Status of *Vitis vinifera* 'Thompsons seedless' transformants

No.	Signal peptide	Plasmid	Callus	Embryos	Plants
1	Mature	pDU05.1002	yes	Yes	Yes
2	Ramy	pDU05.0401	yes	Yes	Yes
3	XSP	pDA05.XSP	yes	Yes	Yes
4	Chi	pDU06.0201	yes	Yes	Yes
5	Nt	pDU05.1910	yes	Yes	Yes

As a result of the above transformation callus, embryos and plants have been obtained for all five vectors (**Table 2**). Callus cultures that were embryogenic were selected on kanamycin and grown into plants. We have tested all the plants containing vectors 4 and 5 using PCR (**Table 3**). DNA was isolated from leaves using DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Primers used for detection of nptII were Aph3: 5' ATGATTGAACAAGATGGATTGCACGCA and Aph4: 5' GAAGAACTCGTCAAGAAGGCGATAGA. Primers for detection of PGIP were 5' Mature PGIP: 5' ATGGATCTCTGCAACCCCGACGAC and 3'PGIP: 5' TTACTTGCAGCTTGGGAGTG. Tissue from these plants has been tested individually for PGIP activity using the zone inhibition assay with PG (**Table 3, Figure 1**)(Taylor and Secor 1988). PG preparations were obtained from *Botrytis cinerea* strain Del 11 isolated from grape (Aguero et al. 2005). Protein from leaf tissue (~100mg) was extracted in extraction buffer (Dandekar, et al. 1998) at a ratio of 1 ul/mg. Tissue was ground in a 2 ml tube containing a 5mm stainless steel bead in a TissueLyzer (Qiagen). The homogenate was centrifuged at 16000 xg for five minutes. Protein concentration of the supernatant was determined according to Bradford (1976). The inhibition of endo-PG activity from culture filtrates of *B. cinerea* was determined by zone inhibition assay in a 1% agarose gel in a 0.1M sodium acetate buffer pH 5 supplemented with 100mg/L pectin (modified from Taylor and Secor, 1988).

Twenty four of the plants transformed with Nt and ChiPGIP were tested for PGIP activity using the zone inhibition assay with PG (**Table 3, Figure 4**). Select lines were looked at quantitatively. All 11 ChiPGIP plants were assayed and had a range of inhibition from 6-62 %. The 10 remaining NtPGIP plants were also assayed and had a range of 0-45 % inhibition. The ChiPGIP plants had a greater number of lines with strong inhibition than the NtPGIP plants, 6 vs 2, respectively. Also, there were more, 3 NtPGIP vs 0 ChiPGIP, that had no inhibition activity. The 6 ChiPGIP plants with strong inhibition and 5 of the NtPGIP with strong to medium inhibition are being micropropagated to obtain 40 clones of each line for testing with *Xylella* to determine efficacy of the PGIP protein. *In vitro* plants have also been received from the Parsons Plant Transformation Facility transformed with the plasmids pDU05.1002 (mature PGIP, mPGIP) and pDA05.XSP (PGIP with xylem sap protein signal peptide, XSP). They have been PCR tested for PGIP using the primers 5' Mature PGIP: 5' ATGGATCTCTGCAACCCCGACGAC and 3'PGIP: 5' TTACTTGCAGCTTGGGAGTG.



**Figure 4.**

**Table 3.** Evaluation of *Vitis vinifera* ‘Thompsons seedless’ transformed plants

No	Signal peptide	Plasmid	Plant	Positive PCR for PGIP	Positive for PGIP Activity	Lines Cloned	Lines grafted	Moved greenhouse
1	Mature	pDU05.1002	Yes	2/7	2	2		
2	Ramy	pDU05.0401	Yes	To be tested	To be tested			
3	XSP	pDA05.XSP	Yes	8/10	In progress			
4	Chi	pDU06.0201	Yes	11/11	10	6	2	In progress
5	Nt	pDU05.1910	Yes	17/22	5	5		In progress

**Table 4.** Construction of vectors for the expression of HNE-CECB and pgipHNE-CECB

No	Signal Peptide	Binary Plasmid Map	Reporter Gene	Promoter	Marker Genes	Vector
1	HNE-CECB		HNE-CECB	CaMV35S	GUS and KAN	pDU04.6105
2	pgipHNE-CECB		HNE-CECB	CaMV35S	GUS and KAN	pDA05.0525

In the meantime individual clones of Chi and nt PGIP are being acclimated to the soil for transfer to the greenhouse for initial experiments with *Xylella*. The vines will be allowed to grow up to 6'-12" (about 10 nodes long), then inoculated with *Xf* by hand and by insect and evaluated for symptoms of Pierce's disease (PD) after three months. Since we found in earlier research that pPGIP with its endogenous signal peptide is xylem competent, we are using a grapevine successfully transformed with this construct and highly expressed as a positive control in the inoculation experiments (Aguero et al. 2005). The screening of mature-PGIP and XSP-PGIP plants has been initiated. We have also initiated grafting experiments where selected transformed lines will be grafted with wild type TS scion; we have done this type of experiment previously to evaluate the movement of the PGIP protein from the rootstock up into the xylem of the wild type scion (Aguero et al. 2005).

## 2. Validate expression of chimeric antimicrobial proteins in transgenic grapevines, test for anti-*Xf* activity *in planta*, and test for graft transmissibility:

*In vitro* grape plants transformed with the constructs, pDU04.6105 (Elastase-Cecropin = HNE-CECB) and pDA05.0525 (pgipSP-Elastase-Cecropin= pgipHNE-CECB) (Table 4), have been received from the Parsons Plant Transformation Facility. 69 HNE-CECB plants and 18 pgipSP-HNE-CECB plants have been screened by PCR to verify the individual transformation events. PCR was performed on DNA isolated from leaves using the Qiagen DNeasy Plant Mini Kit. Primers used for detection of elastase were: CaMV355-2: 5' GACGTAAGGGATGACGCACAAT and 3HNEb: 5' TTACTAGAGTGCTTTTGCTTCTCCAG. As an additional screen, the micro-propagated plants were tested for methylation of the elastase gene using digestion with McrBC. Methylated DNA is not expressed and, therefore, we would not be interested in these plants. The McrBC enzyme cleaves DNA containing methylcytosine on one or both strands and was obtained from New England BioLabs Inc., catalog #M0272S. This assay involves preparing a reaction mix of 100ng DNA, 1X NEBuffer 2, 1X BSA, 100mM GTP and 5 units of McrBC in a volume of 25ul. Following 4 hour incubation at 37°C, a 10 ul aliquot of this reaction was tested by PCR for elastase using the above primers; no product will be amplified if the gene is methylated. The resulting 33 HNE-CECB plants (**Table 5**) are being micropropagated for future transfer to the greenhouse and for RNA and protein analysis. This will be followed up with a more significant evaluation of disease susceptibility using both needle and insect inoculations in the greenhouse.

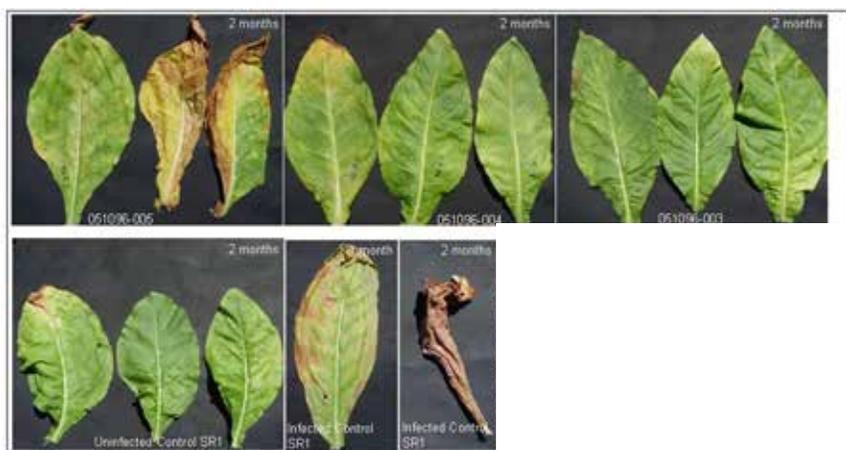
**Table 5.** Current status of testing transgenic lines of *Vitis vinifera* var. Thompson Seedless grapevines with HNE-CECB and pgipHNE-CECB genes.

Signal peptide	Plasmid	Plant	Positive PCR	Negative for methylation	Lines Cloned	Lines grafted	Moved greenhouse
HNE-CECB	pDU04.6105	yes	37/69	33	In progress		
pgipHNE-CEPB	pDA05.0525	yes	7/18	In progress	In progress		

**Table 6.** Current status of testing transgenic lines of N Tabacum SR1 with HNE-CECB and pgipHNE-CECB genes.

Plasmid	Plant	Positive PCR	Lines Cloned	Moved greenhouse
pDU04.6105	20	15	15	15
pDA05.0525	34	24	29	29

We also obtained transgenic tobacco transformed with either the HNE-CECB or pgipHNE-CECB constructs. We have tested 13 tobacco SRI lines transformed with pgipHNE-CECB, of which 11 performed measurably better than controls after inoculation with *Xf* (**Table 6; Figure 5**).



**Figure 5.** *In planta* testing of 8SRone tobacco transformed with pgipHNE-Cecropin vector (pDA05.0525). Three leaves on three plants each line (9 leaves) were inoculated with hypervirulent HXfB strain of *Xf*. Plants were scored after two months. Infected controls had no surviving leaves (0/9), one line was low (3/9) five were moderate (5/9) and two showed a strong response of leaf survival (6/9). Line 051095-005 is an example of moderate and 051095-004 and 051095-003 are examples of transgenic lines showing strong response indistinguishable to the uninfected controls.

## CONCLUSIONS

The main objective of this project is to develop a potent therapy against *Xf* by utilizing the principles of innate immunity by which plants counteract virulence factors like PG with PGIP or that recognize pathogens using their surface characteristics and then rapidly clear them by cell lysis. Because *Xf* is xylem-limited, xylem-targeted expression of transgenic therapeutic proteins, such as PGIP and the antimicrobial chimeric proteins, may be used to prevent and control PD. Five different vectors were successfully constructed to test four signal sequences to target PGIP to the xylem of grapevine. Plants have been obtained for all five constructs and PG inhibiting activity for two constructs has been tested. We are testing two

constructs containing two versions of the chimeric protein Elastase-Cecropin and have transformed tobacco and grapevine. Expression in tobacco indicates that protection against *Xf* looks promising. Transgenic grapevines expressing these two constructs have been obtained and are being screened and propagated for greenhouse testing. Next we will validate the efficacy of these two types of proteins to ensure that our signal sequences are essential and sufficient to mobilize proteins into grapevine xylem and that the targeted chimeric proteins control *Xf* in grapevine tissues. Such transgenic proteins, if synthesized in a rootstock, could confer resistance to xylem-specific infections such as Pierce's disease and assist in control of *Xf* infestations.

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# ANALYSIS OF THE BACTERIAL COMMUNITY ASSOCIATED WITH SHARPSHOOTERS, INSECT VECTORS OF *XYLELLA FASTIDIOSA* SUBSP. *PAUCA*

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## ABSTRACT

*Xylella fastidiosa* subsp. *pauca* (*Xfp*) causes citrus variegated chlorosis (CVC) disease in Brazil, resulting in significant production problems in the citrus industry. *Xfp* is mainly transmitted by three species of sharpshooters (Hemiptera: Cicadellidae) in Brazil, *Dilobopterus costalimai* (Young), *Acrogonia citrina* Marucci & Cavichioli and *Oncometopia facialis* (Signoret). Endophytic bacteria have been defined as those that do not visibly harm the host plant but can be isolated from surface-disinfected plant tissue or the inner parts of plants, showing potential benefits in the biocontrol of pathogens causing diseases. Some endophytes colonize the same niche of phytopathogens, such as *Xfp*, allowing interaction with the phytopathogen during plant colonization and transmission. We evaluated the bacterial communities associated with the heads of the insect vectors of *Xfp* that were collected from CVC affected citrus groves in Brazil. Bacteria were isolated from the heads of three insect species (*O. facialis*, *D. costalimai* and *A. citrina*). Total DNA of insect heads was analyzed by denaturing gradient gel electrophoresis (DGGE). The composition of the microbial community was found to be characteristic of the insect species and period of evaluation. Specific polymerase chain reactions (PCRs) for detection of two important citrus endophytes, *Curtobacterium flaccumfaciens* and *Methylobacterium mesophilicum* were performed, and the highest frequency of detection was 89.6% for *C. flaccumfaciens*, which has been described as a citrus endophyte that interacts with *Xfp*.

## INTRODUCTION

Citrus variegated chlorosis (CVC) was first reported in Brazil in 1987 (Rossetti et al. 1990) and has spread over at least 90% of the orchards in Brazil (Lambais et al. 2000) and is caused by the xylem-limited gram-negative bacterial pathogen, *Xylella fastidiosa* subsp. *pauca* (*Xfp*) (Schaad et al. 2004). In Brazil, CVC is responsible for losses of US \$100 million per year to the citrus industry (Della-Coletta et al. 2001). Endophytic microorganisms, not visibly harmful to the host plant, can be isolated from surface-disinfected plant tissue or the inner parts of plants (Hallmann et al. 1997). Endophytes were reported to be contributing to host plant protection and ultimately survival (Azevedo et al. 2000). Furthermore, endophytes can colonize an ecological niche similar to that of phytopathogens, which makes them possible candidates as biocontrol agents (Hallmann et al. 1997). Araújo et al. (2002) found that the endophytic bacteria *Methylobacterium* spp. and *Curtobacterium flaccumfaciens* were present in asymptomatic citrus trees. Lacava et al. (2004) reported that the growth of *Xfp* was inhibited by endophytic *C. flaccumfaciens* and stimulated by *Methylobacterium* sp. and Lacava et al. (2007) demonstrated that *C. flaccumfaciens* reduced the severity of CVC symptoms when co-inoculated with *Xfp* in planta. Cicadellinae leafhoppers, commonly named sharpshooters, are xylem-feeders (Young 1968). In Brazilian citrus groves, *Dilobopterus costalimai*, *Oncometopia facialis* and *Acrogonia citrina* are the most common sharpshooters found (Lopes et al. 1996). After acquisition of *Xf* by the insects, colonies of bacterial cells were visible in the cibarium and pre-cibarium of transmitting insects attached to the foregut walls (Purcell & Finlay 1979; Newman et al. 2003). Many aspects can influence the transmission of a pathogen by an insect vector such as the low concentration of *Xfp* cells in the citrus plants (Almeida et al. 2003) and the low number of colonized vessels in affected plants (Alves et al. 2003). The interaction between different bacteria inside the insect foregut can also influence the transmission, as once inside the foregut, bacterial interaction, such as competition for nutrients, space and other complex interactions, could occur.

## OBJECTIVES

The aims of this work were:

1. Access the bacterial population associated with the main sharpshooters responsible for the transmission of *Xfp* in citrus.
2. Compare the bacteria collected from insects to endophytic bacteria collected from citrus by denaturing gradient gel electrophoresis (DGGE).

## RESULTS

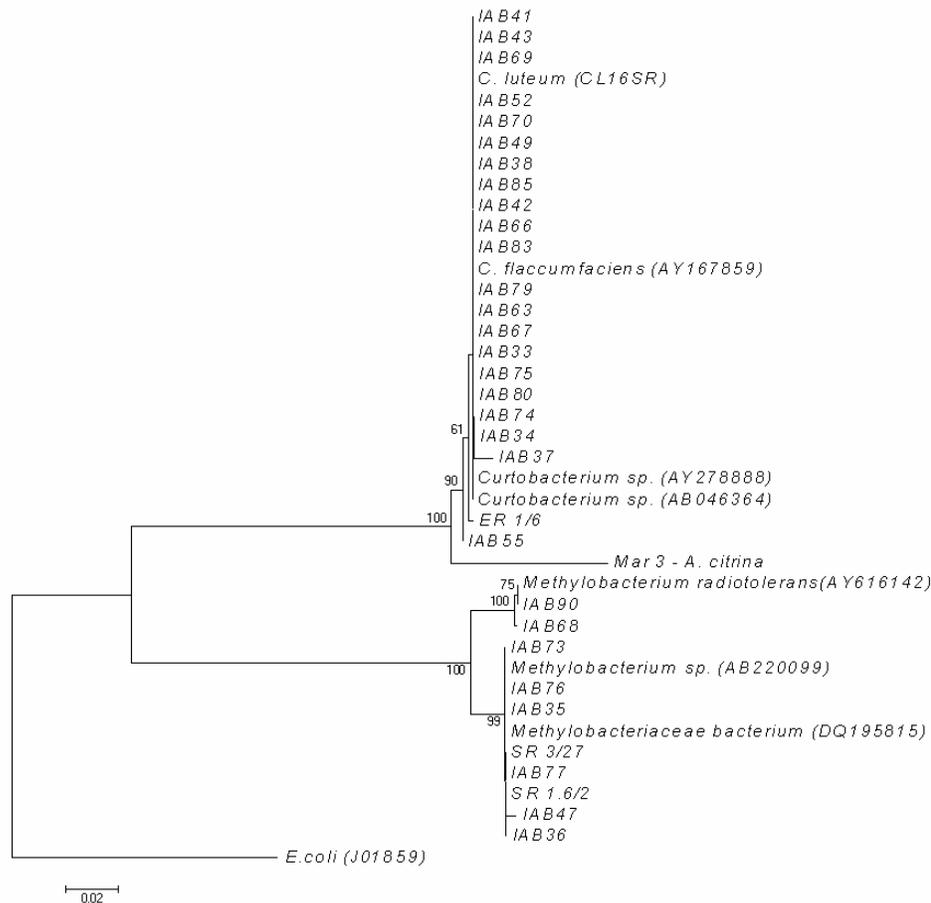
A total of 17,230 bacteria were isolated and they were classified according to morphological groups. The morphological groups found during isolation and the results of the sequencing of one representative of each group were: G1) actinomycetes, G2) dark pink colonies (*Curtobacterium* sp.), G3) light pink colonies (*Methylobacterium* sp.), G4) yellow colonies (*Sphingomonas* sp.), G5) white colonies (*Bacillus* sp.), and G6) transparent colonies (*Microbacterium* sp.). From the heads analyzed, 51.7% of insects from the species *O. facialis* were positive for the presence of the endophytic bacteria *M. mesophilicum*, 8.7% of the *D. costalimai* and 20% of the *A. citrina*. *C. flaccumfaciens* was found in 89.6% of *O. facialis*, 39.1% of *D. costalimai* and 70% of *A. citrina*. A summary of the results comparing to previous data of *Xfp* transmission by the insect vectors and the presence of *Curtobacterium* sp. is presented in **Table 1**. The DGGE analysis showed considerable variability between the different insect species and also between sampling periods. **Figure 1** shows that the samples from March are more similar to samples from May than the ones from July. The bacteria isolated from insects are represented by the code IAB (Insect Associated Bacteria) described on **Table 2**. In the present study, *Curtobacterium* sp. was the most important bacteria colonizing insect heads. *Curtobacterium flaccumfaciens* was implicated in playing an important role in the prevention of CVC symptoms in citrus trees. The presence of the citrus endophyte, *Curtobacterium* sp., colonizing the insect heads could explain why the transmission efficiency of *Xfp* by vectors is low (5 to 10%), when compared to the transmission of *Xf* subsp. *piercei* by GWSS, which transmit Pierce's disease (PD) (45%). **Table 1** illustrates that *Curtobacterium*, can play an important role in the transmission of *Xfp*, as it could be influencing pathogen adhesion to the vector foregut or inhibiting growth of the pathogen.

## CONCLUSIONS

The bacterial communities associated with insects appear to change with changes in environmental conditions. Endophytic bacteria could influence disease development by reducing the insect transmission efficiency due to competition with pathogens in host plants and also in insect foreguts. In addition the bacterial communities in the foregut of insect vectors of *Xfp* changed with time, environmental conditions and in different insect species. However, since members of the genus *Curtobacterium* were consistently detected in the insect vectors of *Xfp*, they maybe candidates for biological control of *Xfp*, which requires endophytic bacteria that can colonize both the insect vectors of CVC and citrus plants.

**Table 1.** Resume of the results of the present work, comparing to previous data of *Xfp* transmission by the insect vectors and the presence of *Curtobacterium* sp.

	<i>O. facialis</i>	<i>A. citrina</i>	<i>D. costalimai</i>
<i>Curtobacterium</i> sp. frequency of isolation (G2)	1.01 x 10 <sup>3</sup>	2.16 x 10 <sup>2</sup>	4.33 x 10 <sup>1</sup>
<i>Curtobacterium</i> sp. positive specific PCR	89.6%	70%	39.1%
Presence of Haplotype 1 ( <i>Curtobacterium</i> sp.) from 120 total isolates	45	17	0
Transmission rate of <i>Xfp</i> (Krügner et al., 2000)	1%	2%	5%



**Figure 1.** Dendrogram representing the insect associated bacteria clustered with citrus endophytes. Bootstraps of 1,000 repetitions.

**Table 2.** Average of the number of colony forming units per head of insect (CFU/head) found in each isolation experiment. Groups: G1) actinomycetes, G2) dark pink (*Curtobacterium* sp.), G3) light pink (*Methylobacterium* sp.), G4) yellow (*Sphingomonas* sp.), G5) white (*Bacillus* sp.), G6) transparent (*Microbacterium* sp.).

Vector insect	Isolation	G1	G2	G3	G4	G5	G6
<i>O. facialis</i>	March	100,4 (±44,6)	1225,7 (±253,2)	51 (±11,1)	627,7 (±176,3)	185,13 (±33,8)	48 (±21,4)
	May	23 (±11,1)	0	101,7 (±46,5)	2,75 (±1,2)	82,25 (±36,3)	0
	June	12,73 (±5,03)	1,5 (±0,47)	0,4 (±0,10)	0,8 (±0,26)	1,6 (±0,5)	3,1 (±1,38)
<i>A. citrina</i>	March	0	16,2 (±5,18)	2 (±0,89)	0	0	0
	May	0	0	0	1 (±0,35)	6,5 (±1,16)	176,9 (±48,1)
	June	0	0,6 (±0,17)	0	1,5 (±0,5)	0,2 (±0,08)	0,4 (±0,17)
<i>D. costalimai</i>	March	0,2 (±0,08)	584,6 (±258,8)	1 (±0,44)	121,1 (±49,6)	8,5 (±3,8)	4 (±1,78)
	May	0	1 (±0,35)	0,25 (±0,12)	1,625 (±0,65)	1,5 (±0,47)	1,25 (±0,37)
	June	0,4 (±0,1)	0	0	0	0,2 (±0,08)	0

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## BACTERIOPHAGE AND BACTERIOCINS OF *XYLELLA FASTIDIOSA*: POTENTIAL BIOCONTROL AGENTS

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### ABSTRACT

The development of a bacteriophage (phage) and/or high molecular weight bacteriocin-based biocontrol strategy offers a novel approach for the control of Pierce's disease caused by *Xylella fastidiosa* (*Xf*). We have isolated, propagated and characterized a functional *Xylella* phage, designated *Xfas53*. Phage morphology was examined by electron microscopy and was classified as belonging to the family *Podoviridae* with a head diameter of 55 nm and a short non-contractile tail having a diameter of 12 nm. The dsDNA genome of *Xfas53* was determined to be 36,673 base pairs with a GC content of 57%. Bioinformatic analysis of the *Xfas53* genome predicted a total of 46 protein coding genes.

### INTRODUCTION

Pierce's disease of grapes, caused by the xylem-limited, fastidious bacterium *Xylella fastidiosa* (*Xf*), is a limiting factor in the cultivation of high quality wine grapes. This disease is particularly damaging to Texas vineyards. The Texas Wine Marketing Institute in 2007 reported that the Texas wine industry currently consists of 2,900 bearing acres which produced 8,500 tons based on 2005 year end data ([http://www.depts.ttu.edu/hs/texaswine/docs/TX\\_ECONOMIC\\_IMPACT\\_2007.pdf](http://www.depts.ttu.edu/hs/texaswine/docs/TX_ECONOMIC_IMPACT_2007.pdf)). The full economic benefit of Texas wine and grape industry is estimated at \$997.3 million to the state's economy and provides over 8000 jobs. It is estimated that the disease has resulted in several millions of dollars in damage due to lost vines, replanting costs, and the closure of numerous productive vineyards. Pierce's disease has also become a major threat to the nation's most valuable wine producing regions in California, with the potential to have an economic impact in the billions of dollars. Other strains of the same bacterium cause disease in oleander, peach, plum, and several shade trees including sycamore and a number of oak species native to Texas. *Xf* has a wide host range and is transmitted by several species of sharpshooter leafhoppers.

The search for new ways to combat microbial pathogens is an ongoing process in both medicine and agriculture. The long-term goal of our project is to ultimately develop a phage and/or bacteriocin-based biocontrol agents to control *Xf*, the causal agent of Pierce's disease in grapes. The double-stranded DNA (dsDNA)-containing phages are very likely the most numerically abundant group of similar organisms in the biosphere (Hendrix et al. 1999). Treatment of a disease with phages, a practice also termed phage therapy, involves the use of bacterial viruses that can only attack specific bacteria to kill the targeted pathogenic microorganism. Despite a controversial legacy arising from the pre-DNA era of microbiology, there is growing interest in reconsidering therapy as an additional weapon against both human (Chibani-Chennoufi et al. 2004, [Bruttin and Brussow 2005](#)) and plant bacterial pathogenesis ([Federal Register /Vol. 70, No. 248/Wednesday, December 28, 2005](#), Balogh et al. 2003, 2008). Phage therapy pre-dates antibiotics by decades, but was largely abandoned when chemical antimicrobials became readily available. Now, however, the emerging threat posed by antibiotic-resistant pathogens is spurring a resurgence of interest in phage as a potential therapy to cure or prevent infections, and as a tool to kill food-borne pathogens. A combination of six phages were recently approved by the FDA to be sprayed on ready-to-eat meat and poultry products, including sliced ham and turkey ([Federal Register Vol. 71, No. 160, Friday, August 18, 2006](#)). Multiple commercial efforts to develop phage therapeutics are underway ([www.evergreen.edu/phage/companies.htm](http://www.evergreen.edu/phage/companies.htm) for a list). We submit that it is necessary to address experimentally the key scientific issues that are involved to establish practical phage and/or bacteriocin therapy for Pierce's disease.

The genomes of the *Xylella* strains have a high number of phage-related sequences dispersed in their chromosomes, constituting 7% of the CVC strain 9a5c genome and 9.02% of the Temecula strain genome (Simpson et al. 2000) suggesting that bacterial viruses have contributed to the evolution of *Xylella*. The 9a5c genome exhibits five potential prophage regions (Simpson et al. 2000, Canchaya, 2004) with a different GC content (57%) and several other phage-related genes dispersed throughout the sequence, which result in a high percentage of repeated fragments. These regions and genes are organized

differently in the Temecula strain genome. A total of eight clusters of phage-related genes have been identified in the Temecula strain, none of which is present in strain 9a5c genome (Moreira et al. 2005). A more recent analysis using the “Prophage Finder” program indicates four and three potential prophage clusters in the strain 9a5c and Temecula genomes, respectively (Bose and Barber 2006). Using manual annotation, we have identified three and four potential prophage clusters in the draft sequence contigs of the Dixon and Ann-1 strains, respectively. Presumptive phage particles associated with the Temecula strain grown in PW broth were recently observed by transmission electron microscopy (Chen and Civerolo 2008).

## OBJECTIVES

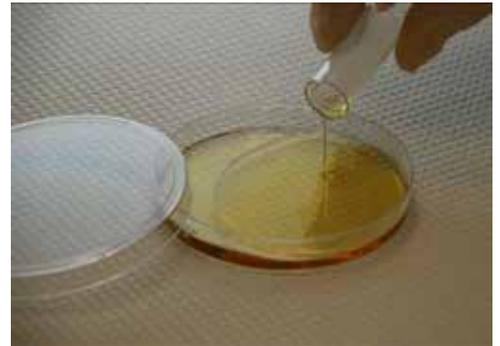
1. To develop a method for the isolation and propagation of *Xylella* phage.
2. To isolate lysogenic and/or virulent phage.
3. To characterize the isolated phage.

## RESULTS AND DISCUSSION

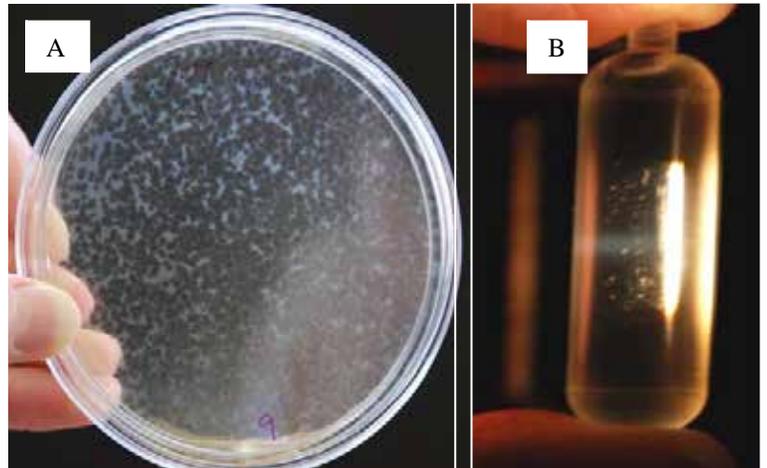
The formulation of a semi-solid medium that is conducive to even dispersal and confluent growth of *Xf*, a technique which is required for the efficient manipulation and study of phages and bacteriocins, has not been previously reported in the literature. We have established such techniques and developed an efficient plate assay for detection of phage and bacteriocins (**Figure 1**). Using this novel method we were able to screen a 30 X 30 matrix using each isolate as an indicator to test supernatants of isolates grown in PW-M broth. California *Xf* isolates included in the study were Temecula, Ann1 and Dixon (Feil and Purcell 2001). Texas isolates included one each from American Sycamore (*Plantanus occidentalis*), seacoast sumpweed (*Iva annua*), annual sunflower (*Helianthus annuus*), redspike mexican hat (*Ratibida columnifera*), and western ragweed (*Ambrosia psilostachya*), black Spanish grape (*Vitis aestivalis* hybrid), mustang grape (*Vitis mustangensis*), as well three giant ragweed (*Ambrosia trifida* var. *texana*) isolates, two oleander (*Nerium olenander*), and 15 grape isolates (*Vitis vinifera*) isolated from different commercial varieties of grapevines grown in Texas.

Using the overlay method, we were able to identify plaque production on plates seeded with hosts. Phage activity was indicated by plaque formation. Serial dilutions of supernatants indicating activity were plated using the overlay method in which the bacterial suspensions and phage were added and mixed before being applied. After incubation for five-seven days at 28 °C, individual plaques were excised from the overlay, suspended in phage buffer and titered. This procedure was repeated twice to obtain a single clonal plaque isolate. High titer lysates ( $10^{10}$  PFU/ml) were prepared by harvesting overlays of plates exhibiting confluent lysis (**Figure 2A**). To perform further analysis, high titer lysates were purified using a CsCl gradient (**Figure 2B**).

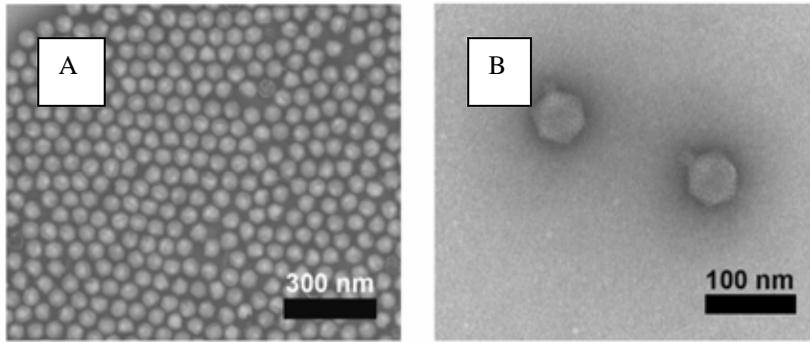
Transmission electron microscopy of purified phage revealed that phage *Xfas53* belonged to the family *Podoviridae* with a head diameter of 55 nm and a short non-contractile tail having a diameter of 12 nm (**Figure 3 A and B**).



**Figure 1.** Plate overlay method for isolation and propagation of *Xylella* phage.



**Figure 2.** **A.** Dilution series of phage *Xfas53* exhibiting confluent lysis of host. **B.** CsCl gradient showing phage band (red arrow).

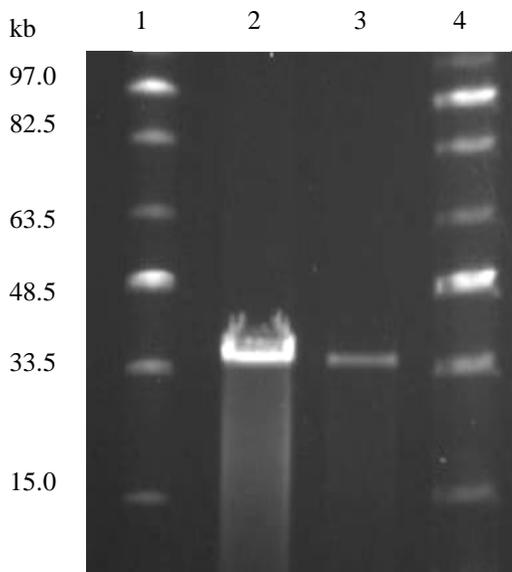


**Figure 3A and B.** Electron micrograph of CsCl purified phage Xfas53 stained with 2% (w/v) aqueous uranyl acetate.

The adsorption of *Xfas53* was characterized by measuring the disappearance of free phage after mixing with the susceptible host cells. We observed 95% adsorption to susceptible host in 15 min at an MOI of  $10^{-3}$ . Both sensitive and resistant *Xf* isolates from a variety plant hosts were identified in a host range study. Representative *Xf* isolates from a range of hosts or grape varieties that exhibited sensitivity to phage *Xfas53* are listed in Table 1.

**Table 1.** Representative isolates, plant source and origin exhibiting sensitivity to phage *Xfas53*.

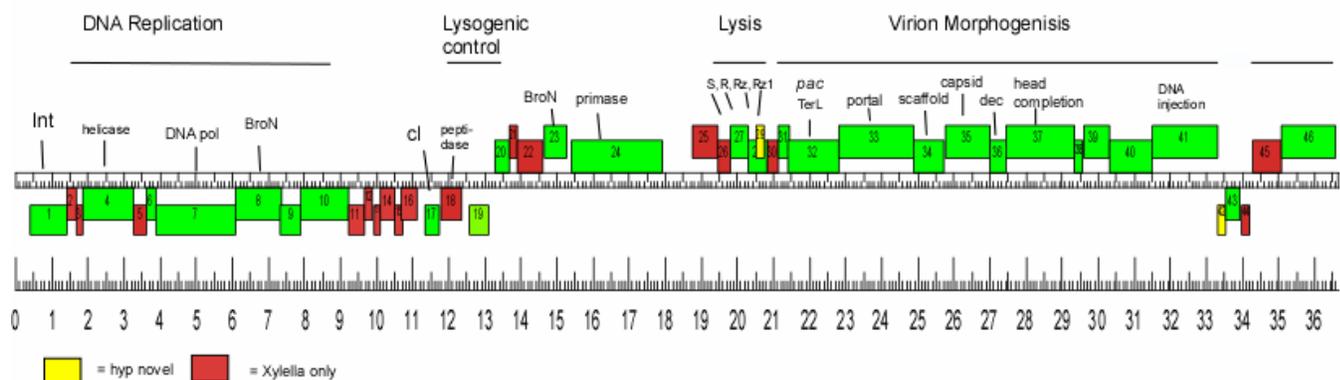
Plant source-variety	Isolate	Origin
Oleander	<i>Xf</i> 95	M. Black (TX)
Black Spanish Grape	<i>Xf</i> 39	M. Black (TX)
Mustang Grape	<i>Xf</i> 41	M. Black (TX)
Grape-Chambourcin	<i>Xf</i> 48	Apple/Torres(TX)
Grape-Sangiovese	<i>Xf</i> 76	Gonzalez/Enderle (TX)
Grape-Zinfandel	<i>Xf</i> 67	Apple/Torres(TX)
Grape-Zinfandel	<i>Xf</i> 78	Gonzalez/Enderle (TX)
Grape-Syrah	<i>Xf</i> 66	Gonzalez/Enderle (TX)
Grape	Temecula	Temecula (CA)



**Figure 4.** Ethidium bromide stained gel showing 0.5 µg (lane 2) and 0.05 µg (lane 3) of genomic DNA from phage *Xfas53*. MidRange PFGE Marker I (lanes 1 and 4). Marker sizes are indicated.

The genome size of phage *Xfas53* was estimated to be approximately 36 kb by pulsed-field gel electrophoresis (**Figure 4**), and no genomic ladder, indicating end annealed multimers, was observed, suggesting that the phage uses pac-type rather than cos-type DNA packaging.

A random library of the phage DNA was sequenced with eight-fold coverage. The assembled reads resulted in the production of a single contig of 36,673 base pairs with a GC content of 57%. A total of 46 protein coding genes were predicted. The genes are organized into two transcription units with the first 19 genes being transcribed from the reverse strand and all but three of the remaining 27 genes being transcribed from the forward strand (**Figure 5**). Genes at the divergent promoter region include some with similarity to proteins implicated in lysogenic control in other temperate phage. *Xfas53* has a lysis cassette that includes a holin, endolysin, Rz, and Rz1 equivalent. Functional annotation of the 46 genes indicates these include genes for DNA replication and metabolism, lysogenic control, host cell lysis, and virion morphogenesis. Comparison of the *Xfas53* encoded proteins to those from other phages indicated that the predicted structural proteins are most closely related to the Bpp-1 – like podophages as well as *Thalassomonas* phage BA3.



**Figure 5.** Map of phage Xfas53. Boxes are predicted genes drawn to scale and placed above or below the genome scale bar, based on transcription orientation. Gene numbers are listed in the boxes and homolog or functional assignments, where possible, are indicated. Yellow boxes indicate novel hypothetical proteins. Red boxes indicate proteins unique to *Xylella*. Green boxes indicate conserved hypothetical proteins.

## CONCLUSIONS

We have isolated and characterized a functional lysogenic phage of *Xf*. This is a significant step forward in understanding the biology of *Xf* and its phages, which will allow us to study the phage-*Xylella* interaction and the potential use of phages as biocontrol agents. Our results increase the probability of success in identifying functional virulent phage and/or bacteriocins for the implementation of a control strategy that is not currently available against this economically important pathogen.

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**BIOLOGICAL CONTROL OF PIERCE'S DISEASE OF GRAPEVINE  
WITH BENIGN STRAINS OF *XYLELLA FASTIDIOSA***

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

**ABSTRACT**

This project includes the evaluation of the biological control of Pierce's disease (PD) in California with a strain of *Xylella fastidiosa*, EB92-1, which has provided effective control of PD in previous greenhouse and vineyard tests in Florida. On June 26, 2008 in greenhouses at UC Davis, fifty plants of Orange Muscat, Cabernet Sauvignon, Reisling, Chardonnay, Barbera, and Viognier were inoculated with strain EB92-1 and fifty plants of each were left untreated as controls. The Orange Muscat and Cabernet Sauvignon vines were transplanted into the Bella Vista Vineyard in Temecula on July 21-22. Barbera and Viognier were transplanted into Preston Vineyards in the Sonoma Valley during the last week of July and Reisling and Chardonnay were transplanted into Beringer Vineyard in Napa Valley in October. The development of PD in these trials will be monitored for two-five years. In Florida, different methods of obtaining young plants colonized with strain EB92-1 were evaluated for the effectiveness in biological control of PD. In the greenhouse, rooted Chambourcin cuttings from vines in the UF research vineyard that had been treated with EB92-1 had fewer PD symptoms than rooted cuttings from control vines; however, Chardonnay rooted cuttings from treated vines had similar PD symptoms as from the untreated. In the vineyard at Mid-Florida REC, scion, rootstock, and scion plus rootstock treatments with EB92-1 all had significantly lower incidences of PD than the untreated vines of Merlot and there were no significant differences among these treatments.

**INTRODUCTION**

Pierce's disease (PD) of grapevine is a chronic problem for the California grape industry and has become more of a threat to the industry with the introduction of the glassy-winged sharpshooter. PD is especially damaging in the southeastern USA where it is endemic and is the primary factor limiting the development of a grape industry based on the high-quality European grapes (*Vitis vinifera* L.). The only feasible control for Pierce's disease is resistance. Through 10 years of research on the biological control of Pierce's disease of grapevine in Florida by cross protection with weakly virulent strains of *Xf*, we demonstrated that this also is a potential means of controlling this disease. In a vineyard study, *Xf* strain EB92-1, benign to grapevine, provided excellent control of PD in *V. vinifera* cv. Cabernet Sauvignon for four years in the vineyard in central Florida (Hopkins, 2005). Strain EB92-1 was introduced into the vines only once at the beginning of the 4-year trial and was still controlling the disease at the end; whereas, all unprotected vines were dead. These treated vines were still healthy and producing fruit in 2008, 11 years after treatment. The overall goal of this project is to develop a biological control system for Pierce's disease (PD) of grapevine that would allow the production of *V. vinifera* in California and other areas where PD and the glassy-winged sharpshooter (GWSS) are endemic.

**OBJECTIVES**

1. To evaluate strain EB92-1 of *Xf* which has provided effective biocontrol of PD in previous greenhouse and vineyard tests in Florida for possible commercial application for the biological control of Pierce's disease of grapevine in the vineyard in California.
2. To compare different methods of treatment with strain EB92-1 of *Xf* for the biocontrol of PD in *V. vinifera* in the vineyard.

**RESULTS**

**Establishment of field trials of strain EB92-1 for biological control of PD in vineyards in California**

It took from July to December 2007 to obtain the USDA Permits to test the biocontrol strain in California. The field plot locations are in the Bella Vista Vineyard in Temecula, CA, in the Beringer vineyard in the Napa Valley, and in Preston Vineyards in the Sonoma Valley.

All plants for the vineyard tests were planted in April in greenhouses at UC Davis. The cultivars were Orange Muscat (propagated by the grower, Imre Cziraki, and starting budbreak when planted April 6), Cabernet Sauvignon/110R (dormant rooted vines from Vintage Nursery, planted April 30), Reisling/3309 (dormant rooted vines from Vintage Nursery, planted April 30), Chardonnay/3309 (dormant rooted vines from Vintage Nursery, planted April 30), Barbera/110R (dormant rooted vines from Sunridge Nursery, planted April 30, and Viognier/110R (growing potted vines from Vintage, planted April 30).

The biocontrol strain, EB92-1 was recovered from storage in glycerol at -70 C. Five and six-day cultures of second transfer of the bacterium from storage on PD3 solid medium were hand-carried by Don Hopkins on a flight to California. For biocontrol treatment of the grape plants, a slightly cloudy solution of EB92-1, approximately 0.25 OD at 600 nm ( $10^7 - 10^8$  CFU/ml) was prepared in 75 ml of SCP buffer (disodium succinate, 1.0 g/L; trisodium citrate, 1.0 g/L;  $K_2HPO_4$ , 1.5 g/L;  $KH_2PO_4$ , 1.0 g/L; pH 7.0) in Bruce Kirkpatrick's laboratory at UC Davis. A pin pricking technique was used to inoculate the biocontrol into the xylem vessels of the treated grapevine. A drop (0.02 ml) of the biocontrol suspension was placed onto each of two lower internodes of the plants. The stem was pierced three-five times through the drop with a syringe needle. The inoculum was pulled into the plant by the negative pressure of the pierced xylem vessels. Approximately  $5 \times 10^5$  to  $5 \times 10^6$  bacteria were inoculated into each node.

For transplanting into the Bella Vista Vineyard in Temecula, 50 Orange Muscat were inoculated with the biocontrol strain (EB92-1) on June 26, and 50 were left untreated as controls. Fifty Cabernet Sauvignon/110R were treated and 50 were untreated controls. These plants were transported to Temecula and transplanted into plots in the Bella Vista Vineyard on July 21-22..

For Preston Vineyards in Sonoma, 50 Barbera/110R and Viognier/110R from were inoculated with EB92-1 and 50 vines of each were left as untreated controls. These plants were transported to Sonoma and transplanted the last week of July, 2008. For transplanting into the Beringer Vineyard in Napa, 50 Reisling/3309 and 50 Chardonnay/3309 were treated with EB92-1 on June 25 and 50 vines of each were left untreated as controls. The vines were transplanted in Beringer Vineyard in October.

#### Comparison of treatment methods with strain EB92-1 for biocontrol of PD

Cuttings of the cultivars Chardonnay and Chambourcin (French/American hybrid) in the UF Mid-Florida REC vineyard were taken both from vines that are colonized by biocontrol strain EB92-1 and vines not colonized by *Xf*. Rooted cuttings of these vines were potted in the greenhouse and 12 of the cuttings from untreated vines were injected with strain EB92-1. Two weeks later all plants were inoculated with pathogenic PD strains and observed weekly for symptoms. In both cultivars, plants injected with strain EB92-1 in the greenhouse had significantly lower PD rating than the untreated plants (**Table 1**). There did not seem to be any effect of taking the cuttings from an infected vine in the vineyard with Chardonnay, but PD was significantly less severe in the plants derived from cuttings of biocontrol vines of the cultivar Chambourcin than in plants derived from untreated vines. This may mean that the biocontrol strain is not consistently carried over into propagated plants. Recent experiments have indicated that the 0.25 OD inoculum of pathogen can overcome the biocontrol strain in some cases; therefore, this experiment is being repeated with lower pathogen inoculum levels.

**Table 1.** Comparison of treatment method with EB92-1 on control of PD in the greenhouse.

Source of EB92-1 treatment	PD rating after 8 wks: <sup>1,2</sup>	
	Chardonnay	Chambourcin
Untreated rooted cuttings	2.9 b	4.1 b
Rooted cutting from field EB92-1, biocontrol plant	3.0 b	2.8 a
Injected EB92-1 untreated rooted cuttings	2.2 a	2.6 a

<sup>1</sup>Plants were rated on a 0 - 5 scale with 0 = no symptoms and 5 = a dead plant. Ratings were averaged for treatments.

<sup>2</sup>Mean separation in columns by Duncan's New Multiple Range Test, 5% level.

Experiments to evaluate different methods of treatment with EB92-1 were established in the Mid-Florida REC vineyard during the summer, 2007. Four treatments were applied to the cultivar Merlot/101-14 (dormant rooted vines from Vintage Nursery planted in pots in mid-April) on May 29 and the plants were transplanted into the vineyard on June 21. The treatments were 1) injection of EB92-1 into the new growth of the scion only, 2) injection of EB92-1 into the rootstock only, 3) injection of EB92-1 into both the rootstock and scion, and 4) nontreated. Five treatments were applied to the cultivar Chardonnay CL96/330914 (dormant rooted vines from Vintage Nursery planted in pots in mid-April) on June 13 for the three greenhouse treatments and on July 26 for the scion field injection.. The plants were transplanted into the vineyard on July 3. The treatments were 1) injection of EB92-1 into the scion only in the greenhouse, 2) injection of EB92-1 into the rootstock only in the greenhouse, 3) injection of EB92-1 into both the rootstock and scion in the greenhouse, 4) nontreated, and 5) injection of EB92-1 into the scion only in the vineyard. In a third experiment, Chardonnay cuttings from the MREC vineyard were grafted onto Salt Creek rootstock rooted cutting from the vineyard. The grafted plants were transplanted into the vineyard on August 14. The treatments included 1) Chardonnay cuttings from mature vines that had been treated three years ago with EB92-1 on Salt Creek, 2) Chardonnay cuttings from mature nontreated vines on Salt Creek, and 3) Chardonnay cuttings from mature nontreated vines on Salt Creek, with the scion injected with EB92-1 in the vineyard on August 29.

One year after these trials were established, PD incidence was still low and there were not any significant differences between treatment methods and the untreated in Chardonnay/3309 (**Table 2**). However, on October 7, 2008, scion, rootstock, and scion plus rootstock treatments with EB92-1 all had significantly lower incidences of PD than the untreated vines of Merlot. This early, preliminary data indicates that it may not be critical whether the strain EB92-1 is injected into xylem of rootstock, scion, or both. Disease development in these trials in years two and three is most important to any conclusions on the most effective treatment methods. In the first year, there were no significant differences among the Chardonnay/Salt Creek treatments. This evaluation of cuttings from treated vines is especially significant, because rooting cuttings from infected mother vines would be a preferred treatment method over having to inject every vine by the pin pricking method.

**Table 2.** Effect of methods of treatment of grape plants with *Xylella fastidiosa* strain EB92-1 on biological control of PD.

Treatment	% PD incidence in: <sup>1</sup>	
	Merlot/101-14	Chardonnay/3309
Scion injection	0 a	0
Rootstock injection	0 a	0
Scion & Rootstock injection	11 a	11
Scion field injection	NT	11
Untreated	33 b	0

<sup>1</sup>Mean separation in columns by Duncan's New Multiple Range Test, 5% level.

## CONCLUSIONS

There are no results or conclusions for the California field trials, since the vines treated with strain EB92-1 were not established in vineyards until mid-summer to fall of this year. Preliminary results in Florida indicated that rooted cuttings from EB92-1 mother vines did not consistently have reduced incidence of PD in greenhouse tests. This method of using the biocontrol strain may not be feasible, but field tests are underway to evaluate this treatment method, because rooting cuttings from infected mother vines would be a preferred treatment method over having to inject every vine by the pin pricking method. As symptoms of PD began to develop in Merlot in Florida trials, the strain EB92-1 appeared to reduce PD incidence whether it was applied to rootstock or scion.

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## FUNDING AGENCIES

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## IDENTIFICATION OF FACTORS MEDIATING COLD THERAPY OF *XYLELLA FASTIDIOSA* INFECTED GRAPEVINES.

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**Reporting Period:** The results reported here are from work conducted February 2008 to October 2008.

**ABSTRACT**

Pierce's disease (PD) is currently found in many regions of California and the southeastern United States. One factor that has been shown to be associated with the observed limited geographical distribution of PD in North America is the severity of winter temperatures in those regions. For example, PD does not occur in New York, the Pacific Northwest or at high altitudes in South Carolina, Texas and California where the winter temperatures on average drop below zero degrees Celsius (Hopkins & Purcell, 2002). Purcell (1977, 1980) and Feil's (2002) research suggested that some factor(s) expressed in the intact plants helps eliminate *Xylella fastidiosa* (*Xf*) from grapevines.

To elucidate the mechanism(s) of the "cold curing" phenomenon, it is necessary to determine the cold curing temperature threshold that maximizes PD recovery and minimizes vine mortality. This research should also allow us to generate projection maps to determine if vineyards in cold boundary areas (i.e., foothills of the Sierra and northern-most California) are at risk for developing PD. The information obtained from these experiments will facilitate basic research on mechanisms causing cold therapy and provide data that could be used by grape growers for risk assessment and management purposes. It has been well documented that xylem sap contains many metabolites such as mono- and disaccharides, organic acids, plant growth regulators and other organic compounds (Andersen et al., 1989; Bollard, 1960; Pate, 1976; Wormall, 1924; reviewed by Seyedbagheri & Fallahi, 1994). Though it is well known that these compounds are in the sap, little is known about the effects of cold temperatures on the synthesis or quantity of these compounds in sap. Also, little is known about the effect of cold temperatures on factors such as pH and osmolarity of xylem sap and how these factors may be contributing to the cold curing phenomenon. Assessing the effect of pH and osmolarity on the viability of *Xf* cells *in vitro*, could provide insight into the factors that contribute to the cold curing phenomenon.

Previous research has shown that herbaceous and woody plants exposed to sub-lethal cold conditions have significantly elevated levels of plant hormones, such as abscisic acid (ABA), which induce the synthesis of a number of cold shock proteins (Guy, 1990; Bravo, et al., 1998; Thomashow, 1998). Kuwabara et al. (2002) elicited cold-shock proteins at 23°C in winter wheat using an exogenously applied 100ppm ABA solution. The ABA treated plants elicited the synthesis of proteins that inhibited the *in vitro* growth of a wheat fungal pathogen. Although nothing is known about the effects of these cold-induced proteins on the growth of *Xf*, if they were antagonistic the application of ABA could lead to a potentially novel approach for managing Pierce's disease.

**INTRODUCTION**

*Xylella fastidiosa* (*Xf*) is a xylem-limited, gram-negative bacterium that causes Pierce's disease (PD) in grapevines. The *Xf* strains that cause PD in grapevines also cause alfalfa stunt and almond leaf scorch, while other strains of *Xf* cause citrus variegated chlorosis, oleander leaf scorch, phony peach, and several other diseases (Purcell, 1997). Little is known about host specificity of strains or the mechanisms by which *Xf* causes plant disease (Purcell & Hopkins, 1996). Symptoms of this "mysterious disease" were first described by Newton Pierce in 1882. Today, typical symptoms of PD in grapevines include leaf margin necrosis, leaf blade drop, irregular lignification of canes, "raising" of fruit clusters, dieback and death of grapevines (Hopkins & Purcell, 2002; Varela, et al., 2003).

PD is currently found in many regions of California and the southern United States. One factor that has been shown to be associated with the observed limited geographical distribution of PD in North America is the severity of winter temperatures in those regions. For example, PD does not occur in New York, the Pacific Northwest or at high altitudes in South Carolina, Texas and California where the winter temperatures on average drop below zero degrees Celsius (Hopkins & Purcell, 2002). Purcell (1977, 1980) demonstrated that relatively brief exposures to sub-freezing temperatures eliminated *Xf* in cold treated *Vitis vinifera* grapevines. Purcell also found that moderately susceptible 'Cabernet Sauvignon' had a higher curing rate following cold treatment compared to the PD-susceptible variety 'Pinot Noir'. More recently, Purcell's group also showed that whole, *Xf* infected potted vines that were exposed to low temperatures had a higher rate of recovery than PD-affected detached bud sticks exposed to the same cold temperatures (Feil, 2002). This implies that some factor(s) expressed in the intact plant, but not in detached bud sticks, helped eliminate *Xf* from the plants. Despite documentation of the cold curing phenomenon, little is known about the physiological/biochemical basis that mediates cold therapy. To further understand the basis of this phenomenon, we are conducting several studies to identify the

physiological/biochemical factor(s) that occur or are expressed in cold treated vines that contribute to the elimination of *Xf*. If such a factor(s) is/are found, it may be possible to induce their expression under non-freezing temperatures and potentially provide a novel approach for managing PD.

## OBJECTIVES

1. Develop an experimental, growth chamber temperature regime that can consistently cure Pierce's disease affected grapevines without causing unacceptable plant mortality.
2. Analyze chemical changes such as pH, osmolarity, total organic acids, proteins and other metabolites that occur in the xylem sap of cold-treated versus non-treated susceptible and less susceptible *Vitis vinifera* varieties.
3. Assess the viability of cultured *Xf* cells growing in media with varying pH and osmolarity and cells exposed to xylem sap extracted from cold- and non-treated grapevines.
4. Determine the effect of treating PD-affected grapevines with cold-induced plant growth regulators, such as abscisic acid (ABA), as a possible therapy for PD.

## RESULTS AND CONCLUSIONS

Objective 1: The results described in previous reports show that our field plots and cold chamber plants showed lower disease ratings and higher curing rates in the colder temperature treatments. In 2005-2007 sites, vine mortality was minimal due to better cold acclimation of the grapevines prior to establishing the plots in the fall.

The data collection for the field and cold chamber studies is complete and analysis of the data to determine the critical temperature thresholds for inducing the cold curing phenomenon is underway. We are continuing to work with Len Coop from the University of Oregon to generate a cold temperature model to determine if vineyards in cold boundary areas (i.e., foothills of the Sierra and northern-most California) are at risk for developing PD. The information obtained from these models could provide data that could be used by grape growers for risk assessment and management purposes.

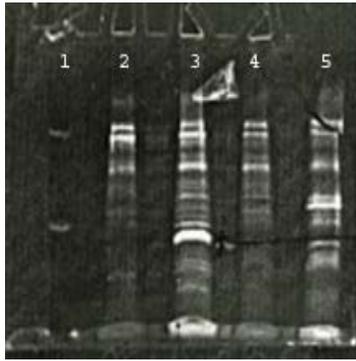
Objective 2: Xylem sap was extracted from vines from each field location and cold chamber treatment using a pressure bomb. The samples were then tested for potential changes in pH, osmolarity, protein profiles, total sugars, and calcium and magnesium concentrations in xylem sap. The results for the 2005-2006 field and cold chamber grapevine xylem sap pH, osmolarity, and calcium and magnesium concentrations can be found in previous reports. The 2006-2007 xylem sap samples were analyzed for sugars, calcium and magnesium levels. The results of these analyses will be reported in our 2008 Pierce's Disease Meeting poster in December once the statistical analyses are completed.

Xylem sap protein profiles were analyzed for the 2005-2007 samples. The sap proteins were concentrated by acetone precipitation and the proteins were electrophoresed in a 12% Tris-HCl 1-dimensional polyacrylamide gel (PAGE). Protein profiles of the PAGE gels were compared for each treatment. Unique protein bands that were found in the cold treated plants were cut from the gel, and end terminally sequenced by the UCD Molecular Structure Facility. Some of the constitutively expressing xylem sap proteins were also sequenced to determine their identity.

### Cold Chamber Experiment Results:

The results reported in previous reports show that the pH of Cabernet Sauvignon (CS) xylem sap was significantly higher than Pinot Noir (PN) sap overall. Sugar and select ion concentration analysis of CS grapevines showed greater amounts of glucose and fructose in  $-5^{\circ}\text{C}$  cold chamber vines, whereas  $\text{Ca}^{+}$  levels were greater in the warmest treatments. Osmolarity was greatest in the coldest treatments and decreased with increasing temperature. Conversely, in PN grapevines, glucose and fructose levels were the lowest in the coldest treatments.  $\text{Ca}^{+}$  levels showed a similar trend with CS vines, with increased  $\text{Ca}^{+}$  levels in the warmer temperature treatments. Temperature appeared to have a less direct effect on osmolarity in Pinot Noir grapevines. ABA concentrations in the spring xylem sap collections were the lowest in the coldest cold chambers.

Protein profiles of grapevine xylem sap exposed to various temperatures were determined by PAGE (**Figure 1**). Most of the proteins were similar for the various temperatures, but a few unique proteins were found in the cold stressed and/or *Xf*-inoculated plants and these proteins were end terminally sequenced by the UCD Molecular Structure Facility. Sequencing of xylem proteins from cold-treated vines identified proteins that had high sequence homology with stress proteins that are produced by Cabernet Sauvignon berries under water deficit stress conditions, proteins that are similar to proteins produced in Pinot Noir roots, trypsin inhibitors and a thaumatin-like protein which is reported to have anti-fungal properties.



Lane 1: Dual Color SDS-ladder.  
 Lane 2: CS +5°C xylem sap from control vines.  
 Lane 3: CS -5°C xylem sap from control vines.  
 Lane 4: CS +5°C xylem sap from inoculated vines.  
 Lane 5: CS -5°C xylem sap from inoculated vines.

**Figure 1.** Protein profiles of grapevine xylem sap as determined by PAGE analysis. 150 uL of xylem sap was precipitated with cold acetone. Proteins were suspended in 30 uL of SDS-loading buffer and electrophoresed in a 12% Tris-HCl polyacrylamide gel.

### Field Experiment Results:

ABA concentrations in the spring xylem sap collections were the lowest in the coldest field locations. ABA levels were higher in the late winter sap collections than in the spring collections for the field locations. Osmolarity, pH, calcium and magnesium levels show similar trends to those seen in the cold chamber experiments.

Objective 3: The solutions used for these viability experiments included: water, extracted *V. vinifera* ('Pinot Noir' and 'Cabernet Sauvignon' varieties) xylem sap, PD3, HEPES, sodium and potassium phosphate buffers. All buffers and media were adjusted to pH 6.8. *Xf* cells suspended in the various buffers and media were exposed to various temperatures (28°C, 5°C, 2.2°C, 0°C, -5°C, -10°C and -20°C). Potassium phosphate buffer at various pH values (5.0-6.8) was also used to determine the effects of pH on the survival of *Xf*.  $10^4$  *Xf* cells were suspended in 1ml of the various test solutions which were then incubated at various temperatures. Aliquots of the suspensions were plated on PD3 medium and *Xf* CFUs were counted seven days post plating.

The results of these experiments were reported in detail in the 2007 progress report. To summarize the results, these experiments indicate that *Xf* can survive at 28°C in most media except water. The mortality rate was the lowest in PD3 medium in the 5°C and 2.2°C temperature treatment. The deionized water treatment had the highest mortality rate followed by potassium phosphate at pH 6.2. The highest survival at 0°C occurred with PD3 media and in xylem sap collected from grapevines growing in a cold climate (Placer County, CA). These experiments showed that *Xf* can survive at -5°C in all buffers at pH 6.8, media and xylem sap for at least four days. No cultivable *Xf* was recovered from any of the media, buffers or xylem sap after 24 hours at -10°C or at -20°C.

Objective 4: To assess the possibility of using a plant hormone to artificially induce cold curing, we contacted Valent Bioscience Corporation who has an active research and development program on the use of ABA on agricultural crops. In November of 2005, 2006, and 2007, healthy and *Xf*-inoculated Cabernet Sauvignon and Pinot Noir vines grown and inoculated with *Xf* as described in Objective 1 were foliar sprayed or soil drenched with solutions of ABA in the fall. The 2005-2006 results showed interesting trends and were repeated in the 2006-2007 and 2007-2008 seasons. Our applications of ABA in the 2005-2006 season appeared to have a curing effect in PD-infected grapevines. ABA application that was the most effective was VBC-30030 applied as a drench, but some of the other forms and concentrations of ABA also had some curing effect. For this first application in 2005-2006, there was no rain until a week following the application. In 2006-2007, this experiment was replicated with some modifications to the treatments used in 2005-2006. The resulting curing rates were not the same as in the 2005-2006 treatments. The only treatment that seemed to have more curing than the control treatment was the VBC-30030 drench in Pinot Noir grapevines, but the curing rate was not as high as in the 2005-2006 season. This difference could possibly be due to a rain event that occurred a few hours after the ABA application, possibly diluting, washing off, or leaching out the applied ABA.

To evaluate the reproducibility of the 2005-2006 results, a third ABA trial was conducted in the 2007-2008 season. In 2007, there were four treatments with *Xf*-infected vines and healthy controls. The curing rates for the various treatments are currently being evaluated by IC-PCR.

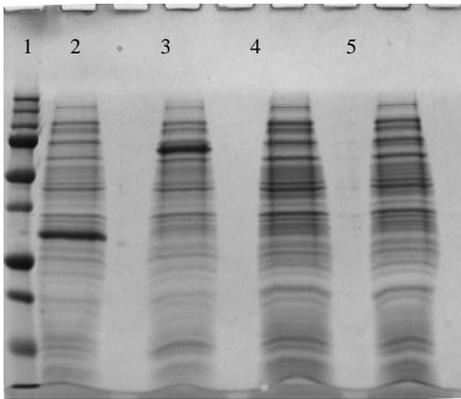
To examine the mechanism behind the possible curing due to ABA application, the xylem sap of the grapevines was extracted using a pressure bomb four days after the application of the ABA treatments. To examine the proteins produced when grapevines are exposed to ABA, protein profiles were made of each treatment. The 150 ul of xylem sap was precipitated with cold acetone to concentrate the proteins. The proteins were resuspended in 30 uL of SDS-loading buffer and electrophoresed in a BioRad 12% Tris-HCl gel. Some of the proteins that were sequenced in the ABA treated vines were similar to those found in our cold treated vines.

## 2008-2009:

To evaluate the reproducibility of the 2006-2007 Chardonnay results a third ABA spray trial is being prepared for the 2008-2009 season. In the fall, treatments will be applied to healthy and *Xf*-infected Chardonnay vines as follows:

Control:	16 Chardonnay plants sprayed with water
2000 ppm spray:	16 Chardonnay plants sprayed with VBC-30054
100 ppm spray:	16 Chardonnay plants sprayed with VBC-30030
100 ppm drench:	16 Chardonnay plants drenched with VBC-30054
10 ppm drench:	16 Chardonnay plants drenched with VBC-30030

Our first unique cold expressed protein found in Objective 2 has been cloned and expressed (**Figure 2**). Expressed and purified xylem sap proteins will be used to determine if the proteins demonstrate any anti-*Xf* activity *in vitro*. If anti-*Xf* activity is shown, future work would focus on expressing the anti-*Xf* proteins in transgenic rootstocks as a possible Pierce's disease control method.



1. Molecular size markers.
2. Cloned and expressed Cabernet TLP protein.
3. Cloned and expressed PG protein (positive control).
4. Cloned, non-induced Cabernet TLP protein.
5. Cloned, non-induced PG protein.

**Figure 2.** Cloning and expression of an *Xf* polygalacturonase and *Vitis vinifera* thaumatin-like protein in *E. coli*.

## CONCLUSIONS

The results of our field and cold chamber experiments show lower disease ratings and higher curing rates in the colder temperature treatments. The coldest treatments had the highest rate of recovery from PD, but also the highest grapevine mortality. These findings will be used to determine a Pierce's disease Risk Assessment Model based on curing rates and winter temperatures.

Analysis of the biochemical factors in sap revealed some interesting results. For the cold chamber experiments, the pH of CS xylem sap was significantly higher than PN sap overall. Sugar and select ion concentration analysis of CS grapevines showed greater amounts of glucose and fructose in  $-5^{\circ}\text{C}$  cold chamber vines, whereas  $\text{Ca}^{+}$  levels were greater in the warmest treatments. Osmolarity was greatest in the coldest treatments and decreased with increasing temperature. Conversely, in PN grapevines, glucose and fructose levels were the lowest in the coldest treatments. Interestingly, the osmolarity of PD3 media is 113 mmol/kg, whereas the osmolarity of xylem sap was 25-45 mmol/kg.  $\text{Ca}^{+}$  levels showed a similar trend with CS vines, with increased  $\text{Ca}^{+}$  levels in the warmer temperature treatments. Temperature appeared to have a less direct effect on osmolarity in Pinot Noir grapevines.

ABA concentrations in the spring xylem sap collections were the lowest in the coldest field locations and coldest cold chambers. ABA levels were higher in the late winter sap collections than in the spring collections for the field locations. PAGE protein profile analysis showed that most of the proteins we found were similar for the various temperatures, but a few unique proteins were found in the cold stressed and/or *Xf*-inoculated plants. Sequencing results of xylem proteins from cold-treated vines showed proteins that are similar to stress proteins that are produced by Cabernet Sauvignon berries developing under water deficit stress conditions, proteins that are similar to proteins produced in Pinot Noir roots, and trypsin inhibitors. One of the proteins that was expressed at comparatively high concentrations in cold-exposed vines is a thaumatin-like protein which has been reported to have anti-microbial activity. We will assess the potential anti-*Xf* properties of this protein by cloning, expressing, purifying and using this protein in *Xf* growth inhibition assays in the future.

The *in vitro* culture experiments indicate that *Xf* can survive at  $28^{\circ}\text{C}$  in most media except water. At  $28^{\circ}\text{C}$ , the survival rate was the highest in PD3 media followed by potassium phosphate at pH 6.8, sodium phosphate, and xylem sap. At the coldest temperatures, the highest survival at  $0^{\circ}\text{C}$  occurred with PD3 media and in xylem sap collected from grapevines growing in a cold climate (Placer County, CA), whereas survival was the lowest in deionized water and potassium phosphate at pH 6.2. Interestingly, *Xf* can survive at  $-5^{\circ}\text{C}$  in all buffers at pH 6.8, media and xylem sap for at least four days. No cultivable *Xf* was

recovered from any of the media, buffers or xylem sap after 24 hours at -10°C or at -20°C. *Xf* in potassium phosphate buffers with pH values at 5.0, 5.4 and 5.8 died rapidly at all temperatures.

The results the ABA application experiments in the 2005-2006 season indicate that ABA appears to have a curing effect when applied as a drench. The synthetic ABA had the most interesting result with 100% curing in Pinot Noir vines. Disease ratings for both ABA drench treatments were significantly less than untreated controls.

In 2006-2007 this experiment was replicated with some modifications to the 2005-2006 treatments. Curing rates were not as high as what we saw in the 2005-2006 treatments. The only treatment that seemed to have more curing than the control treatment was the VBC-30030 drench in Pinot Noir grapevines. The difference observed in the 2006-2007 ABA application could possibly be due to a rain event that occurred a few hours after the ABA application, possibly diluting, washing off, or leaching out the applied ABA. We are repeating the ABA experiment this season to determine if ABA applications could be used as a possible tool in the management of Pierce's disease.

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#### FUNDING AGENCIES

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# INHIBITION OF *XYLELLA FASTIDIOSA* POLYGALACTURONASE TO PRODUCE PIERCE'S DISEASE RESISTANT GRAPEVINES

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**Reporting Period:** The results reported here are from work conducted July 2008 to September 2008.

## ABSTRACT

Polygalacturonases (PG) (EC 3.2.1.15), catalyze the random hydrolysis of 1, 4-alpha-D-galactosiduronic linkages in pectate and other galacturonans. *Xylella fastidiosa* (*Xf*) possesses a single PG gene, *pglA* (PD1485) and *Xf* mutants deficient in the production of PG lose pathogenicity and have a compromised ability to systemically infect grapevines. We have cloned the *pglA* gene into a number of protein expression vectors and a small amount of active recombinant PG has been recovered, unfortunately most of the protein expressed is found in inclusion bodies in an inactive form. The goal of this project is to use phage panning to identify peptides or single chain fragment variable (scFv) antibodies that can bind to and inhibit *Xf* PG. Once peptides or scFvs are discovered that can inhibit PG activity *in vitro* these peptides will be expressed in grapevine rootstock to determine if the peptides can provide protection against Pierce's disease (PD).

## INTRODUCTION

Polygalacturonases (PG) have been shown to be virulence factors of a number of plant pathogenic bacteria including *Ralstonia solanacearum*, *Xanthomonas campestris*, and *Erwinia carotova* (Huang and Allen 2000; Dow et al. 1989; Lei et al. 1985). *Xylella fastidiosa* (*Xf*) possesses a single PG gene *pglA* (PD1485), and mutation of this gene results in a loss of pathogenicity and reduced ability to systemically infect grapevines (Roper et al. 2007). In order for *Xf* to systemically infect a grapevine, it must break down the pit membranes that separate individual xylem elements. Pectic polymers determine the porosity of the pit membrane (Baron-Epel, et al. 1988; Buchanan et al. 2000) and *Xf* PG allows the bacterium to breakdown the pectin in these membranes. The premise of this research is to identify a peptide that can be expressed in the xylem of a grapevine that can suppress *Xf* PG activity thus limiting the ability of *Xf* to spread systemically through grapevines and cause PD.

To accomplish this we will use phage display of a random dodecapeptide library and a scFv antibody library attached to the coat protein gp38 of M13 phage in a phage panning experiment using active recombinant *Xf* PG as the target. After three rounds of panning, phage that show a high binding affinity for *Xf* PG will be screened for their ability to inactivate PG activity *in vitro* in reducing sugar assays. Once a suitable inhibitory peptide, or the peptide embedded in a small protein carrier, is discovered it will be cloned into an Agrobacterium binary vector and used to transform tobacco and grapevines by the UCD Plant Transformation Facility. These transgenic plants will then be inoculated with *Xf* and the progression of PD symptoms will be compared to non-transgenic plants. If significant disease inhibition is shown, we will use these transgenic grapevines as rootstock to determine if they can also provide resistance to grafted non-transgenic *Vitis vinifera* scions.

## OBJECTIVES

1. Isolate a sufficient amount of biologically active *Xf* polygalacturonase (PG) enzyme to conduct phage panning and PG-inhibition assays.
2. Isolate M13 phage that possess high binding affinities to *Xf* and/or *Aspergillus aculeatus* (AA) PG, or synthetic peptides specific for the active sites of several PGs, from a M13 random peptide and scFv library.
3. Sequence candidate binding phage and determine if selected M13 phage and the gp38 M13 protein which mediates phage binding to *Xf* PG can inactivate PG activity *in vitro*.
4. Clone anti-*Xf* PG gp38 protein into an Agrobacterium binary vector and provide this construct to the UCD Plant Transformation facility to produce transgenic SR1 tobacco and Thompson seedless grapevines.
5. Determine if anti-*Xf* PG gp38 protein is present in xylem sap of transgenic plants.
6. Mechanically inoculate transgenic plants with *Xf* and compare Pierce's disease development with f-inoculated, non-transgenic control plants.

## RESULTS

### Objective 1.

Although we now have in hand a PG enzymatic activity assay, we would still like to obtain greater amounts of active *Xf* PG. The first attempt at using a recently developed agroinfection-compatible tobacco mosaic virus protein expression system (Lindbo, 2007) did not provide us with active *Xf* PG. However, we have produced a new *Xf* PG plant expression construct to help improve our yields using the plant expression system. This construct employs the use of a Rice Alpha Amylase signal peptide that will export *Xf* PG to plastids and extracellular compartments (Chen et al. 2004). Targeting the *Xf* PG to these

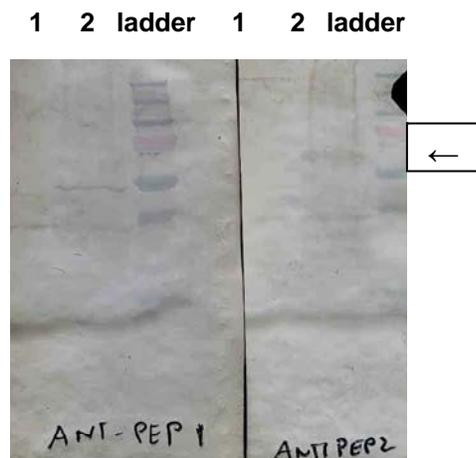
areas could be important if the reason we are not getting active *Xf*PG is because the plant is recognizing it and degrading it in the cytoplasm. In addition to the plant expression system, we are also generating constructs for an *E. coli* expression system that fuses *Xf*PG to Maltose Binding Protein (MBP) in the hopes the MBP will help overcome some of the insolubility issues we have encountered with other *E. coli* protein expression systems. The method we described previously for generating active *Xf*PG remains the method that delivers the most protein in active form, however we hope that one of these new strategies will provide us with a greater amount of active protein.

As reported in the previous PD/GWSS Proceedings, we feel confident that the reducing sugar assays that we are using to detect *Xf*PG activity dinitrosalicylic acid (Wang et al. 1997) and 3-Methyl-2-benzothiazolinonehydrazone methods (Anthon and Barrett 2002), will be suitable for the PG-inhibition assays.

#### Objective 2.

We have done extensive *in silico* analyses of the enzymatic active sites of several phytopathogenic bacterial and fungal PGs such as *Pectobacterium carotovora* ssp. *carotovora* and *Aspergillus aculeatus* (Pickersgill et al. 1998, Cho et al. 2001). The PGs from these other microbes have been well studied and structural studies have shown that the active site consists of roughly eight amino acids and the tertiary structure of the PGs are highly conserved across all fungal and bacterial PGs (Pickersgill et al. 1998, Cho et al. 2001, Shimizu et al. 2002, Abbott and Boraston 2007). Furthermore, previous research using phage display technologies showed that many of the phage that bound to a variety of enzymes also bound to and inactivated the enzyme (Hyde-deRuyscher et al. 1999).

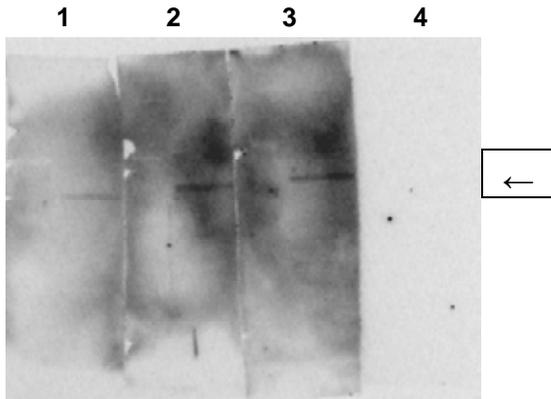
Having a very good idea of where the *Xf*PG active site is likely located on the PG protein, and which amino acids are involved in catalysis and substrate binding, we had synthesized two 14-mer peptides derived from the *Xf*PG sequence, one which will target the active site directly and a second that will target an area providing substrate entry into the active site. Additionally, these peptides were injected into rabbits to create polyclonal antibodies. These polyclonal antibodies were used in Western blot analyses that confirmed that the antibodies created against each 14-mer peptide could also recognize full length *Xf*PG (**Figure 1**).



**Figure 1.** Western blot analysis of polyclonal antibodies to *Xf*PG peptides 1 and 2. Lane 1 is *E. coli* lysate containing no *Xf*PG. Lane 2 is *E. coli* lysate containing recombinant *Xf*PG. Arrow represents location of *Xf*PG band.

We have completed the phage panning procedure for peptide 2 using the Tomlinson I and J scFv libraries. At the end of the third round of selection a polyclonal ELISA with BSA conjugated peptide 2 as the target was run which showed that each library (I and J) of scFv's showed a higher binding affinity to BSA conjugated peptide 2 than to BSA alone, or to other negative control wells in the plate. With this knowledge, 90 individual colonies from each library were picked from the third round phage pool and used in a monoclonal based ELISA to determine which monoclonal scFvs had the highest binding efficiencies for the *Xf*PG peptides. Eight clones from each library (I and J) providing the highest ELISA absorbance readings were chosen for sequencing. We have currently only sequenced the heavy chain variable portion of the scFv and although none of the eight clones from each of libraries shared the exact same sequence they did have similarities to each other. We are in the process of sequencing the light chain portion of these clones.

The eight monoclonal phages from each library (I and J) were then used individually as the primary antibodies in a Western blot to confirm that monoclonal phage raised against the 14-mer peptide 2 conjugated to BSA would also be able to identify full length recombinant PG (**Figure 2**) (Tanaka et al. 2002). Now that we have monoclonal phage that can bind to *Xf*PG, we will finish sequencing the variable regions and begin testing the efficacy of each monoclonal phage to inhibit *Xf*PG activity *in vitro*.



**Figure 2.** Western blot analysis of three representative monoclonal scFv phages from the J-library. Lanes 1-3 are *E. coli* lysate containing recombinant Xf PG, each membrane strip was reacted with a single monoclonal phage from the third round of panning. Arrow shows the location of the Xf PG protein. Molecular weight markers are on the left side of each gel strip. Lane 4 is a conjugate control that was not reacted with any monoclonal phage.

### Objectives 3-6.

We have sequenced the heavy chain variable regions of the 16 candidate monoclonal phage and although none of the eight clones from each of libraries shared the exact same sequence, they did have similarities to each other. We are in the process of obtaining the sequences of the light chain variable portions. Once all variable region sequences have been determined we will use the monoclonal phages in Xf PG inhibition assays. Once a candidate phage is found that can inhibit Xf PG *in vitro* we will then express the scFv protein alone and determine if the protein itself can also inhibit Xf PG activity *in vitro*. We will then be able to clone the anti-Xf PG protein into an Agrobacterium binary vector and provide this construct to the UCD Plant Transformation facility to produce transgenic SR1 tobacco and Thompson seedless grapevines. Once we have transgenic plants, we will be able to complete Objectives 5 and 6.

### CONCLUSIONS

We have made good progress thus far in identifying suitable PG activity assays to use in the PG-inhibition assays. We are currently exploring different plant and *E. coli* protein expression systems to generate more active PG to use in phage panning and activity assays. We have identified 16 candidate scFv phage, by panning against peptide 2 conjugated to BSA, that are capable of binding to full length Xf PG in Western blot analyses. These phage will now be used in *in vitro* Xf PG inhibition assays identified in Objective 1. If one of the candidate phage can inhibit Xf PG activity *in vitro*, then we will transform tobacco and grapevines with the peptide(s) and determine if the expressed anti-PG peptides are present in xylem sap and evaluate their potential for providing resistance to Pierce's disease.

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# ISOLATION, CHARACTERIZATION, AND GENETIC MANIPULATION OF *XYLELLA FASTIDIOSA* HEMAGGLUTININ GENES

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

## ABSTRACT

*Xylella fastidiosa* (*Xf*) possesses genes for hemagglutinins (HAs), large adhesion proteins involved in cell-cell aggregation and biofilm formation. Mutations in either one of the functional HAs, HxfA (PD2118) or HxfB (PD1792), result in hypervirulent strains that move faster and cause more severe disease in grapevines. We generated antibodies against portions of the HA proteins, used them in Western blot analyses and showed that HA proteins are secreted into the supernatant as soluble proteins, associated with membrane vesicles, and inserted into the outer membrane of *Xf*. Native HA proteins are processed from a predicted size of 360 kD to 220 kD. We identified two N-terminal portions of the HA proteins that will be expressed in transgenic tobacco and grapevines where we hope the protein will act as a “molecular glue” to aggregate insect-inoculated *Xf* cells, retard their ability to systemically colonize plants and potentially provide a unique form of resistance against PD.

## INTRODUCTION

*Xylella fastidiosa* (*Xf*) HAs are large secreted proteins that play important roles in mediating cell-cell aggregation and plant pathogenicity. Mutations were made in both *Xf* HA genes, HxfA (PD2118) and HxfB (PD1792), by transposon mutagenesis. The resulting mutants did not aggregate in liquid culture and they had reduced biofilm formation *in vitro* and *in planta* (1). When inoculated into grapevines the mutant cells showed hypervirulence and more rapid colonization of xylem vessels (1). The premise of this research is to determine if by expressing *Xf* HA adhesion domains in the xylem of transformed grapevines, the HA can act as a “molecular glue” to clump *Xf* cells and retard their ability to systemically colonize grapevine and cause Pierce’s disease (PD).

Because of the large size of the HA genes (10 kb), it is difficult to transform grapevines with the whole HA gene. Therefore we have been trying to identify the active adhesion domains (ADs) responsible for cell-cell aggregation by dividing the HA genes into several smaller fragments that should contain the cell-cell AD. Recombinant proteins derived from these fragments were expressed in *E. coli*, purified and injected into rabbits to produce AD specific antisera. The resulting antisera were used in ELISA, Western blot analysis, immunolocalization studies and cell-cell clumping experiments to determine which of the HA fragment(s) contain functional ADs that could be transformed into plants.

## OBJECTIVES

- 1a. Use antibodies we have prepared against a conserved, putative binding domain (AD2) that is present in both *Xf* hemagglutinins (HA) to determine the native size and location of *Xf* HA in cultured *Xf* cells and PD-affected grapevines.
  - b. Determine if these antibodies (Fab fragments) can prevent cell-cell clumping in liquid *Xf* cultures.
  - c. Prepare an affinity column using HA domain antibodies and isolate native *Xf* HAs from culture cells. Establish the identity of affinity purified, putative HAs by N-terminal sequencing.
  - d. Determine if native HAs and HA domain fusion proteins can bind to *Xf* cells.
  - e. Inject affinity purified HA proteins into rabbits and obtain HxfA and B specific-antibodies. Determine if HxfA and B specific antibodies can block cell-cell clumping of *Xf* grown in liquid medium.
- 2a. PCR-amplify, clone and express as fusion proteins, additional hypothetical adhesion domains of HxfA and B.
  - b. Prepare rabbit polyclonal antibodies against each Hxf A/B domain fusion protein. Use antibodies to determine native size and location of *Xf* HAs in cultured cells.
  - c. Determine if antibodies against various HxfA/B domain fusions can block cell-cell clumping of *Xf* grown in liquid medium.

- 3a.** Transform Thompson seedless grapevines and tobacco, an experimental host of *Xf* and an easily transformable plant, with *Xf* HA binding domains. Use antibodies prepared in Objective 2 to determine if *Xf* HA proteins can be found in tobacco and grapevine xylem fluid.
- b.** Mechanically inoculate HA-transgenic grapevines and tobacco with wild type (wt) *Xf* cells. Compare disease progression and severity in transgenic plants with non-protected controls.

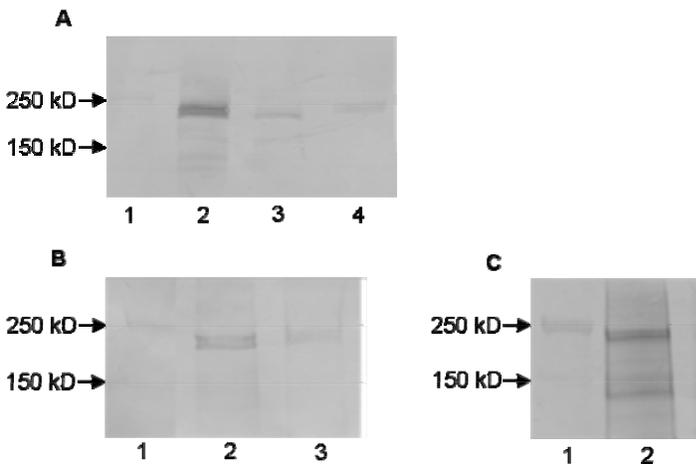
## RESULTS

### Objectives 1a-d, 2a

The results of these objectives have been reported in the Proceedings, 2007 Pierce's Disease Research Symposium, CDFA, Sacramento, CA. Because of the low quality of the AD2-antibodies, we repeated Objectives 1a-d with high quality antibodies that were generated against AD1-3 and AD4 in Objective 2a (2).

### Objective 2b

Determination of native size and location of *Xf* HAs in cultured cells.



**Figure 1 A.** Western blot analysis of soluble proteins extracted *Xf* media supernatant and probed with anti-AD4 antibodies. Lane 1: Molecular weight standards; lane 2: Wild type Temecula; lane 3: HxfA-, lane 4: HxfB-. A doublet of bands in the wild type Temecula is detectable. The larger protein band corresponding to HxfA is missing in the HxfA- mutant, the slightly smaller protein band corresponding to HxfB is missing in the HxfB- mutant strain.

**Figure 1 B.** Western blot analysis of *Xf* vesicle preparation developed with anti-AD4 antibodies. Lane 1: Molecular weight standards; lane 2: Wild type Temecula vesicle preparation; lane 3: TCA-precipitated supernatant after isolation of vesicles, proteins represent soluble HA proteins. **C:** Western blot analysis of an *Xf* outer membrane protein preparation. Lane 1: Molecular weight standards; Lane 2: outer membrane preparation of wild type Temecula.

The antibodies that were raised against AD1-3 and AD4 of the HA proteins (2), were used in Western blot analysis of isolated *Xf* soluble, secreted proteins (**Figure 1A**), HA proteins associated with secreted vesicles (**Figure 1B**) and HAs present in the outer membrane (**Figure 1C**); HA proteins were detected in all three fractions.

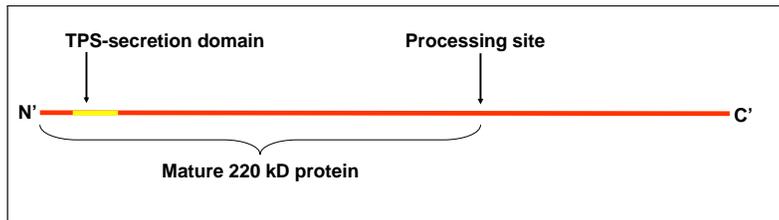
A doublet of proteins at approximately 220 kD that corresponds to HxfA and HxfB was observed in wild type supernatant (**Figure 1A**, lane 2), and only one protein band was detected in the HxfA- and HxfB- mutant supernatant samples (**Figure 1A**, lanes 3 and 4). The large protein corresponding to HxfA is missing in the HxfA- mutant, the slightly smaller protein band corresponding to HxfB is missing in the HxfB- mutant strain (**Figure 1A**).

To distinguish between secreted soluble HA proteins, and HA proteins that are associated with membrane vesicles, the vesicles were isolated by centrifugation and proteins remaining in the supernatant were precipitated using trichloroacetic acid (TCA). Western blot analysis of the precipitate confirmed the presence of HA proteins as secreted soluble proteins (**Figure 1B**). Interestingly, 220 kD proteins in the vesicle fraction were also detected by the anti-AD4 antibody (**Figure 1B**). Vesicles that are released from the envelope of growing bacteria may contain virulence factors in many Gram-negative bacteria (3).

Western blot analysis of isolated outer membrane proteins of *Xf* wild type Temecula also revealed a doublet of bands at 220 kD corresponding to both HA proteins (**Figure 1C**). To ensure that the outer membrane were indeed being isolated, Western blots were also probed with anti-MopB antibodies (kindly provided by the Bruening lab). MopB is the major outer membrane protein in *Xf* (4), and a 38.5 kD protein corresponding to MopB was detected in the membrane fraction thus confirming that *Xf* HAs are inserted into the outer membrane (data not shown).

The size of the mature protein detected by Western blot analysis (220 kD) was smaller than the predicted size based on the amino acid sequence of the protein (360 kD). To identify the processing site of the mature HA proteins, we isolated secreted

HA proteins by size exclusion chromatography and subjected the native HA proteins to LC MS/MS mass spectrometry (Genome Center Proteomics Core, University of California, Davis). Identified peptides were associated only with the N-terminal portion of the HA protein, suggesting that the C-terminal portion is cleaved off in the mature protein and does not play a role in cell-cell aggregation(**Figure 2**).



**Figure 2.** Secreted HA proteins were isolated by size exclusion chromatography and analyzed by LC MS/MS to estimate the location of processing site to produce the mature 220kD protein.

### Objectives 2c

The results of these objectives were reported in the 2007 Pierce's Disease Research Symposium Proceedings.

### Objectives 3

AD1-3 and the entire processed 220kD HA protein (protein220) will be used for transformation of tobacco SR-1 and Thompson seedless grapevines. These constructs were both cloned into the vector pCR-2.1 resulting in pCR2.1-AD1-3 and pCR2.1-220. Several clones of both constructs were sequenced to confirm the integrity of the cloned genes. We obtained suitable clones for both pCR-2.1-AD1-3 and pCR-2.1-220 that can be used for plant transformation. We obtained the pGIP-signal sequence, which directs the secretion of proteins into the plant apoplast and xylem, fused to the N-terminal portion of AD1-3 from a biotech company (DNA2.0) in a plasmid called pJ202:21008. This construct can be used to insert the pGIP-signal sequence (5) in front of AD1-3 as well as for protein220 to direct the secretion of the expressed proteins outside the plant cell. This secretion signal sequence has been successfully inserted into pCR2.1-220 and we are in the process of putting the secretion signal on pCR-2.1-AD1-3. After successful fusion of the pGIP-signal peptide to the HA proteins, the constructs will be cloned into a Agrobacterium binary vector that is available in the Dandekar lab (5) and used to transform *Agrobacterium tumefaciens*. The Agrobacterium cultures will then be used by the UC Davis Plant Transformation facility to transform tobacco SR-1 and Thompson seedless grapevine with the two HA protein constructs. Xylem sap will be expressed from transgenic tobacco and grapevines and analyzed by ELISA and Western blots to determine if *Xf* HA proteins are present in the sap of transgenic plants. The transgenic plants will be mechanically and insect inoculated with *Xf* and the plants will be evaluated for the presence of *Xf* and the severity of PD symptoms.

### CONCLUSION

Our data suggests that HA proteins are needed for efficient aggregation of *Xf* cells because *Xf* cells that have a mutation in either HxfA or HxfB lose the ability to aggregate and to form biofilms. Also, *Xf* cell cultures that were incubated with Fab fragments against AD1-3 and AD4 of HxfB were inhibited in their ability to aggregate (2). We showed that HA proteins are secreted and processed to a mature 220 kD protein and that contain N-terminal hemagglutination domains. Taken together, this suggests that the secreted N-terminal portion of the HA proteins is responsible for cell-cell aggregation and biofilm formation. We hope that free *Xf* HA protein in the plant xylem may mediate increased cell-cell aggregation of insect inoculated *Xf* cells and increase the agglutination of *Xf* cells in the plant xylem, thereby retarding the systemic colonization of grapevines and possibly providing a novel resistance to Pierce's disease.

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# CONTROL OF PIERCE'S DISEASE BY METHODS INVOLVING PATHOGEN CONFUSION

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**Reporting Period:** Most of the results reported here are from work conducted October 2007 to October 2008.

## ABSTRACT

*Xylella fastidiosa* (*Xf*) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF) that changes its gene expression in cells as they reach high numbers in plants. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease (PD). We have investigated both the role of DSF-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, performed genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control. *Xf* mutant strains that overproduce DSF cause disease symptoms in grape, but only at the site of inoculation and the cells do not move within the plant as do wild-type strains. Thus elevating DSF levels in plants should reduce movement of *Xf* in the plant and also reduce the likelihood of transmission by sharpshooters. When co-inoculated into grape with *Xf* DSF-producing strains such as *Rhizobium etli*, harboring *rpfF* from *Xf* greatly reduced the incidence and severity of disease in grape; lesser effects were observed when these strains were inoculated into plants separately, suggesting that the biological control strains did not move efficiently within the plant and hence were not coincident with *Xf*. Topical application of DSF extracted from over-producing strains of *Erwinia herbicola* harboring *rpfF* cloned from *Xf* reduced the severity of Pierce's disease when applied shortly before inoculation with *Xf*. We have transformed tobacco, tomato and grape with the *rpfF* gene of *Xf* to enable DSF production in plants. While expression of *RpfF* in the cytoplasm has yielded modest levels of DSF that were sufficient to greatly reduce the movement of *Xf* in grape, and thus reduce Pierce's disease, targeting of *RpfF* to the chloroplast of plants has led to much higher levels of DSF production that should provide even high levels of disease control. Grafting studies are underway to determine if DSF produced by rootstocks can move to scions and confer disease control.

## INTRODUCTION

We have found that the virulence of *Xylella fastidiosa* (*Xf*) is strongly regulated in a cell density-dependent fashion by accumulation of a signal molecule called DSF encoded by *rpfF* and involving signal transduction that requires other *rpf* genes. We now have shown that *Xf* makes a DSF molecule that is recognized by *Xanthomonas campestris* pv. *campestris* (*Xcc*) but slightly different than the DSF of *Xcc* (**Figure 1**).

In striking contrast to that of *Xcc*, *rpfF*- mutants of *Xf* blocked in production of DSF, exhibit dramatically increased virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors since they do not form a biofilm within the insect. These observations of increased virulence of DSF-deficient mutants of *Xf* are consistent with the role of this density-

dependent signaling system as suppressing virulence of *Xf* at high cell densities. Our observations of colonization of grapevines by *gfp*-tagged *Xf* are consistent with such a model. We found that *Xf* normally colonizes grapevine xylem extensively (many vessels colonized but with only a few cells in each vessel), and only a minority of vessels are blocked by *Xf*. Importantly, *rpfF*- mutants of *Xf* plug many more vessels than the wild-type strain. We thus believe that *Xf* has evolved as an endophyte that colonizes the xylem; blockage of xylem would reduce its ability to multiply since xylem sap flow would cease and thus the DSF-mediated virulence system in *Xf* constrains virulence. That is, *Xf* would benefit from extensive movement throughout the plant where it would partially colonize xylem vessels but would have evolved not to grow to excessively within a vessel, thereby plugging it and hence blocking the flow of necessary nutrients in the xylem sap. Given that the DSF signal molecule greatly influences the behavior of *Xf*, we are investigating various ways by which this pathogen can be "confused" by altering the local concentration of the signal molecule in plants to disrupt disease and/or transmission. We thus are further exploring how DSF-mediated signaling occurs in the bacterium as well as ways to alter DSF levels in the plant. Our work has shown that the targets of *Rpf* regulation are genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel as well as adhesins that modulate movement. Our earlier work revealed that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf*. In this period we have extensively investigated both the role of DSF-production by *Xf* on its behavior within plants, the patterns of gene regulation mediated by DSF, the extent to which other endophytes can modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, obtained genetic transformation of grape and other hosts of *Xf* to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control.

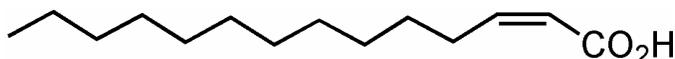


Figure 1

## OBJECTIVES

1. Evaluate plants with enhanced production of DSF for disease control.
2. Determine if DSF is transferable within plants – eg. whether DSF production in rootstocks can confer resistance to Pierce’s disease in the scion.
3. Evaluate enhanced DSF-producing endophytic bacteria for control of Pierce’s disease.
4. Investigate DSF-overproducing strains of *Xf* as biocontrol agents for Pierce’s disease and whether *Xf* strains previously identified with biocontrol potential exhibit an elevated production of DSF.
5. Determine if resistance to Pierce’s disease is associated with low rates of degradation of DSF by plants.
6. Determine those plant factors that confer induction of virulence genes in *Xf* and whether susceptibility to Pierce’s disease is due to differences in induction of virulence factors in the pathogen by the plant.

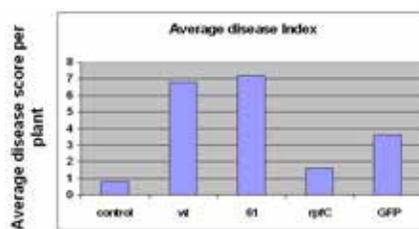
## RESULTS

### Objective 1. Production of DSF in transgenic plants for disease control.

We have expressed the *rpfF* gene in several different plant species to investigate whether DSF excess can lead to reduced disease caused by *Xf*. In addition to grape, we have transformed genes conferring DSF production into tobacco since this species is colonized by *Xf* and disease symptoms can be produced (**Figure 2**). Because transformation of tobacco is much quicker than grape, we have used studies of *Xf* infection of tobacco as a surrogate for studies in grape to speed our assessment of different ways to produce DSF in grape. The various mutants of *Xf* that are hyper and hypo virulence on grape yield similar reactions on SR1 tobacco (**Figure 3**).



**Figure 2.** Symptoms caused by *Xf* on SR1 tobacco.



**Figure 3.** Severity of disease on SR1 tobacco inoculated with WT *Xf* and an *rpfF* mutant (61), an *rpfC* mutant, and a *gfp*-marked strain.

Further tests of SR1 tobacco as a surrogate host to evaluate transgenic expression of *rpfF* as a means to increase DSF abundance in plants were performed. SR1 tobacco which had been transformed with the untargeted *rpfF* genes from either *Xf* or *Xcc* were inoculated with *Xf*; the incidence of disease was dramatically reduced in *rpfF*-expressing SR1 compared to untransformed tobacco (**Table 1**). Some of the more mature leaves on the base of the plant had exhibited leaf scorching even on uninoculated plants (**Table 1**), suggesting that the extent to disease control conferred by expression of *rpfF* was much greater than 50%.

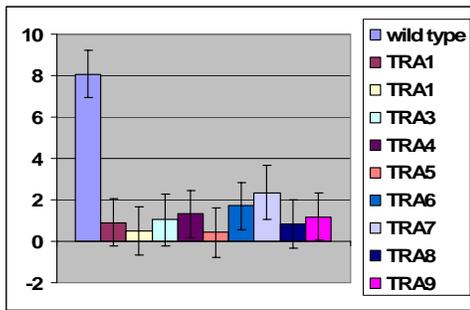
Grape has been transformed at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California at Davis with a non-targeted *rpfF* construct. These plants produced only very low levels of DSF but are MUCH less susceptible to Pierce’s disease (**Figure 4**). While *Xf* spread throughout non-transformed plants causing disease on petioles located great distances from the point of inoculation, disease was observed only very close to the point of inoculation in *rpfF*-expressing plants. We thus expect to find that *Xf* is limited in its movement in plants having even higher levels of DSF due to the expression of *rpfF*, in a manner similar to what we have observed in DSF-overproducing strains of *Xf*.

**Table 1.** Proportion of leaves of wild-type and DSF-producing SR1 tobacco with marginal leaf scorch after inoculation with *Xf*

Treatment	Fraction of leaves
Wild-type SR1	0.52 a
<i>Xf rpfF</i> -expressing SR1	0.38 b
<i>Xcc rpfF</i> -expressing SR1	0.27 b
No <i>Xf</i> control	0.22 c

We have recently transformed tobacco and *Arabidopsis* with an *rpfF* gene that has been modified to direct the protein product to the chloroplast where fatty acid synthesis (and DSF synthesis) should be much enhanced compared to its production in the cytosol, the presumed location of *RpfF* in the current transformed plants. Assay of DSF in transgenic SRI tobacco plants—where the *RpfF* is targeted to the chloroplast, indicates that the DSF levels as well as expression of *rpfF* are much higher as compared to the plants in which the *RpfF* is expressed in the cytosol. Transcription analysis of the chloroplast targeted *rpfF* transformed plants indicates high level expression of the *rpfF* gene (**Figure 5**). We have generated seeds from the transgenic

SRI tobacco plants and we are conducting pathogenicity assays with *Xf* comparing these enhanced producing plants with normal and untargeted *RpfF* plants.



**Figure 4.** Disease severity (# symptomatic leaves/plant) on Freedom grape transformed with the *rpfF* gene encoding DSF production and inoculated with *Xf*.



**Figure 5.** DSF extracted from transgenic tobacco SRI plants harboring a chloroplast targeted *RpfF* (left) or from WT tobacco (center) or purified DSF from *Xcc* (right). DSF is spotted on a paper disc on the right side of each image and the *Xcc* DSF bioindicator is to the left. gfp fluorescence is evidence of DSF.

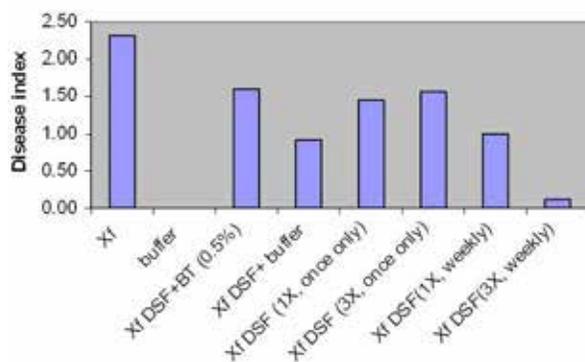
Further tests of the efficacy of chloroplast targeting of *rpfF* implants were performed by evaluating DSF production in transgenic MoneyMaker tomato. Substantial levels of DSF could be detected in the chloroplast-targeted tomato and sufficient amounts of DSF were present to alter the behavior of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) that was inoculated onto leaves. While an average of 323 lesions formed when *Xcv* was inoculated onto normal tomato, 570 lesions formed per leaf on the DSF-producing plants, a finding expected if DSF was present since virulence of *Xcv* is enhanced by DSF. We have also initiated transformation of grapes with a chloroplast targeted *rpfF* construct. We expect to receive the transformed plants by December, 2008, and then will grow them to larger sizes, make green cuttings to produce enough plants for pathogenicity testing by mid-2009. Although *RpfB* is not required for DSF synthesis in *Xf*, it presumably aids in DSF synthesis by encoding long chain fatty acyl CoA ligase which might increase availability of the appropriate substrates for DSF synthesis by *RpfF*. We expected that co-expression of *RpfB* and *RpfF* in the chloroplast will further enhance the DSF levels in plants. We have produced transgenic *Arabidopsis* plants with such a construct and find evidence of high levels of DSF production. Pathogenicity assays with the *rpfF* mutant of *Xcc* indicated that the transgenic plants can complement the virulence of the non pathogenic *rpfF* mutant of *Xcc*

(Table 2). Importantly, transgenic plants expressing both *rpfB* and *rpfF* were more susceptible to the *rpfF* mutant of *Xcc*, indicating enhanced DSF levels. Given this evidence of enhanced DSF production in transgenic *Arabidopsis*, and recent results with similarly-transformed tomato, we are initiating transformation of grape with similar constructs.

Direct application of DSF to non-transgenic grape can also confer disease control. While we have very recently determined the chemical structure and have synthesized DSF of *Xf*, for these studies we used crude ethyl acetate extracts of a DSF-producing *E. herbicola* strain as a source of DSF. The DSF was either topically applied as well as needle inoculated into the stems of grape either once a day before inoculation with *Xf* or weekly. While a single needle application of DSF reduces disease index, a weekly application of DSF into the stem of the plant was much more effective (Figure 6). These results are exciting in that they suggest that disease control from external applications of DSF might be a practical means of disease control. We have recently been successful in determining the structure of *Xf* DSF and have synthesized gram quantities of DSF. Plants have recently been treated with topical and injected synthetic DSF and then inoculated with *Xf*; disease assessment will commence in mid-November.

**Table 2.** Disease severity from topical application of bacteria varying in DSF production to *Arabidopsis*. Bacteria were inoculated on different *Arabidopsis* genotypes transformed with *rpfF* or with both-*rpfB* and *rpfF*

<i>Arabidopsis</i> genotype	<i>Xcc</i> strains	
	Wild type	<i>rpfF</i> -
Col (WT)	++++	-
<i>rpfF</i> transformed	++++	+
<i>rpfF</i> & <i>rpfB</i> transformed	++++	++



**Figure 6.** Disease incidence-severity relation (disease index) for grape inoculated with *Xf* and to which DSF was topically applied or introduced into the stem.

*Objective 2. Graft transmissibility of DSF.*

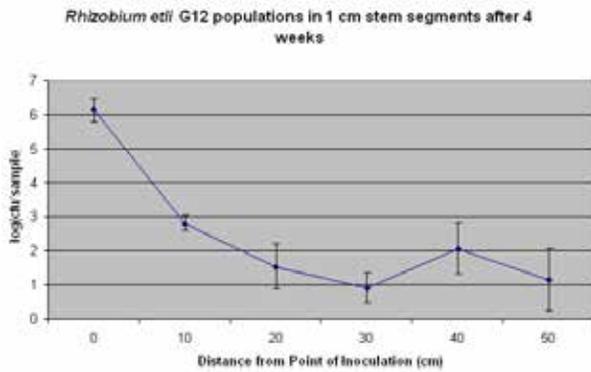
To test whether DSF is mobile within the plant we are performing grafting experiments in which DSF-producing tobacco transformed with the *rpfF* of *Xf* are used as rootstocks to which normal SR1 tobacco is grafted as a scion (**Figure 7**). Over 100 of such grafted plants have now been made, and they have been inoculated with *Xf* to test whether normal SR1 scions on DSF-producing rootstocks have a lower susceptibility to *Xf* colonization; disease will be rated by mid-November. Non-chloroplast targeted *RpfF*-expressing transgenic Freedom grape plants have been propagated in sufficiently large numbers to produce enough plants to serve as rootstocks to test with *Xf* inoculations in larger scale studies. Over 100 such plants have now been propagated and green-grafting of Cabernet Sauvignon has been successfully employed to produce grafted plants with a normal Freedom and a DSF-producing Freedom rootstock (**Figure 7**). Initial attempts at green grafting of grape produced a low frequency of successful grafts, but a new procedure has provided a satisfactory level of graft success; the grafted plants have been inoculated with *Xf* to test for graft transmissibility of DFS as evidenced by reduced movement of *Xf* and disease severity.



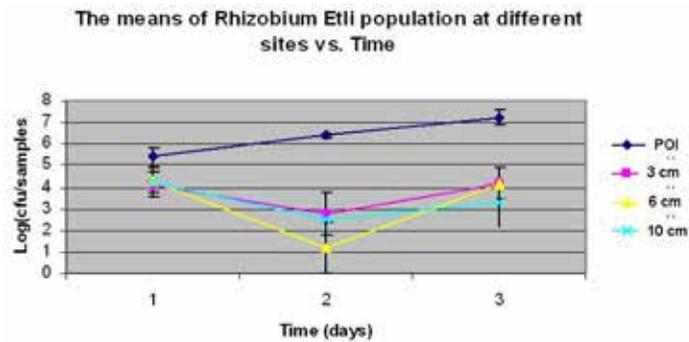
**Figure 7.** Grafted SR1 tobacco plants (left) and Cabernet Sauvignon grape grafted onto DSF producing Freedom rootstocks (right) onto which *Xf* has been inoculated. The plants are as yet asymptomatic.

*Objectives 3 and 4. Disease control with endophytic bacteria.*

We have been successful in producing large quantities of DSF in endophytes like *Erwinia herbicola* and also in lab strains of *E. coli* (**Table 2**). We recently were able to transform a putative efficient endophyte of plants, *Rizobium etli* G12 with both the *Xcc* and *Xf rpfF* (DSF biosynthetic gene) and have obtained production of DSF in this strain. This DSF-producing endophyte has been inoculated into grape to determine both its ability to move and multiply within grape as well as its ability to interfere with the disease process. The *R. etli* strain G12 was found to move within grape tissue after inoculation into either the stem or the leaves. When measured four weeks after inoculation by puncture inoculation into one site in the stem, measurable populations of *R. etli* were seen as far as 50 cm away from the point of inoculation (**Figure 8**). While the population size away from the point of inoculation were relatively low in this short time interval since inoculation, this strain clearly has the ability to move within grape; we are excited about this result since no other of several bacterial strains that we have investigated for the ability to move within grape has ever exhibited any ability to move beyond the point of inoculation. Further studies are underway to determine the population sizes to which *R. etli* will grow given more time after inoculation. *R. etli* also has the ability to move within grape leaves and multiply to high population sizes. When applied as a point source to leaves using a penetrating surfactant, cells of *R. etli* could be found up to three cm away within one week, and population sizes of this strain increased 100-fold within three weeks after inoculation (**Figure 9**). Studies are continuing to determine the maximum population size that this strain can achieve in grape leaves.

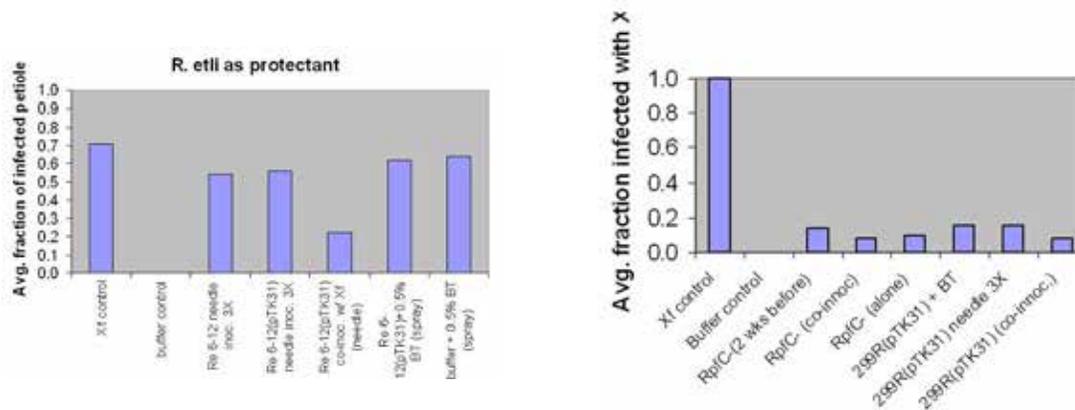


**Figure 8.** Population size of *R. etli* in stems four weeks after inoculation at one point.



**Figure 9.** Population size of *R. etli* in leaves one, two, and three weeks after inoculation at a single point.

Various DSF-producing bacteria were tested for their ability to control Pierce’s disease when applied to grape in different ways. DSF-producing *R. etli* were both needle inoculated one or more times at sites near where *Xf* was subsequently inoculated, as well as co-inoculated with *Xf* into grape stems and sprayed onto leaves with 0.5% of the penetrating surfactant Breakthru 1 week before *Xf* was inoculated into stems. The co-inoculation of *R. etli* with *Xf* greatly decreased the incidence of colonization of grape petioles compared to control plants inoculated with *Xf* alone (Figure 10) while topical application or injection elsewhere in the stem provided little control. We presume that the relatively slow movement of *R. etli* in the stems of plants (Figure 8) explains why co-inoculation was most effective. *R. etli* was somewhat susceptible to damage from Breakthru and its population sizes were reduced during application with this detergent. We will continue to test different ways in which *R. etli* can be introduced into plants to determine its ability to control PD. We expect that introduction of *R. etli* into stems far in advance of *Xf* will provide much better disease control. *RpfC*- mutants of *Xf* greatly over-produce DSF so we tested them for their ability to control PD when applied in various ways as discussed above for *R. etli*. The incidence of colonization of grape petioles with *Xf* was greatly reduced when plants were needle inoculated into grape either two weeks before plants were inoculated with *Xf* or when coinoculated with the pathogen (Figure 11). While the *RpfC* mutant does not move as well within grape as the wild-type *Xf*, its presence locally in plants can suppress the movement of wild-type *Xf* and thus lead to control of PD. These studies are promising and are being repeated.



**Figure 10.** Incidence of colonization of petioles of grape by *Xf* when plants were treated with DSF-producing *R. etli* in various ways.

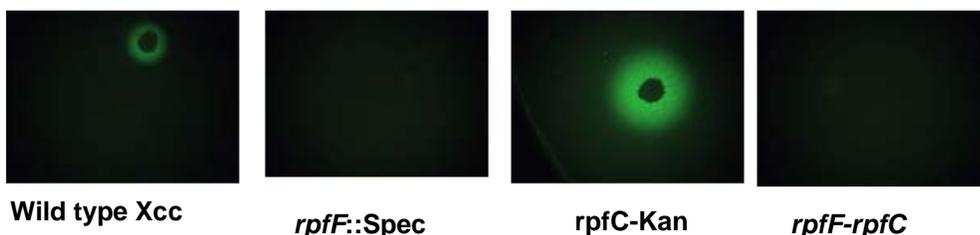
**Figure 11.** Incidence of colonization of petioles of grape by *Xf* when plants were treated with *RpfC* mutants of *Xf* in various ways.

**Objective 5. Degradation of DSF by plants. Development of an *Xcc* biosensor efficient in detecting *Xylella* DSF.**

For many of the objectives of this project, in addition to the study of DSF degradation in plants an improved bioindicator for DSF would be very valuable. We are presently using an *Xcc*-based biosensor in which the endoglucanase gene is linked to a green fluorescent protein (GFP) reporter gene. Previous studies have shown that this biosensor is able to detect the DFS made by *Xf* but that it detects *Xf* DSF with a lower efficiency than the *Xanthomonas* DSF since the two molecules apparently differ slightly. We have devised a strategy to develop a surrogate *Xcc* biosensor system which will express all the

components of DSF signal transduction of *Xf*. This should give rise to a system which is close to DSF signal transduction system in *Xcc*. We have made two different *Xanthomonas* strains in which the endogenous signal synthesis as well as signal recognition system (consisting of the hybrid two component *RpfC* and *RpfG* response regulators) has been knocked out. In one of these strains the DSF signal synthase *RpfF* and the DSF signal sensor *RpfC* has been knocked out (**Figure 12**). We have also made an *Xcc* strain in which the DSF synthase gene *RpfF* has been knocked out in a background of a *RpfCHG* deletion.

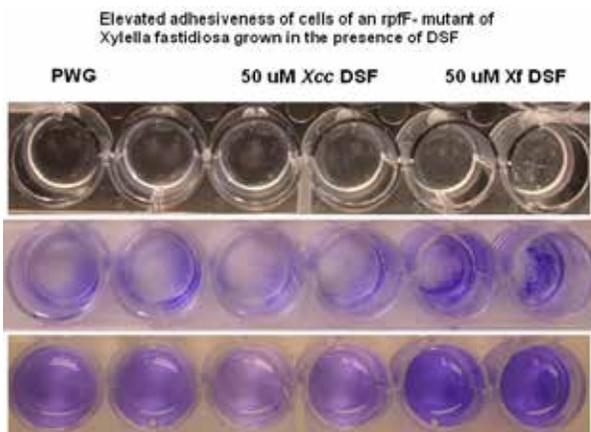
These mutants will enable us to express the *Xf RpfC-RpfG* two component system and should serve as a more sensitive surrogate host biosensor. Completion of the biosensor is expected within three more months. It then will be applied to the study of *Xf* DSF stability in plant extracts as originally proposed.



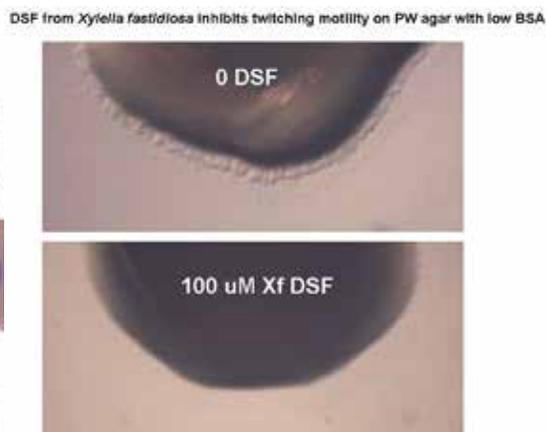
**Figure 12.** Different *Xcc* mutants constructed to serve as surrogate host for expressing the *Xf RpfC-RpfG* two component DSF signal transduction system. The presently used *Xcc* biosensor 8523/pKLN55 is sprayed over the colonies. Presence of DSF is detected by the GFP fluorescence of the biosensor.

We also are investigating the use of *Xf* itself to detect DSF. Among the several genes that we know to be regulated by DSF, those genes most strongly regulated include *pil* genes involved in twitching motility, several genes such as *fimA* and *hxfA* and *HxfB* which are involved in cell-surface adhesion, and gum genes involved in production of EPS. We thus have examined the phenotypes of an *rpfF*- mutant of *Xf* exposed to different amounts of DSF to determine if it can be used to bioassay for the presence of DSF. Initial results are encouraging. Suppression of twitching motility of the *rpfF*- mutant was observed when DSF was added at concentrations greater than about 10  $\mu$ M (**Figure 13**). Likewise, cells of the *rpfF*- mutant which were not adherent, and thus which did not form cell-cell aggregations became much more adherent to each other when DSF was added at concentrations greater than about 10  $\mu$ M (**Figure 14**). Thus it appears that we can assess the concentration of DSF in samples using either a cell twitching assay or a cell adhesion assay using *Xf* cells, although both assays are time consuming and somewhat qualitative.

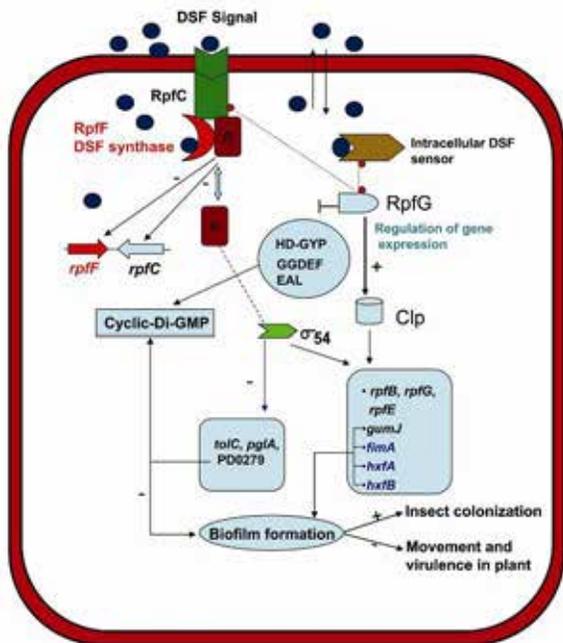
Initial results have shown relatively little induction of EPS production in an *rpfF*- mutant of *Xf* by the addition of DSF; little EPS was observed whether DSF was added to culture medium or not. We are investigating, in cooperation with Rodrigo Almeida, other medium contents which might be needed for EPS production and have very preliminary evidence that EPS production can be stimulated by DSF under the correct culture conditions. EPS abundance will then be measured both chemically and immunologically as an estimator of DSF abundance.



**Figure 13**



**Figure 14**



**Figure 15.** A proposed model for DSF-mediated cell-cell signaling regulation in *Xylella fastidiosa*.

**Table 3.** Relative quantification of gene expression regulated by *rpfF* and *rpfC* by real-time RT-PCR.

Gene name	Fold change $\pm$ SE*		
	<i>rpfF</i> <sup>-</sup>	<i>rpfC</i> <sup>-</sup>	<i>rpfF</i> <sup>-</sup> <i>rpfC</i> <sup>-</sup>
<i>fimA</i>	0.4 $\pm$ 0.04	2.15 $\pm$ 0.18	0.73 $\pm$ 0.19
<i>hxfA</i> ( <i>xadA</i> )	0.56 $\pm$ 0.07	3.2 $\pm$ 0.1	0.7 $\pm$ 0.17
<i>hxfB</i>	0.15 $\pm$ 0.05	5.2 $\pm$ 0.52	0.49 $\pm$ 0.3
<i>gumJ</i>	0.56 $\pm$ 0.02	2.6 $\pm$ 0.2	0.4 $\pm$ 0.04
<i>rpfF</i>	n.d.	6.6 $\pm$ 0.71	n.d.
<i>rpfC</i>	4.9 $\pm$ 0.4	n.d.	n.d.
<i>rpfE</i>	0.73 $\pm$ 0.06	2.2 $\pm$ 0.17	0.7 $\pm$ 0.12
<i>rpfB</i>	0.6 $\pm$ 0.09	2.13 $\pm$ 0.07	0.50 $\pm$ 0.3
<i>rpfG</i>	0.7 $\pm$ 0.06	1.8 $\pm$ 0.04	1.13 $\pm$ 0.45
(PD0279)	5.3 $\pm$ 0.3	3.5 $\pm$ 0.23	0.62 $\pm$ 0.06
<i>toIC</i>	5.5 $\pm$ 0.7	3.8 $\pm$ 0.6	0.6 $\pm$ 0.09
<i>pglA</i>	1.9 $\pm$ 0.17	1.8 $\pm$ 0.04	0.7 $\pm$ 0.07

\*Amount of RNA relative to that in the wild-type *X. fastidiosa* cells is equal to 1.0 and is normalized for cellular abundance by using 16S ribosomal RNA as an endogenous control. n.d. indicates not determined. Standard errors were calculated based on at least two independent experiments.

#### Objective 5. Plant regulation of *Xf* virulence factors.

Before investigating the effects of plant extracts on gene expression in *Xf*, we have further examined the complex pattern of gene regulation in *Xf* that is DSF dependent to better understand which virulence genes might be most informative to examine. Analysis of the genome sequence of *Xf* revealed that several genes encoding proteins potentially involved in intracellular signaling are present. Gene expression of several genes was thus examined in both an *rpfF* and *rpfC* mutant background as well as a double mutant (Table 3). The results have enabled the production of a more complete model of DSF-dependent gene expression in *Xf* (Figure 15). The several genes identified in Table 3 will be examined by RT-PCR in cultures of *Xf* to which plant extracts have been applied as proposed.

## CONCLUSIONS

Several methods of altering DSF levels in plants, including direct introduction of DSF-producing bacteria into plants, and direct application of DSF itself to plants appear promising as means to reduce Pierce's disease. Given that DSF overabundance appears to mediate an attenuation of virulence in *Xf* we have transformed grape with the *rpfF* gene of *Xf* to enable DSF production in plants; such grape plants produce at least some DSF and are much less susceptible to disease. Higher levels of expression of DSF have been obtained in plants by targeting the biosynthetic enzymes to the chloroplast. Studies are underway using grafting experiments to determine if DSF produced by rootstocks can move to scions and confer disease control. Transgenic DSF-producing plants appear particularly promising and studies should soon indicate whether they could serve as a rootstock instead of a scion. Several genes encoding traits such as exoenzyme production, type IV pili involved in twitching motility, and a variety of fimbrial and non-fimbrial adhesins are most strongly regulated by the accumulation of DSF in bacterial cultures as well as in planta. The expression of these genes will be assessed when *Xf* is within different plant species to determine the host plant specificity of expression of such virulence genes. While the principle of disease control by altering DSF levels has been demonstrated, more work is needed to determine how to achieve this by the most practical means.

## FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

## EXPLOITING PATHOGEN SIGNAL MOLECULES FOR CONTROL OF PIERCE'S DISEASE

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**Reporting Period:** The results reported here are from work conducted July 1, 2008 to October 1, 2008.

### ABSTRACT

The movement of *Xylella fastidiosa* (*Xf*) in plants and insect transmission is controlled by a small diffusible signal factor (DSF) that accumulates when cells are at high cell densities. Pathogen behavior can be dramatically changed and disease reduced by altering the abundance of DSF in plants in a form of "pathogen confusion." To enable new strategies of pathogen confusion we have chemically characterized the DSF produced by grape strains of *Xf* as 2-Z-tetradecenoic acid (hereafter called C14-cis). The DSF is structurally related to, but distinct from, the DSF made by *Xanthomonas campestris* pv. *campestris* (*Xcc*). While an *Xcc* *eng:gfp* based biosensor for DSF can detect as little as about 1  $\mu$ M of DSF produced by *Xcc*, more than about 100  $\mu$ M of C14-cis is required for detection. Biological assays for the presence of C14-cis are being developed in *Xf*. As the expression of genes conferring type IV pili and thus twitching are suppressed while those involved in extracellular polysaccharide (EPS) production and production of various cell adhesins are induced in the presence of DSF in *Xf*, we are developing bioassays for C14-cis using an *rpfF* mutant of *Xf* that cannot produce DSF but which can respond to exogenous C14-cis. Twitching motility of the *rpfF* mutant was suppressed in the presence of as little as 1  $\mu$ M exogenous C14-cis while cell-cell adhesiveness and cell-surface adhesiveness was enhanced. Preliminary results indicate that *Xf* responds to C14-cis concentrations that are at least 10-fold less than that of the DSF produced by *Xcc* suggesting that the responsiveness of different DSF-producing bacteria is likely species specific; eg. they respond best to the DSF that they produce. Further bioassays based on immunological detection of cell surface adhesins or EPS as well as by quantifying mRNA associated with these genes in *Xf* are being developed. Sufficiently large amounts of C14-cis, as well as the sodium salt of this fatty acid, which is highly water soluble, have been produced and have been used as topical and injected treatments of grape that have subsequently been challenge inoculated with *Xf* for tests of disease control. We have designed and will soon initiate synthesis of DSF-analogs and test them for their ability to alter pathogen gene expression and behavior in culture as well as control disease.

### INTRODUCTION

Research in the Lindow lab has provided considerable evidence for a diffusible signal factor (DSF) encoded by *rpfF*, which was considered likely to be a fatty acid derivative, that operates in quorum sensing and biofilm initiation in *Xylella fastidiosa* (*Xf*). *Xf rpfF*- mutants, blocked in production of DSF, exhibit increased virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors. We found that *Xf* colonizes grapevine xylem extensively, with many vessels harboring relatively few *Xf* cells and only a minority blocked by *Xf*. We thus believe that *Xf* has evolved as an endophyte that colonizes the xylem; blockage of xylem would reduce its ability to multiply and thus the DSF-mediated virulence system in *Xf* constrains virulence when cell density increases to high levels in the plant. Preliminary data indicate that DSF perception is central to the expression of a large number of genes in *Xf*, including those that are involved in virulence to plants as well as acquisition by insect vectors. DSF accumulation results in the expression of several fimbrial and afimbrial adhesins, resulting in the cells becoming "sticky" in the plant. DSF accumulation also results in the suppression of expression of extracellular enzymes such as polygalacturonases and endoglucanases that are required for erosion of pit membranes and hence movement through the plant. As the pathogen apparently acquires substantial nutrition from the degradation products of the pit membranes, DSF thus suppresses the multiplication in vessels as cell numbers, and hence DSF, accumulate. *Xf* thus appears to coordinate its behavior in a plant to have both an "exploratory" phase (non-sticky cells highly expressing pit membrane-macerating enzymes) that enable it to spread widely through the plant but not be easily acquired and transmitted by insect vectors, that occurs until cells start to become locally abundant. This phase is followed by an "acquisition phase" (sticky cells that no longer express extracellular enzymes) in a subset of the cells that are maximally transmitted, by insects. Thus, because the plant lifestyle (as an endophyte) conflicts with its ability to adhere to insects and be transmitted the pathogen apparently takes on a "bi-polar" lifestyle of two different physiologies that are adapted for plant invasion and insect transmission, respectively. DSF serves as the switch to coordinate the plant lifestyle and convert cells into the insect acquisition phase.

Our earlier work demonstrated that the severity of Pierce's disease is reduced when the levels of DSF are increased in the plant in various ways. For example, the severity of Pierce's disease is greatly reduced when DSF-producing bacteria are co-inoculated with *Xf* into grape or when DSF expression is enhanced in *Xf* itself. In a direct approach to altering DSF levels in plants we have transformed grape with the *rpfF* gene from *Xf*. Large numbers of clonal *rpfF*-expressing grapes have been produced and inoculated with *Xf* to test for susceptibility to Pierce's disease. In very exciting results, the DSF-expressing grapes are MUCH less susceptible to Pierce's disease. The severity of disease was reduced over 10-fold compared to non-

transformed plants. While *Xf* spread throughout non-transformed plants causing disease on petioles located great distances from the point of inoculation, disease was observed only very close to the point of inoculation in *rpfF*-expressing plants. A major goal of this proposal is to determine the structure of *Xf* DSF so that it and analogs can be evaluated in a strategy of control of diseases caused by *Xf* that rely on “pathogen confusion”. Synthetic DSF and analogs will be made and tested for efficacy in controlling Pierce’s disease by introducing these materials on or into the plant in various ways.

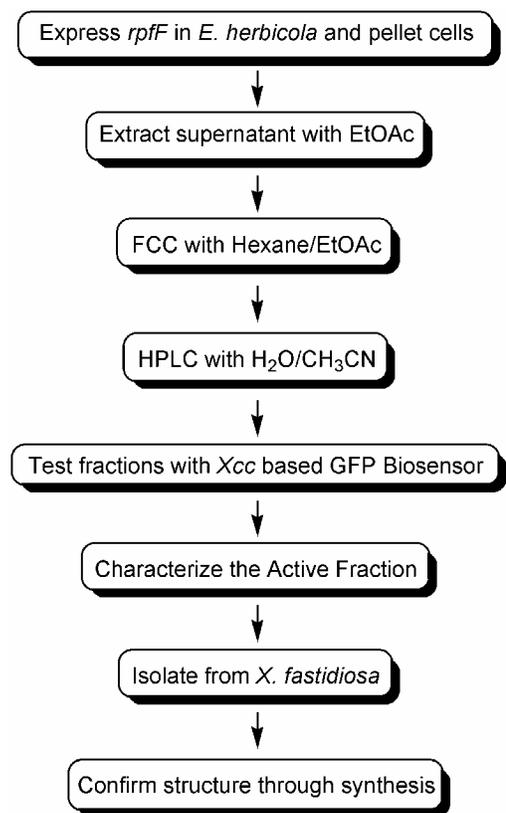
## OBJECTIVES

1. Identification and characterization of low molecular weight signaling molecule (DSF) central to behavior of *Xf*.
2. Design and synthesize low molecular weight compounds capable of interfering with signal molecule function in *Xf*.
3. Evaluate efficacy of signal analogs for control of disease and insect transmission of *Xf*.

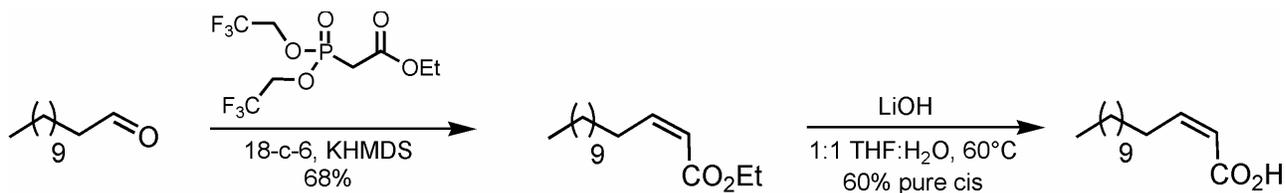
## RESULTS AND DISCUSSION.

**Objective 1. Characterization of DSF.** We determined the conditions that led to optimum production of DSF by *Xf* and surrogate hosts. An *rpfC* mutant of *Xf* that is de-repressed for DSF production was cultured in defined media for the harvest of signal molecules. We found that an *RpfC*- mutant of *Xf* produces about 11-fold more DSF than a wild type strain. We also expressed *rpfF* from *Xf* in *E. coli* and *Erwinia herbicola* strain 299R under strong promoters. The yield of DSF from these surrogate hosts was much larger than even from the *rpfC* mutant of *Xf* because of the much larger number of cells that could be produced in culture. We obtained more than 100-fold more DSF than normally produced by a comparable number of *Xf* cells in such surrogate hosts, and found that that *E. herbicola* is a superior surrogate host.

The scheme depicted in **Figure 1** was used to isolate and characterize the DSF from *Xf*. Initial characterization of DSF was made from the large amounts of DSF produced in these surrogate hosts. DSF was extracted from culture media using ethyl acetate partitioning. Among several fractions from separations of materials made from these crude extracts made by flash column chromatography, the fraction containing organic acids showed higher activity in an *Xcc* DSF bioassay than other fractions above the background. The *Xf* DSF isolated from reverse phase HPLC of the active fraction showed NMR spectral data consistent with a fatty acid containing one site of unsaturation. The DEPT 135 indicates that this is a straight chain acid with no branching. Spectral data suggest the *Xf* DSF has a molecular formula of  $C_{14}H_{26}O_2$ . The methyl ester was synthesized for GCMS analysis. The methyl ester has a molecular formula of  $C_{15}H_{28}O_2$  which means the *Xf* DSF has a formula of  $C_{14}H_{26}O_2$ . DSF was then extracted from *Xf* and used to verify that the compounds made by *Xf* and the surrogate hosts are the same. *Xf* was grown on periwinkle wilt (PW) gel in solid culture. From 200 plates (~4 L volume), we were able to obtain 0.8 mg of the *Xf* DSF. The gel medium was cut into 0.4 x 0.4 cm squares and sonicated with twice the volume of ethyl acetate. Extracts were purified by flash column chromatography and HPLC as described above. The isolable active compound (DSF) from *Xf* was identified as 2-*Z*-tetradecenoic acid (hereafter called C14-*cis*). Isolates from an *rpfF* mutant of *Xf* strain did not produce C14-*cis*. The putative *Xf* DSF was synthesized using a Still-Gennari olefination followed by saponification (**Figure 2**). The spectral data for the acid isolated from *E. herbicola* match those obtained for the synthetic 2-*Z*-tetradecenoic acid.

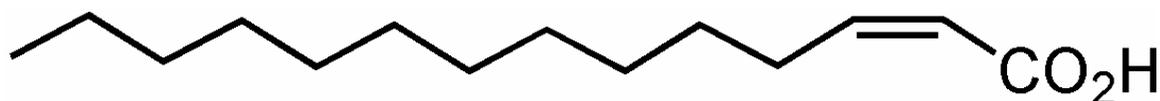


**Figure 1.** Process by which *Xf* DSF was isolated and characterized.

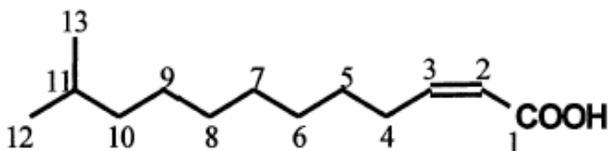


**Figure 2.** Synthesis of C14-cis.

Based on the finding that the DSF from the *E. coli* and *E. herbicola* surrogate hosts harboring *Xf rpfF*, and that isolated from *rpfC* mutants of *Xf* were the same and that all matched that the synthetic material, we conclude that DSF from *Xf* is C14-cis (**Figure 3**). The putative DSF from *Xf* differs somewhat from the DSF made by *Xcc* in that it has a longer, but unbranched acyl chain (**Figure 4**).

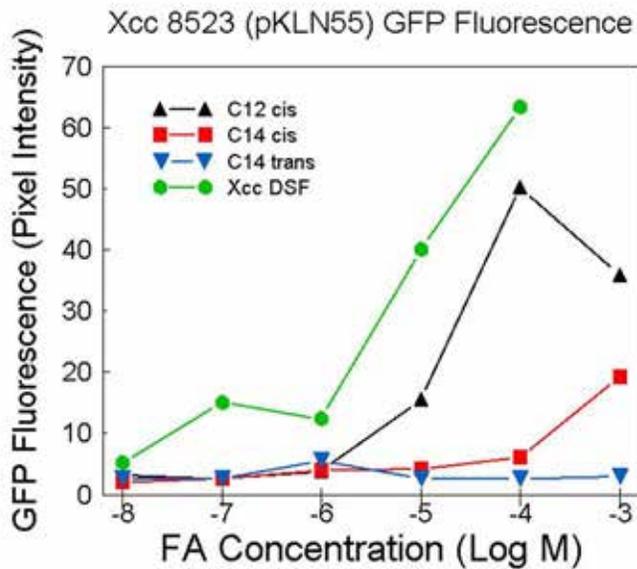


**Figure 3.** Putative structure of C14-cis, the DSF made by *Xf*.



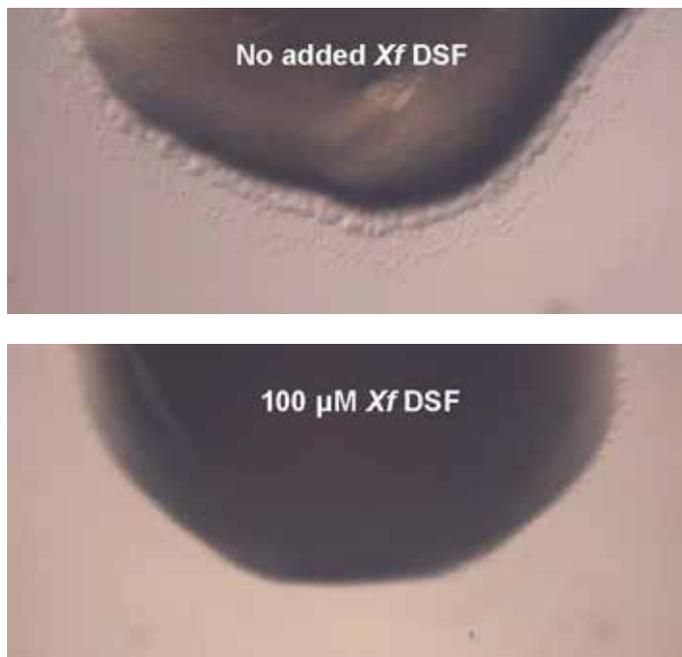
**Figure 4.** Structure of DSF made by *Xanthomonas campestris*.

The biological activity of C14 cis was assessed using the *Xcc* based biosensor *Xcc* 8523 (pKLN55). In this biosensor *gfp* fluorescence conferred by cells harboring an *eng:gfp* reporter gene fusion that is responsive to *Xcc* DSF is measured. While the *Xcc* based biosensor for DSF can detect as little as about 1  $\mu$ M of DSF produced by *Xcc*, more than about 100  $\mu$ M of C14-cis is required for detection. (**Figure 5**). It is important to note that the biological activity of C14-cis was much less than that of *Xcc* DSF; this was expected as earlier work had revealed that while the *Xcc* biosensor could detect DSF from *Xf* the signal was much lower than from a corresponding amount of cells of *Xcc*. It is also clear that the trans form of the C14 enoic acid has no biological activity in this assay (**Figure 5**).

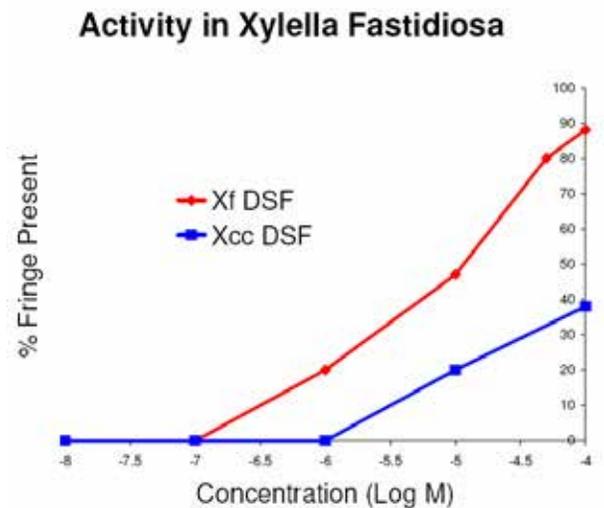


**Figure 5.** Dose response relationship for DSF from *Xcc* and that from *Xf* as well as other related enoic acids.

Biological assays for the activity of C14-cis are also being developed in *Xf*. As the expression of genes conferring type IV pili and thus twitching are suppressed while those involved in EPS production and production of various cell adhesins are induced in the presence of DSF in *Xf*, we are developing bioassays for C14-cis using an *rpfF* mutant of *Xf* that cannot produce DSF but which can respond to exogenous C14-cis. Twitching motility of the *rpfF* mutant was suppressed in the presence of as little as 1  $\mu$ M exogenous C14 cis while cell-cell adhesiveness and cell-surface adhesiveness was enhanced (Figure 6). Preliminary results indicate that *Xf* responds to C14-cis concentrations that are at least 10-fold less than that of the DSF produced by *Xcc* (Figure 7) suggesting that indicating that the responsiveness of different DSF-producing bacteria is likely species specific; eg. they respond best to the DSF that they produce.



**Figure 6.** Twitching motility of *Xf* evident as a fringe around the colony of an *rpfF* mutant (top) on PWG medium but not around the colony when grown on medium containing C14-cis.

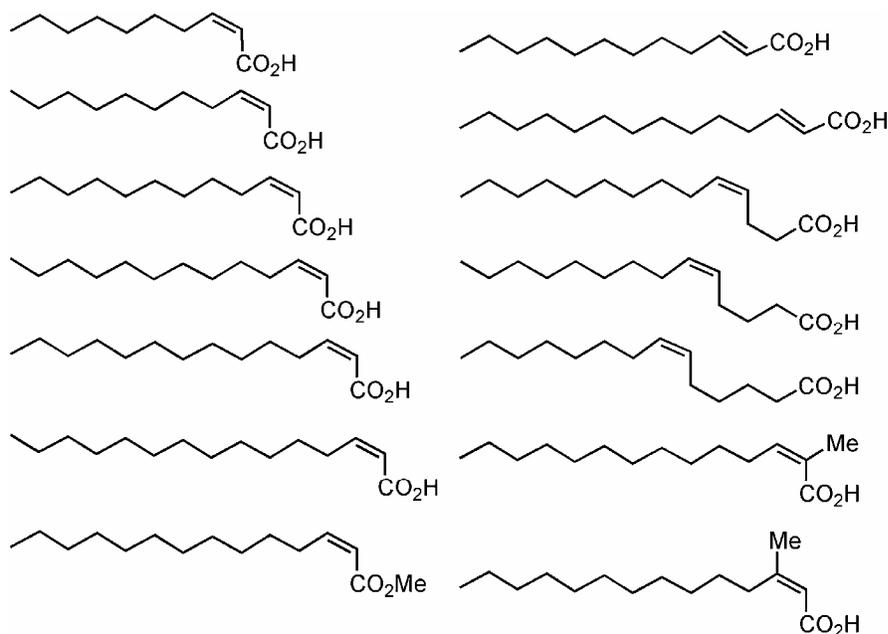


**Figure 7.** Inhibition of twitching activity of an *rpfF* mutant of *Xf* in the presence of different concentrations of DSF from *Xf* and *Xcc*.

Further bioassays based on immunological detection of cell surface adhesins or EPS as well as by quantifying mRNA associated with these genes in *Xf* are being developed to better assess the activity of DSF and synthetic analogs in future

experiments. The current bioreporter for DSF that we developed earlier is based on an *eng:gfp* fusion that is expressed in *Xanthomonas campestris* pv. *campestris* (*Xcc*) (it was known that the endoglucanase gene of *Xcc* was induced in the presence of DSF). The *Xcc* DSF biosensor (8523/PKLN55) will detect DSF of *Xf* but we have now shown it to be much less responsive to C14-cis. This may be due to considerable differences in the components involved in DSF sensing like *RpfC* and *RpfG* which are hybrid two component sensor and response regulators in *Xcc* and *Xf*. We thus will produce improved biosensors by two different means: A) The *rpfC* and *rpfG* genes from *Xf* that are believed to be required for signal transduction in the presence of DSF will be used to replace these homologs in *Xcc*. To increase the sensitivity of *Xcc* biosensor for *Xylella* DSF, we will express the whole DSF signal transduction component (*RpfC*, *RpfG* and *RpfE*) of *Xf* in an *rpfF*-*Xcc* mutant background. In this strategy, we will clone the entire operon of *rpfC*, *rpfG* and *rpfE* of *Xf* and insert the operon in a construct containing the flanking sequence of the *Xcc* *rpf* genomic region. The entire region will be recombined in the *rpfF*- and wild type *Xcc* background. We have already made constructs which can express high levels of *Xf* *rpfC*. Thus this *Xcc* bioreporter should respond more efficiently to DSF from *Xf*; and B) as an alternative, we will take advantage of the fact that we now know what genes in *Xf* are induced in the presence of DSF. For example, we now know that *gumJ*, involved in extracellular polysaccharide (EPS) biosynthesis is strongly induced in the presence of DSF from *Xf* and that DSF-deficient strains produce noticeably less EPS in culture. We will fuse this gene to a *gfp* reporter gene and introduce it into the genome of *Xf* by homologous recombination to yield cells of *Xf* that will become green fluorescent in the presence of DSF. Such cells should be much more responsive to *Xf* DSF and be useful in assaying biochemical fractions for DSF in the purification processes below and in assaying DSF analogs. Alternatively, we can detect EPS production by *Xf* both in culture and in plants by use of antibodies that recognize the EPS of *Xf*. Such antibodies have recently been described by the group of Bruce Kirkpatrick. We expect that DSF-deficient *RpfF*- mutants of *Xf* will exhibit little or no EPS production as monitored by use of fluorescently-labeled antibodies directed against EPS. A GFP-marked *RpfF*- strain of *Xf* could be used as a DSF detector both in culture and *in planta* by examining co-localization of constitutive GFP fluorescence and red fluorescence when a red-fluorophore-labeled anti-EPS antibody is applied to a sample; GFP fluorescent cells that were not also labeled with the antibody stain would indicate lack of DSF availability while cells that were both GFP and red fluorescent would indicate the presence of DSF.

**Objective 2. Design and synthesize DSF analogs.** We have made several synthetic analogs of C14-cis for testing for biological activity in *Xf* (**Figure 8**). As these materials have only recently been synthesized, the biological activity of most have not yet been assayed. As noted above and as expected, the trans variant of the C14 enoic acid exhibited no activity in any of the biological assays performed today. In addition to the DSF analogs noted in **Figure 8**, various halogenated variants will also be synthesized.



**Figure 8.** Analogs of the DSF produced by *Xf* that have been synthesized to date.

**Objective 3. Synthesis of sufficient DSF analogs for *in planta* evaluations.** We have synthesized gram quantities of C14 cis as well as the Sodium salt of this fatty acid which is highly water soluble. These quantities are sufficiently large for initial greenhouse studies. These materials have been sprayed onto leaves as well as injected into stems and used as a soil drench in

initial studies to determine their efficacy for disease control. After treatment plants have been challenge inoculated with *Xf* and disease incidence will be measured; the first disease symptoms are expected by mid-November.

#### **CONCLUSIONS**

The DSF produced by grape strains of *Xf* has tentatively been characterized by C14-cis. Both its relatively higher biological activity as assessed in *Xf* than that of the DSF from *Xcc* and lesser activity in an *Xcc* bioassay is as expected, indicating that there is considerable specificity in the structure-function relationships between different bacterial DSF signal molecules. The production of sufficient *Xf* for testing for pathogen confusion has been shown to be possible and we are anxiously awaiting initial tests to determine if topical applications of the material can lead to disease control via pathogen confusion.

#### **FUNDING AGENCIES**

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

***Section 5:  
Crop Biology  
and  
Disease Epidemiology***





## WHICH GRAPE VARIETALS ARE SOURCES OF PIERCE'S DISEASE SPREAD? DECOUPLING RESISTANCE, TOLERANCE, AND GLASSY-WINGED SHARPSHOOTER DISCRIMINATION

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**Reporting Period:** This project has just been funded. We have no results to report at this time.

### ABSTRACT

The glassy-winged sharpshooter (*Homalodisca vitripennis*; GWSS) is an important vector of *Xylella fastidiosa* (*Xf*), the etiological agent of Pierce's disease (PD). Grape species and cultivars differ in PD severity, suggesting there is variability among cultivars in resistance or tolerance to *Xf*. Quantifying the relative levels of resistance and tolerance among different varieties is critical because each may impact GWSS spread of Pierce's disease in different ways. Tolerant varieties, especially, may act as *Xf* sources. We will evaluate the feasibility of using existing *Vitis vinifera* cultivars to control PD spread by quantifying resistance, tolerance, and GWSS behavior for several important table and wine grape varieties. This work will provide recommendations to growers in high risk PD areas on which varieties to use to minimize spread.

### OBJECTIVES

We propose to independently quantify *Xylella fastidiosa* (*Xf*) infection level (i.e. resistance), symptom severity (i.e. tolerance), and glassy-winged sharpshooter (GWSS) preference for infected versus healthy plants, for several economically important raisin, table and wine grape varieties. Our specific objectives are:

1. Measure the relative levels of both resistance and tolerance for important California grape varieties.
2. Measure GWSS discrimination against infected vines and *Xf* spread for different grape varieties.
3. Measure overwinter recovery from infection for different grape varieties.

We will address the first objective in both greenhouse and field experiments that evaluate comprehensively *Xf* infection and symptom development in several table and wine grape varieties. The second objective is needed to understand how GWSS movement and feeding preference (for healthy vs. infected) differ among grape varieties, and what are the consequences for *Xf* spread. We will address the third objective with field and greenhouse measurements of recovery from *Xf* infection for different varieties – an important epidemiological determinant of Pierce's disease (PD) prevalence. Collectively, this research will allow us to pinpoint which of the current table and wine grape varieties are most and least likely to promote spread of *Xf*. Such information will allow vineyard managers to temper PD outbreaks with targeted plantings of low risk varieties.

### FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

## EPIDEMIOLOGY OF PIERCE'S DISEASE IN TEXAS VINEYARDS

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**Reporting Period:** The results reported here are from work conducted April 2005 through September 2008.

### ABSTRACT

Multiple studies are being conducted to improve current recommendations for control of Pierce's disease (PD) of grapes in Texas. These studies focus on epidemiology of the pathogen, *Xylella fastidiosa* (*Xf*), in several Texas vineyards. Sequential surveys in one of those vineyards for PD symptoms and vine mortality were conducted annually between 2005 – 2007. Very different disease progress and mortality rates were recorded for four varieties, including Chambourcin, Ruby Cabernet, Shiraz, Primitivo, and Blanc du Bois. Chambourcin exhibited the high mortality rate, while Blanc du Bois had the least mortality. Two different plantings of Shiraz responded very differently to the pathogen. Attempts were made to isolate the pathogen from throughout the vineyard and relate the success to the survey results. The pathogen was most easily isolated from vines in the advanced stages of disease development, but the pathogen could also be isolated from apparently healthy, symptomless vines. Vine recovery from infected plants was common in some varieties. The collection of isolates was analyzed using 5 Single Sequence Repeat (SSR) markers, confirming the population was exclusively in the *Xf* subsp. *fastidiosa* group. Three distinct strains were delineated within the pathogen population. Studies are ongoing to determine the significance of the strains with the population and how they may be influencing disease development. The results of these studies are particularly important to how we recommend roguing for control of within-vineyard spread of the pathogen.

### INTRODUCTION

*Xylella fastidiosa* (*Xf*), the causal agent of Pierce's disease (PD) of grapes, is considered to be a native, endemic pathogen in Texas. PD is a limiting factor for growth of *Vinifera* varieties in many of the winegrape regions in the state. Current recommendations for PD control can be expensive and inconsistent. As a result, growers face a great deal of anxiety over sustained production in existing vineyards, as well as a lack of confidence in selecting varieties for replanting and establishing new vineyards. Information on disease progress in preferred grape varieties is needed to for growers in high risk PD areas.

Another problem for growers concerning PD relates to routine, timely and reliable diagnostic results. Each of the currently available methods for diagnosing PD has strengths and weaknesses when needed for locating diseased vines. This is particularly true when attempting to relate the appearance of visual symptoms to the results of diagnostic testing. The practice of roguing to reduce within-vineyard sources of inoculum is dependent on quickly identifying suspect vines. Failure to do so may lead to additional infections, but removal of falsely identified diseased vines will unnecessarily reduce productivity.

Vine to vine spread of *Xf* appears to be an important element in the development of a PD epidemic in a vineyard. Patterns of disease incidence in some vineyards suggest that there are clusters of diseased vines from which the pathogen is transmitted in distinct directions by sharpshooters (Tubajika et al. 2004). Roguing is intended to prevent this sort of spread. A better understanding of this process might be provided by analyzing the population structure of *Xf* within a vineyard. The existence of multiple subspecies of *Xf*, and the potential for the introduction of different subspecies into vines by sharpshooters, provides an additional source of unknown variation to the epidemiology of PD in Texas. An understanding of strain diversity within a subspecies might also be useful for identifying potential sources of inoculum and subsequent spread of the pathogen through a vineyard.

The goal of this project is to improve current recommendations for control of PD by learning more about the epidemiology of the pathogen. Different approaches are being used to analyze disease incidence and severity over time and space to reveal underlying influences on disease development.

### OBJECTIVES

1. Compare rates of PD development among common grape varieties in a Texas vineyard.
2. Relate symptom development in diseased vines to the isolation of *Xf*.
3. Analyze population structure of *Xf* in a Texas vineyard.

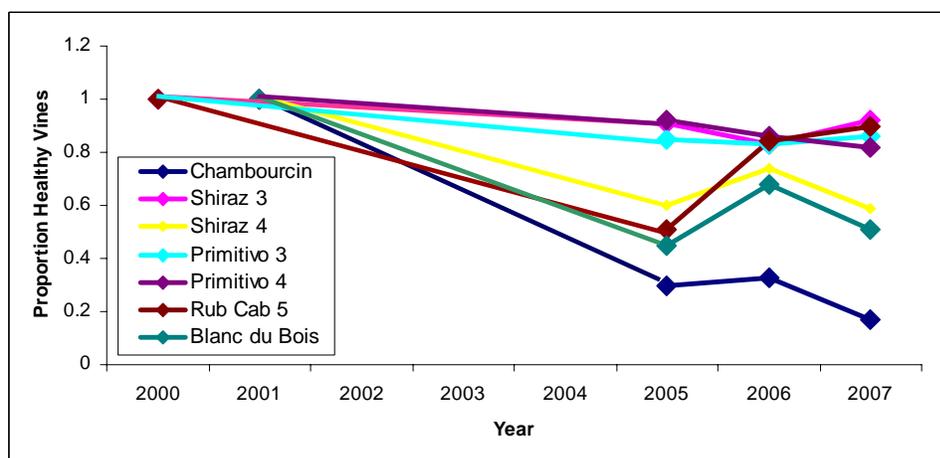
## RESULTS AND DISCUSSION

*Disease progress rates.* Sequential surveys of symptom development in individual vines were carried out annually in a vineyard near Brenham, TX. This vineyard, planted in 2000 and 2001, is located in the south eastern winegrape region of the state. Eight grape varieties were planted in 2000 and 2001 in blocks ranging from 512 – 1270 vines. The results from 7 of these vineyard blocks containing 5 varieties are reported here, including only those blocks containing greater than 1000 vines (**Table 1**). Vines were rated on a 1 – 5 scale, where 1 was a symptomless, healthy vine and 5 was dead. A vine rated 2 had a few leaves with typical foliar scorch, a 3 extensive scorching, and a 4 exhibited various combinations of matchsticks, green islands, and scorching combined with significant dieback.

**Table 1.** Attributes of 7 blocks containing 5 grape varieties in a vineyard near Brenham, TX.

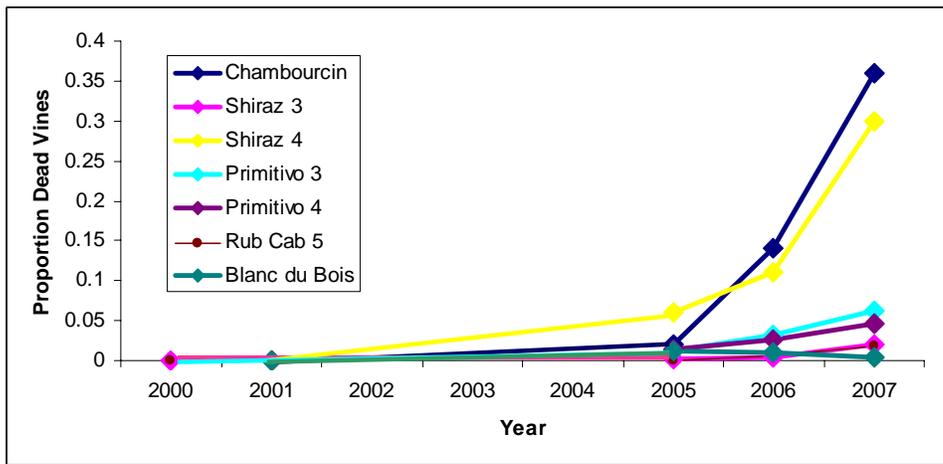
Variety	No. of Vines	Year Planted	Rootstock
Chambourcin	1071	2001	own
Shiraz (4)	1270	2001	101-14
Primitivo (4)	1270	2001	SO4
Primitivo (3)	1280	2000	101-14
Shiraz (3)	1280	2000	101-14
Ruby Cab	1152	2000	101-14
Blanc du Bois	1071	2001	own

The surveys revealed a variety of responses to PD. In **Figure 1**, the decline in vines rated “healthy”, with no scorching (Rating 2) is depicted for each of the varieties. A few of the varieties, such as Chambourcin, Shiraz block 4, and the Ruby Cab block 5 had little decline in the numbers of healthy vines. In contrast, the decline in symptomless vines dropped steeply for the Chambourcin, Blanc du Bois, Ruby Cab block 5, and Shiraz block 4. There was a noted trend of recovery of Chambourcin, Blanc du Bois, Shiraz and Ruby Cab Block 5 in 2006, but the proportions of symptomless vines declined again the following year.



**Figure 1.** Proportions of healthy, or symptomless vines, in 7 vineyard blocks containing 5 grape varieties in a Texas vineyard.

**Figure 2** depicts the mortality curves for the same varieties. The two steepest rates of mortality are exhibited by the varieties Chambourcin and Shiraz Block 4. As would be expected, Blanc du Bois, a muscadine hybrid x French varietal cross, had the lowest mortality rate during the 2 years of the survey. Shiraz Block 3 also had one of the lowest mortality rates, in contrast to Block 3 of the same variety that was planted a year earlier and was located just a few yards away.



**Figure 2.** Disease progress in seven grape varieties as measured by vine mortality.

Chambourcin is a French-American hybrid producing red grapes with an uncertain genealogy. There is some evidence that this variety is prone to overcropping, and without compensating for this tendency can undergo significant vine stress and decline. Blanc du Bois is a wine grape developed in Florida as a result of a cross between another American hybrid and the Cardinal table grape. It is known to be resistant to PD. Although these mortality rates are largely consistent with expectations concerning susceptibility to *Xf*, the differences in mortality between the two blocks of Shiraz were unexpected. The reasons for the dramatic differences in response are unknown. The Shiraz block 3 was planted in 2000, and block 4 in 2001. Although they both came from the same nursery, the different disease responses suggest there are some differences in the genetic backgrounds of the two blocks. They are on the same rootstocks and located adjacent to one another, making site differences unlikely.

*Attempted culturing for isolation of Xf.* Six blocks in the Brenham vineyard were extensively sampled for laboratory culturing and isolation of *Xf* (Table 2). Vines were randomly distributed from throughout each of 6 blocks representing 4 different varieties in order to obtain a representative sample from throughout the block and from vines in various states of health. Samples were collected during the first two weeks of June and rated two months later in August. Samples were surface sterilized and cultured on PW media. From a total of 103 culture attempts in these blocks, the pathogen was successfully isolated from 66 vines after one or two attempts. The health status of the sampled vines is included in Table 2. The 66 vines testing positive for isolation of *Xf* in June of 2007 were largely symptomless in 2005, two years before they were sampled. Twelve of the vines testing positive were ranked symptomless at the end of the growing season, illustrating the ability of some vines to recover from infection and survive. The majority of vines testing negative were rated symptomless or in the earliest stages of disease development in 2007 as well as the two years previous.

**Table 2.** Numbers of vines testing positive or negative for isolation of *Xf* ranked according to health where: 1 = healthy, no symptoms, 2 = incipient symptoms with 1 or a few scorched leaves, 3 = majority of foliage symptomatic, 4 - 6 = scorching, dieback, matchsticks, green islands, dead or removed. Vines were rated at the end of the season after samples were taken.

Year	Numbers of Positive Vines				Numbers of Negative Vines			
	Rating = 1	Rating = 2	Rating = 3	Rating = 4-6	Rating = 1	Rating = 2	Rating = 3	Rating = 4-6
2005	30	33	2	1	24	10	0	1
2006	13	24	21	8	26	5	3	1
2007	12	6	20	28	19	7	3	6

*Strain differentiation of isolates.* Repeated sampling from several vines and sampling of vines from some smaller blocks in the Brenham vineyard resulted in collection of 97 *Xf* isolates. Simple sequence repeat (SSR) markers (Lin et al. 2005) are being utilized to analyze the population structure of the isolates. When 5 SSR markers were used with conventional PCR (polymerase chain reaction), hierarchical clustering analysis resulted in delineation of 3 strain groups containing 14 – 44

isolates per group. There appears to be no selection of strains for different cultivars. Spatial relationships and the association between the occurrence of the strains and variation in disease progress will be analyzed.

## CONCLUSIONS

Differences in susceptibility, tolerance, and resistance to *Xf* are being observed in popular grape varieties in Texas vineyards. However, these differences are not entirely consistent with expectations based on previous observations (Fry and Millholland 1990). Blanc du Bois proved to be extremely resilient, as expected, even though large proportions of the vines exhibit low levels of scorching. Even though we were unable to isolate the pathogen from the Blanc du Bois, vines in this vineyard have tested positive with ELISA (enzyme linked immunosorbent assay) in other studies. Another hybrid, Ruby Cab was also extremely tolerant in the Brenham vineyard. The two blocks of Shiraz responded very differently and illustrate the difficulties that still remain in predicting the course of Pierce's disease. One planting is sustaining heavy losses, while a nearby planting is proving very tolerant. The reasons for these differences are unclear. Insect control, irrigation, and other management practices for the two Shiraz blocks were the same. Since the two blocks were planted in different years, the relative susceptibilities may result from different genetic backgrounds of the grapes.

There are always questions concerning the use of symptoms, particularly scorching, as basis for diagnosing PD. Minor scorching early in the season was prevalent on all varieties throughout the vineyard annually, and was used to select potential vines for sampling and laboratory isolations. These isolations illustrated that the pathogen was widely distributed throughout the six vineyard blocks. Consistent isolation of the pathogen does not occur until the vines are in advanced stages of disease severity. Yet the pathogen was also isolated at lesser frequencies from vines that were entirely symptomless by the end of the growing season. These results show why there are uncertainties in knowing when to rogue for disease control. Although scorching may reflect infection, it is not a good symptom for determining the ultimate fate of the vine. Also, infected vines may be symptomless and continue to yield. Even though they may be colonized at levels too low to serve as inoculum sources, they may unexpectedly collapse and thus become infective. This unpredictability will continue to confound efforts to successfully rogue.

All of the isolates obtained from this vineyard were grape strains in the subsp. *fastidiosa*. There has been some concern in Texas that ELISA results may be identifying other subspecies in grapevines, but in the Brenham vineyard this does not appear to be the case. The significance of the three strains delineated with the 5 SSR markers has yet to be determined. The strains do not, however, appear to be associated with differences in disease development observed in the grape varieties.

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## FUNDING AGENCIES

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## ENABLING TECHNOLOGIES FOR GRAPE TRANSFORMATION

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

### ABSTRACT

The release of new technologies is often hampered by downstream legal and regulatory roadblocks. A thorough analysis of the current intellectual property (IP) rights of commonly used research tools is crucial to avoiding these obstacles, especially in regards to agricultural biotechnology, specifically plant transformation-related technologies. Keeping in mind the intricate patent landscape as well as the strong IP technology portfolio in the public sector, this project looks to combine these available technologies for plant transformation. The goal of this research project is to develop a grape-specific recombinase-based marker excision system for the generation of genetically engineered *Vitis* that is marker free and can be more amenable to market entry. The anticipated construct will provide a convenient means of instituting the various Pierce's disease control strategies. In this reporting period, we present our results in validating key components of the transformation platform.

### INTRODUCTION

PIPRA, Public Intellectual Property Resource for Agriculture, is a nonprofit, public sector organization comprising a multitude of universities and institutions designed to manage the complex Intellectual Property (IP) landscape as well as develop the tools for the deployment of commercial and humanitarian agricultural technologies. Pierce's disease (PD) research has already generated promising long-term transgenic control strategies. Unfortunately, the proprietary nature of these gene transfer tools is unlikely to incorporate features that are compatible with evolving regulatory frameworks. Thus, research output with commercial potential but developed using technologies with limited freedom-to-operate (FTO) may need to be reengineered with legal and regulatory considerations. This research project aims to develop and test a transformation system not only for research, but for commercial development of PD control strategies in grape that address IP and regulatory issues.

### OBJECTIVES

1. Design, develop, and validate a grape-specific transformation system that addresses legal IP, technical and regulatory consideration.
2. Develop alternatives to *Agrobacterium*-mediated transformation for California wine grapes and/or cultivars suitable for generating root stocks.
3. Develop strategies to disseminate biological resources under appropriate licensing agreements for the PD community.
4. Explore collaborative opportunities with researchers developing PD control strategies to link the developed transformation technologies with specific PD resistance technologies.

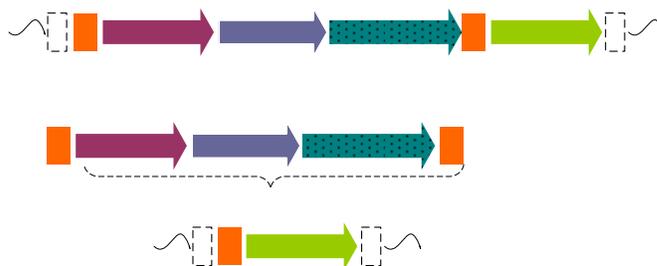
### RESULTS

*Transformation vector system:* There are several marker removal strategies that have been employed in various plants species. However, because of the long generation time of grapes, those strategies which depend on multiple plant generations are not feasible for grape cultivars. Our strategy, which has been tested in several model plant systems, utilizes a recombinase-based excision for removal of the selectable marker, after transgenic lines have been selected for, without the possibility for re-integration of the marker (Dale and Ow 1991, Russell et al. 1992, Gleave et al. 1999, Sugita et al. 1999, Sugita et al. 2000, Hohn et al. 2001, Zuo et al. 2001, Schaart et al. 2004). The recombinase-mediated transformation vector is designed with a three-part system: a plant marker to select for early transformants, a recombinase gene driven by an inducible promoter that can be transiently activated to remove the plant selectable marker, and a negative selectable marker (Perera et al. 1993, Gleave et al. 1999) to kill the cells in which poor or incomplete recombinase-mediated excision has occurred. With this strategy, we will be able to eliminate the selectable marker during the first generation of the plant tissue. Currently, PIPRA's recombinase transformation vector is in its final stages of cloning.

*Selectable markers:* Previous research focused on testing other plant selectable markers such as DEF and Atwbc19; however, neither of these markers is suitable for grape transformation. Thus far, NPTII and hygromycin have shown to be more efficient plant selectable markers. Though there is a pending application, it appears the NPTII marker may have greater FTO in the near future.

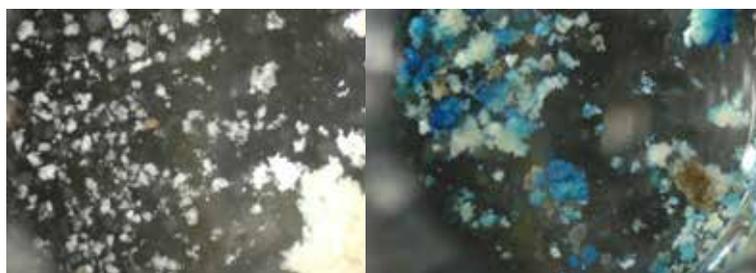
*Negative Selection System:* In addition, PIPRA has proposed using a negative marker selection which will provide a means of eliminating transformants in which the recombinase gene has failed to properly excise the unwanted DNA. Our negative selection system makes use of cytosine deaminase which shows impressive sensitivity when exposed to 5-fluorocytosine. This negative selection system has already been tested with the recombinase-mediated excision strategy for plastid transformation (Corneille, Lutz et al. 2001) and strawberry (Schaart, Krens et al. 2004). IP analysis revealed that we had appropriate freedom to operate (FTO) to isolate the cytosine deaminase gene from *E. coli* K12 MG1655. Cloning of this cassette is complete, with the cytosine deaminase gene being driven by the FMV34S constitutive promoter. The cassette has been successfully cloned into a binary transformation vector and transformed into grape and tobacco callus. The transformed tissue will be treated with increasing amounts of 5-fluorocytosine to identify the concentration necessary to eliminate the tissue. Those experiments are currently ongoing.

A parallel experiment aims to test the efficacy of the Par-A excision activity prior to commercial and humanitarian development of our vector. We will employ another type of negative selection involving DsRed and GUS reporter genes (**Figure 1, panel A**). The recombinase efficiency can be evaluated by comparing expression levels of the two visual markers. A successful recombination event will result in the deletion of the RRS-flanked DNA, which contains the hygromycin plant selection marker as well as the DsRed marker. Therefore, only the GUS marker, placed in the GOI cassette should be expressing in the plant (**Figure 1, panel B**). We are currently in the final stage of the cloning of this construct. The last step is the insertion of the completed Par-A recombinase cassette module.



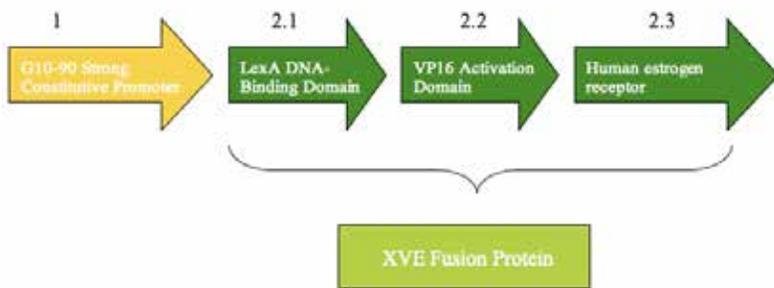
**Figure 1.** Negative selection construct to test efficacy Par-A mediated recombinase activity in tobacco and grape.

*Promoters:* This transformation platform will include a number of constitutive promoters for expression in grapes (Purdue's MAS, University of California's UC FMV34S, G10-90 from Zuo, Niu et al. 2000). In addition, we have incorporated an estrogen-inducible promoter system for tight control of transcription activity. This promoter will be used to regulate the expression of the recombinase gene. Precise control of expression of the recombinase gene is preferred to avoid premature excision which may occur due to the leaky expression of constitutive promoters. The estrogen-induced XVE system has been previously used in a cre-lox-mediated marker free system in Arabidopsis (Zuo, Niu et al. 2001). This system was preferred over another inducible promoter, the glucocorticoid-system (Aoyama and Chua 1997; Ouwerkerk, de Kam et al. 2001), which requires the use of dexamethasone treatment that can often inhibit plant tissue regeneration as well as contain high background levels (Zuo, Niu et al. 2000). The XVE system (Zuo, Niu et al. 2001) with a GUS reporter gene was tested in tobacco and grape transformants with promising results (**Figure 2**).



**Figure 2.** GUS expression in grape callus transformed with pER8:GUS grown on medium without estradiol (left) and grape callus grown for 48 hours on medium containing 50 uM estradiol (right)

In addition, with support from legal counsel, we recently concluded the IP analysis of the XVE system. To conduct the IP evaluation of the XVE system, we divided this system into three parts. Part A considered the three components of the XVE fusion protein: LexA Binding Domain (X), VP16 Transcription-Activation Domain (V), and Estrogen Receptor (E). Part B considered IP related to the LexA Operator sequence. The LexA Operator sequence would be situated before the gene it is regulating, in this case the recombinase Par-A gene. Finally, part C reviewed the legal landscape around the constitutive G10-90 promoter which drives the XVE fusion protein (**Figure 3**). The results from this review show the technology would require licensing from Rockefeller University. PIPRA originally obtained the XVE system from Rockefeller University under a research only material transfer agreement. To consider commercial use of Rockefeller's promoter, we have initiated conversations in order to include this critical component as part of the patent pool that would be made available to the PD community.



**Figure 3.** The XVE fusion protein driven by the constitutive G10-90 promoter. Completed IP analysis of this piece now provided us complete FTO.

## CONCLUSIONS

Research to combat the threat of PD on California's wine grape industry has led to the development of several promising transgenic approaches (Aguero et al. 2005, Reisch and Kikkert 2005). Regardless of the success of these projects, they encompassed proprietary technologies that would hinder their downstream commercial production due to IP restrictions. In order to advance the transgenic grape technology, it is critical that thorough IP analysis be conducted in conjunction with the research such that new control strategies in the lab can be adopted by the commercial sector without unnecessary delays or need to reengineer transgenic plants. Because of the incredibly long generation time of grapes, up to two-three years, it is not feasible to develop technologies that must be repeated during the commercial phase of development because of IP restrictions that could have been avoided at the beginning of the project. For example, the thorough IP analysis on the recombinase-mediated plant transformation system for grape provides a clear legal pathway for commercial applications of these technologies. PIPRA's approach to form a patent pool of the technologies necessary or the PD community has paved the way for the development of technologies with maximum FTO for research on PD and glassy-winged sharpshooter applications.

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## FUNCTIONAL TESTING AND CHARACTERIZATION OF PIERCE'S DISEASE INDUCED PROMOTERS FROM GRAPE

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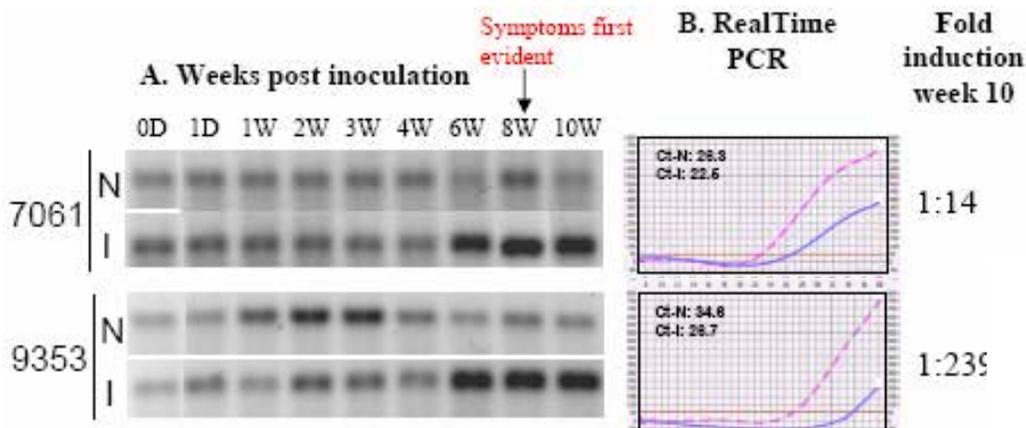
**Reporting Period:** The results reported here are from work conducted October 1, 2007 to September 30, 2008

### ABSTRACT

The goal of this research was to clone and characterize unique DNA sequences from grape that specifically regulate the expression of grape genes in tissues that are infected with *Xylella fastidiosa* (*Xf*) or are receiving systemic signals of pathogen presence. In addition, these promoters, when fused to GFP, are specific tools to non-destructively study the presence and movement of the bacteria in infected grape canes or petioles. This project was initiated in July 2004 as a priority research area by the Pierce's Disease Research Board and will conclude in August 2009 with the delivery of two *Xf*-responsive promoters from grape to a) drive the site-specific expression of any candidate gene at locations where the bacteria reside and b) provide an induced reporter gene expression system that can be used as a powerful tool to study and characterize host responses to *Xf* and *Xf*-secreted effector molecules in intact xylem cells by observing fluorescence due to GFP. The promoters, G9353 and G7061 each will be available as GFP fusion constructs in transgenic Thompson Seedless clonally propagated lines. Illustration of the use of these promoter fusions as diagnostic tools for grape response to *Xf* EF-Tu infiltration can be found in the 2008 PD Symposium Proceedings report from Professor George Bruening's group.

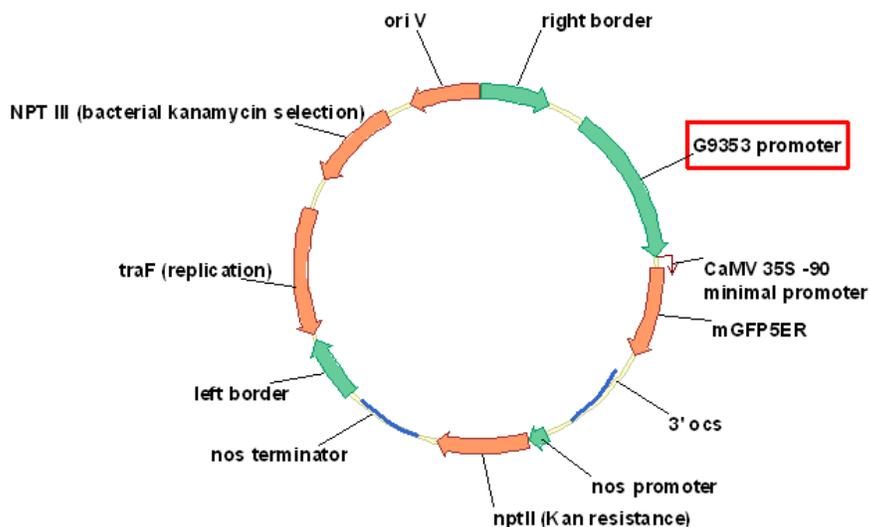
### INTRODUCTION

This project was initiated in July 2004 as a priority research area by the Pierce's Disease Research Board (3). The need for *Xylella fastidiosa* (*Xf*)-inducible promoters was based on the fact that the constitutive promoters, used universally to drive the expression of transgenes, suffer from two disadvantages. Firstly, they are protected by existing patents with the attendant limitation for commercial use, and secondly, the constitutive expression of certain transgenes is widely considered to have deleterious effects. For example, there are recent reports showing a deleterious effect from constitutive expression of disease resistance genes, effects that are remedied by expression from an inducible promoter (5, 6). Specifically, the constitutive expression of genes that normally are under control of stress-responsive promoters (infection responsive and resistance genes) is likely to be disadvantageous to the plant (5). An Affymetrix GeneChip was used to characterize the expression of ~15,000 *Vitis vinifera* genes in response to *Xf* infection and drought stress (1,2) (**Figure 1**).



**Figure 1.** *Xylella fastidiosa* responsive promoters were obtained from genes found to be up-regulated in *X. fastidiosa* infected field grown plants but not up-regulated by water stress alone. Example data used to identify individual genes from which promoters were recovered as illustrated for G7061 and G9353.

Promoters for two of the *Xylella*-responsive vascular tissue localized genes, referred to as G9353 and G7061, were sequenced from bacterial artificial chromosome (BAC) clones. Regions 5' to the coding sequence were isolated by PCR and used to produce promoter-GFP-reporter fusions (**Figure 2**) for transformation into the *Xf* susceptible Thompson Seedless grape background. We received 42 transformants (**Table 1**) from UC Plant Transformation Facility in 2006 from constructs of the promoters G9353 and G7061 fused to GFP. A line bearing each promoter will be selected from the 42 independent transformants for maximum specificity and sensitivity to the presence of *Xf*.



**Figure 2.** Binary plasmid containing the G9353 promoter sequence fused to GFP, which will be available to Pierce's Disease researchers upon request.

In addition to their utility for engineering PD resistance in grape, the availability of *Xf*-induced reporter gene expression can provide an extremely powerful tool to

study and characterize host responses in their intact cellular and tissue context. With such tools, it should be possible to examine the chemical and/or physical cues from the insect or pathogen that trigger host gene expression and the deleterious effect of the disease. Lastly, an immediate and direct application has been identified through our collaboration with the Bruening laboratory wherein, promoter G9353 expression is activated by injection into the grapevine petiole of *Escherichia coli* cells expressing a single *Xf* protein, the protein synthesis elongation factor EF-Tu. Please see article by Dr. Bruening in these Proceedings for specific information on the EF-Tu assay.

**Table 1.** Transgenic plants containing promoters G7061 and G9353

Promoter ID	Putative function of the microarray transcript induced by <i>Xf</i>	total # of transformants with the promoter-GFP fusions	# of transformants tested to date	Results <i>Xf</i> uptake into detached leaf; GFP expression	# transformants petiole inoculated and analyzed	# transformants to be petiole inoculated and analyzed
G7061	unknown	22	12	9 of 12 show GFP expression	4 of 10 show GFP expression	12/22
G9353	Alpha-tubulin	20	10	3 of 10 show GFP expression	3 of 10 show GFP expression	10/20

## OBJECTIVES

1. Evaluation of whole plant transgenics G9353 and G7061 with stem inoculation under greenhouse conditions.
2. Confirm the specificity of response of promoter G9353 to *Xf* vs *Xanthomonas campestris* using intact stem inoculation under greenhouse conditions.
3. Promoter distribution to current researchers and long-term storage.

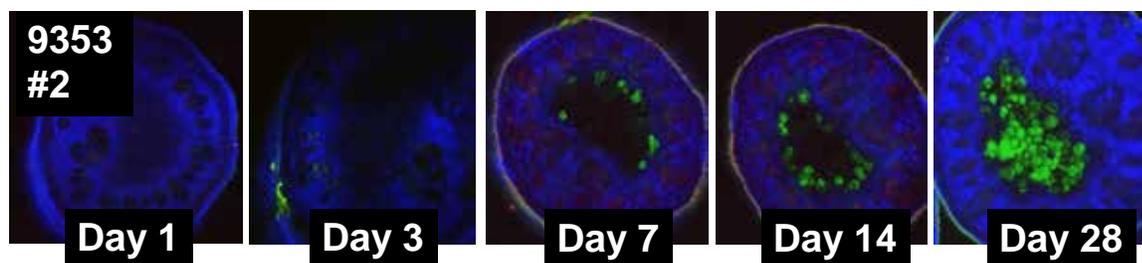
## RESULTS AND DISCUSSION

Two of the original 14 putative promoters, G9353 and G7061, have been advanced to the transgenic stage as GFP (green fluorescent protein) fusions (4) and show a response to infection by *Xf* as visualized by RT-PCR (**Figure 3**) and GFP expression in the inoculated plants using confocal microscopy (**Figure 4**). A total of 42 plants were received (**Table 1**). Prior to any functional analysis, the transgenic grape plantlets were grown in the greenhouse and each primary transgenic was assayed for the presence of the transgenes by genomic PCR prior to any further experimentation. Currently we have tested 50% of the primary transgenic plant lines. While all plants tested to date contain the transgene not all are activated to the level of detectable fluorescence, even though analysis by qPCR confirmed that the promoter was active; just not sufficiently active for GFP detection.



**Figure 3.** RT-PCR analysis of the G9353 transcript from greenhouse grown grape leaves. RNA in lane 1 healthy cv. Freedom; lane 2 *Xf*-infected cv. Freedom; lanes 3 and 5 are healthy Thompson Seedless; and lanes 4 and 6 are *Xf*-infected Thompson Seedless. Lanes 1-4 were amplified with G9353 specific primers and lanes 5-6 were amplified with actin specific primers as a control. Lanes 2 and 4 indicate the induction of the G9353 transcript in the infected plants in the greenhouse.

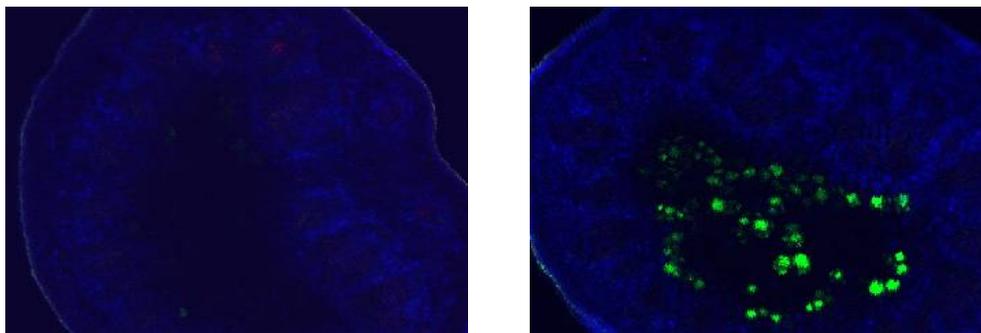
Hence, there is sufficient variation in the strength of the GFP fluorescence response to require that all independent transformants must be tested to identify the plants with the most rapid and strongest response with minimal background. For example, quantitative data indicates that the activation must be greater than four-fold to be visualized as differential by confocal fluorescence microscopy. The remaining plants will be subjected to final testing with whole plant inoculations (**Table 1**). Clonal copies of each line will be made before evaluation by inoculation. Following clonal propagation, 10 transgenic lines of each promoter remain to be inoculated with *Xf* under greenhouse conditions, assessed by confocal microscopy and qPCR for timing and location of response of the respective promoters to the presence of the bacteria. These studies will develop a sufficiently detailed picture of the temporal and spatial aspects of *Xylella*-induced gene expression during bacterial colonization in stems, leaves and petioles of grapes as a base line for use as a diagnostic tool for the effect of pathogen secreted molecules as indicated earlier. Criteria for selecting the prime transgenic lines are that they express a specific, rapid and strong response to *Xf* infection with low background response. RT-PCR of the endogenous genes in Thompson Seedless indicated that this promoter was expressed in the presence of *Xf*. This is confirmation that both the endogenous gene and the promoter gene fusion in transgenic plants are expressed in the presence of *Xf*.



**Figure 4.** Specificity of the response of promoter G9353 fused to GFP in response to the presence of *Xf* in the vascular system of grape compared with *Xanthomonas campestris*. Blue color represents chlorophyll auto-fluorescence; Green color indicates the translation of GFP and, therefore, activation of the promoter 9353 in the presence of *Xf*.

In addition to the *Xf*-activated response of these promoters, it is essential that the response to *Xf* be specific to this bacterium. To assess the specificity, *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*, another xylem dwelling bacteria that closely resembles *Xf* but is not pathogenic on grape served as the bacterial test case. Transgenic G9353 was inoculated with *Xcv* and compared to clonal plants inoculated with pathogenic *Xf* at the same cell density and evaluated by confocal microscopy after 21 days. The presence of *Xcv* in the xylem did not trigger the expression of G9353-GFP whereas the promoter driven GFP was activated by *Xf*, as shown previously. These observations indicate that the promoter was responsive to *Xf in planta* but not to the related *Xcv* (**Figure 5**). In addition, recent results from collaborative efforts between the Gilchrist laboratory and the Bruening laboratory have demonstrated that promoter G9353 expression is activated by injection into the grapevine petiole of *Escherichia coli* cells expressing a single *Xf* protein, the temperature unstable protein synthesis elongation factor EF-Tu. The same *E. coli* strain, not induced to generate *Xf* EF-Tu, and another strain, not bearing the EF-Tu construction, both failed to induce GFP accumulation, indicating that the effect is due to *Xf* EF-Tu and not, for example, to endogenous *E. coli* EF-Tu. In other systems, examples of plant recognition of the EF-Tu from specific plant pathogenic bacteria are well documented.

Please see the report in this volume of the Proceedings of the 2008 Pierce's Disease Research Symposium by George Bruening entitled "Exploiting *Xylella fastidiosa* Proteins for Pierce's Disease Control" for additional research description of the diagnostic application of G9353 transgenic plants.



**Figure 5.** Time course analysis of the expression of promoter G9353 fused to GFP in response to the presence of *Xf* in the vascular system of grape. Bacteria were introduced by puncture inoculation as illustrated in **Figure 2**. Analysis is by confocal microscopy. Blue color represents chlorophyll autofluorescence; Green color indicates the translation of GFP and, therefore, activation of the promoter 9353 in the presence of *Xf*.

## CONCLUSIONS

The activation of these two promoters following infection with *Xf*, but not *Xcv*, confirms the proof of concept that we can supply *Xf*-inducible promoters to PD researchers. Our intention is to package and release the most responsive transgenic lines of each of the two promoters and binary plasmids containing the validated *Xf*-inducible promoters, G9353 and G7061 to all interested researchers. These promoter-GFP fusions are currently being used for studying the timing and incidence of *Xf* infection, action of pathogen effector molecules (Bruening) and could be used to determine plant response to mechanical inoculation or sharpshooter feeding (**Figure 1**).

Lastly, this project will generate intellectual property; specifically promoters that drive expression of grape genes due to the presence of *Xf*. The issue of IP will be handled in the following manner: 1) all sequences derived will be placed in the public domain; 2) IP protection will be sought for functionally verified promoters sequences, and 3) all materials will be made available to qualified researchers, including all those associated with the PD projects through a standard material transfer agreement.

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# SYSTEMIC CONTROL OF PIERCE'S DISEASE BY ALTERED EXPRESSION OF ANTI-APOPTOTIC GENES OR THEIR RNA-BASED REGULATORY ELEMENTS

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**Reporting Period:** The results reported here are from work conducted October 1, 2007 to September 30, 2008

## ABSTRACT

*Xylella fastidiosa* (*Xf*) is both an endophyte and a pathogen. Cell death symptoms associated with the pathogenic state result from the activation of programmed cell death (PCD) pathways with morphological markers of apoptosis in the susceptible grape. The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that are capable of suppression of Pierce's disease (PD) symptoms when constitutively expressed as transgenes. We identified, using a functional cDNA screen, several novel genes from grape and heterologous plants that suppressed PCD when expressed as transgenes. We reported in 2007, several transgenes expressed in the root stock cultivar Freedom that suppressed PD symptoms. In addition, the level of bacteria in the vascular tissue are maintained four orders of magnitude lower than in untransformed control plants, all of which died. We now report that transgene expression in the fruited PD susceptible cultivar Thompson Seedless also affords protection against PD symptoms and limits the bacterial titer up to four to six orders of magnitude below that reached in untransformed plants that are killed within two months after inoculation. The protected plants have remained alive and asymptomatic nine months after inoculation. From the perspective of the grape-bacterial interaction, it appears that the anti-PCD genes tested to date suppress PD symptoms and functionally restore the bacteria to an endophytic ecology in the xylem equivalent to that seen in the asymptomatic host *Vitis californica*.

## INTRODUCTION

At the outset of this project in 2001, little was known about the mechanisms or genes involved in symptoms or death of the grape plants infected with *Xylella fastidiosa* (*Xf*). In the course of these studies, we established that the cell death leading to leaf scorch symptoms in Pierce's disease (PD) is the result of the activation of programmed cell death (PCD) with morphological markers of apoptosis. In addition, Dr. Tom Rost and we, independently, determined that PD symptoms can occur distal to sites where the bacteria are detected suggesting the presence of mobile signals from the bacteria. It also is documented that several relatives of grape, including *Vitis californica*, and other host plants can harbor otherwise lethal titers of *Xf* without exhibiting PD symptoms. The questions posed in this research were: a) are there genes in the plant that respond to *Xf* signals by triggering programmed cell death in certain grape genotypes, b) can this response be blocked genetically; and c) if so, does this then allow the bacteria to maintain the endophytic state, leaving the plant otherwise unaltered but free of disease symptoms; and/or d) does suppressing PD symptoms negatively affect the ability of the bacteria to colonize the vascular system.

Current literature and results from our laboratory indicate a number of plant diseases result from induction of PCD in the host cells in advance of microbial growth (2,11,12). The induction of PCD results in an orderly dismantling of cells that includes maintaining integrity of the plasma membrane until internal organelles and potentially harmful contents including phenolics, reactive oxygen and hydrolytic enzymes have been rendered harmless to contiguous cells. However, when the cell contents are released, they can serve as nutrients for microbial cells when they are present in the immediate environment (2,9). Hence, bacteria like *Xf* could receive nutrients from cells adjacent to the xylem that are triggered to undergo PCD and gradually releasing contents of the grape cell into the apoplastic space surrounding the xylem. The fact that we measure bacterial titers three-six orders of magnitude higher in symptomatic (ultimately dead) grape plants than in either asymptomatic wild grapes or the transgenic asymptomatic grape plants is consistent with enhanced nutrition in the xylem of infected symptomatic plants. The working scenario in this research is; blocking death, limits death dependent nutrient release, and thereby restricts bacteria multiplication but does not act as an antibiotic against the bacteria. If true, this scenario does not apply novel selection pressure on the bacteria any more than residing in *V. californica* or any other asymptomatic host.

Genetic strategies for disease suppression and development of a biological understanding of the bacterial-plant interaction are high priority areas in the PD/GWSS Research Program and the NAS report. The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that, when expressed in grape, will prevent colonization, systemic spread or symptom development due to the presence of *Xf* in the xylem. Recent published information from our laboratory established that susceptibility of several plants to a range of pathogens depends on the ability of the pathogen to directly or indirectly trigger the activation of existing, genetically regulated, pathways leading to apoptosis or programmed cell death (PCD) (1,2,3,4,5,6). These discoveries parallel investigations now widely reported and accepted in human medicine whereby genes, signaling pathways and chemical signals expressed by animal pathogens initiate infection by

activating or blocking apoptosis through constitutive gene expression or signaling pathways present in all cells. These studies are the basis for extensive searches for apoptosis-based therapeutic approaches and agents in human medicine (7,18).

Hence, this research on PD is conducted within a global context in which the process of PCD with apoptotic morphologies is functionally conserved across the animal and plant kingdoms while sharing diagnostic markers of apoptosis including chromatin condensation and segregation into distinct masses referred to as pycnotic DNA bodies 10,13,14,15), oligonucleosomal DNA ladders, externalization of phosphatidylserine, and TUNEL-positive nuclei in the incipient plant disease lesions (2). Also, it is known that many proteins and several regulatory RNAs function in the induction or suppression of animal PCD and exhibit cross-functionality in the plant kingdom. Ectopic expression of known apoptosis-blocking animal and animal virus genes, or treatment with anti-apoptotic pharmacologically active peptides, has been shown to block PCD and suppress disease in plants where cell death is a symptom of disease, as is the case of PD (3,4,16,17).

However, mining of plant genome sequences in the available databases has not revealed plant sequence homologs of either the core pro- or anti-PCD pathway genes found in animals, even though the induction or suppression of PCD in transgenic plants by cross-kingdom expression of pro- and anti-apoptotic animal genes suggested that anti-death homologs likely exist in plant genomes (2,7,18). Consequently, identification of plant genome derived anti-death genes must be based on functional screens and is the premise that defined the direction of our research. Results presented this year and in 2007 (8) indicate that the approach has been successful.

## OBJECTIVES

The objectives of the proposed research for 2008-2010 are as follows:

1. Continue to evaluate recently obtained Thompson Seedless transgenic grape plants expressing the eight candidate anti-apoptotic genes for blocking of PD symptoms (**Table 1**).
2. Measure the effect, over a time course, of blocking PD symptoms with anti-apoptotic transgenes on *Xf* bacterial population levels and movement in the xylem by quantitative PCR (qPCR) and confocal laser scanning fluorescence microscopy to monitor GFP-tagged *Xf*.
3. Determine grape gene expression changes in transgenic compared with non-transgenic plants in response to *Xf* infection by differential transcriptional profiling using quantitative PCR.
4. Produce grape transgenic plants with modified candidate anti-apoptotic genes designed to enhance systemic movement *in planta*.
5. Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape.
6. Graft the resistant transgenic Cv Freedom rootstocks of PR1 and cDNA 456 onto untransformed scions of Thompson Seedless and Chardonnay to monitor movement of either expressed proteins of these genes that contain a secretory leader on VVPR1 or the RNA derived from the 3'UTR from the ortholog of the potato p23 gene that shares stem and loop homology to the Bcl 2 3'UTR. The transformed grafted plants will be inoculated with *Xf* and scored for disease reaction in the untransformed scion.

**Table 1.** Thompson Seedless now available with putative anti-PCD genes under control of the 35S promoter.

Genotype	# Lines or Independent Transformants	# of Plants
TS – CBP14B	24	30
TS - CBP14LD	27	27
TS - CB376	28	29
TS - CB456	27	31
TS - I35	14	15
TS - CBMT	22	24
TS - CBWG23	23	25
TS - CBWG71	20	23
total	185	204

## RESULTS

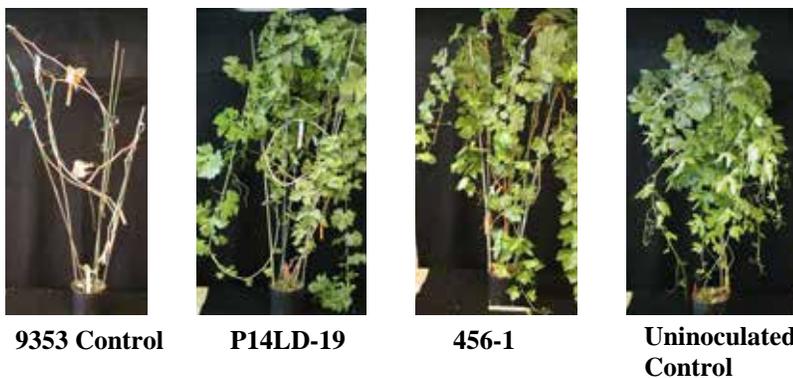
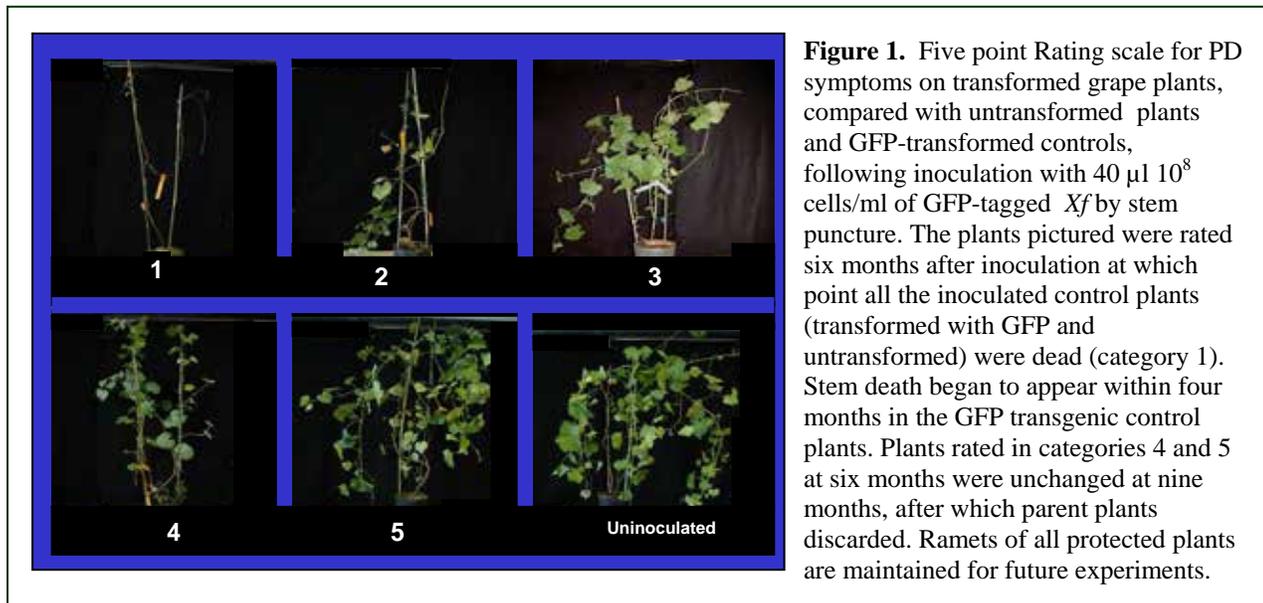
*Genes identified as potential anti-PCD genes from the conditional life-death screens.*

Previous funding on this project led to the development of a functional cDNA screen to identify plant genes, which when over-expressed as transgenes, suppress cell death triggered by chemical inducers of PCD. The genes in **Table 1** have been described in earlier reports to this symposium and the results of inoculation of the first set of transgenic plants of Cv Freedom was reported in 2007 (8). In summary of the 2007 Cv Freedom results, resistance against PD was observed in the susceptible grape rootstock by two anti-apoptotic transgenes (P14LD and MT) and one 350 bp DNA sequence associated with a nematode up-regulated gene designated p23. Furthermore, the expression of these three sequences, not only protected the

transgenic plants against PD symptoms and plant death but maintained the population of *Xf* at four orders of magnitude below the level observed in untransformed plants that died within two months ( $10^8$  bacteria per gram of stem tissue) compared with the asymptomatic transgenic plants that carried a level of  $10^4$  cells/gm stem tissue in the most resistant lines that were alive at nine months. Interestingly, the  $10^4$  titer is equivalent to what we observed in the asymptomatic host *V. californica* 12 months after inoculation. In 2008, we began testing the anti-PCD genes expressed in Cv Thompson Seedless and report the early results of two inoculations of the Thompson Seedless transgenics bearing the CBP14LD and the CB456 genes.

*Thompson Seedless grape plants expressing anti-apoptotic genes.*

After creating clones of these transgenic lines, the plants were trained to grow as two or three canes and maintained by periodic pruning of side and top branches (**Figure 1**). Half of the transformed plants were individually inoculated November and December of 2007 and the second half in April and May of 2008. The inoculation method was the same for both sets however the concentration of *Xf* bacteria was 1000 fold less for the second set. The inoculation method was by needle puncture of the stem to allow uptake of 10-20  $\mu$ l of *Xf* at  $2 \times 10^8$  cfu of the GFP-tagged *Xf*/ml for the 2007 inoculations and a 1000 fold less at  $2 \times 10^5$  cfu of the GFP-tagged *Xf*/ml for the 2008 inoculations. The plants were monitored visually for symptoms and by quantitative PCR (qPCR) for bacterial movement and multiplication. They were scored for disease severity in May 2007 (first set) and in October 2008 (second set), using a five point scale (1=dead and 5= asymptomatic) and photographed. Representative control (scored as 1) and transgenics from the second set (scored as 5) are shown in **Figures 1, 2, and 3**).



**Figure 2.** PD assay on the first set of inoculated Thompson Seedless plants transformed with anti-PCD transcripts P14 and 456 were inoculated with 20 $\mu$ l of  $2 \times 10^8$  *Xf*/ml and photographed three months later. The protected plants are compared with 9353, an *Xf*-inducible promoter fused to GFP, and an uninoculated untransformed Thompson Seedless plant.

The effect of anti-apoptotic transgenes on *Xf* bacterial populations was measured by RealTime quantitative PCR (qPCR) (**Table 2**). Analysis of *Xf* inoculated plants revealed that although bacteria can be detected everywhere in a infected plant, the inoculated cane samples are more consistent than the cane of the stem of primary branches. It is essential to determine the effect of blocking PCD-based symptoms in the transgenic plants on the bacterial multiplication and spread in terms of the overall impact of the transgenes. Based on initial experiments to ascertain which tissue to sample for *Xf* quantization, we sampled the stem of primary branches or petioles of individual plants. Although, this would allow repeated sampling of an individual plant over the course of the experiment, we found that it is not a reliable indicator of the overall bacteria level and could vary by as much as six orders of magnitude. These results indicate that equivalent results were obtained at the two inoculum concentrations. In both cases the mean bacterial load of unprotected control plants reached the same level ( $10^8$ ) after two-three months at which point the plants began to die. The transgenic plants remained healthy appearing (categories 4-5) after assaying at six and nine months with bacterial titres ranging from  $10^2$  to  $10^4$  in the main canes of the inoculated plants (**Table 2**). Representative images of plants in the first inoculation with  $20 \mu\text{l}$  at  $2 \times 10^8$  are shown in **Figure 2** with equivalent images of the second inoculation with  $20 \mu\text{l}$  at  $2 \times 10^5$  are shown in **Figure 3**.

**Table 2.** Thompson Seedless Transgenics Genotype analyzed to date.

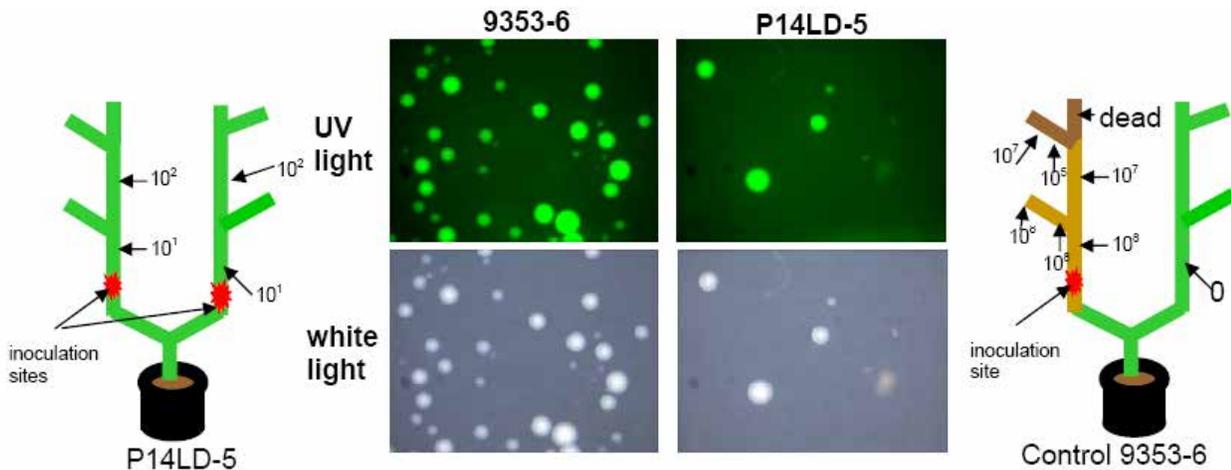
Thompson Seedless Transgenics Genotype analyzed to date	# of Lines evaluated to date	Percent of plants rated as Figure 1 categories 4 and 5 and protected as in Figures 2 and 3	Mean bacterial load per 0.1 gm of stem in each respective line
<b>Inoculation 1 @ <math>2 \times 10^8</math> cfu</b>			
TS - CBP14LD	4	3/4	$10^4$
TS - CB456	4	3/4	$10^4$
TS - 9353 (control)	6	0/6	$10^8$
<b>Inoculation 2 @ <math>2 \times 10^5</math> cfu</b>			
TS - CBP14LD	23	20/23	$10^2$
TS - CB456	26	23/26	$10^2$
TS - 9353 (Control)	6	0/6	$10^8$



**Figure 3.** PD assay on the second set of Transgenic Thompson Seedless plants inoculated in 2008 with  $20 \mu\text{l}$  of  $2 \times 10^5$  *Xf*/ml and photographed three month later. This inoculum is 1000-fold less than that used for the inoculations shown in **Figure 1**. The 9353 control plant has a PD disease score of 1 and all others were scored as 5 on a 5 point scale.

*Bacterial plating for determination of bacterial viability in the control and transgenic plants.*

The pathogenic *Xf* used to inoculate the plants shown in **Figures 1, 2, and 3** and **Table 2** were obtained from Dr. Steven Lindow. These bacteria expressed GFP and were resistant to Kanamycin. Stems sections from the tissue used to generate the data in **Table 2** were further sectioned, incubated in water and centrifuged to pellet the bacteria, re-suspended in water and plated on *Xf* media containing Kanamycin. Bacteria expressing GFP were obtained from the control and transgenic protected plants as shown in **Figure 4**. The schematic illustrations indicate the relative amounts of bacteria estimated by qPCR and the color plates indicate representative fields on the media plates with colonies of GFP-expressing bacteria. These data confirm that many more bacteria were present in the control cane sections and that the bacteria recovered on the plates were viable progeny cells of the inoculated *Xf*. In summary, the qPCR and plating data indicate that the two anti-PCD genes analyzed to date suppress symptoms of PD, do not eliminate the bacteria from the tissue but do reduce the bacterial titre to a level that, while detectable, is orders of magnitude lower than the untransformed control plants.



**Figure 4.** Live GFP-tagged *Xf* bacteria can be isolated from extracts of infected Thompson Seedless plants. Shown are micrographs of *Xf* growing on PD3 plates supplemented with 30 $\mu$ g/ml kanamycin. Consistent with the qPCR data, the control plate has a lot more bacteria. In fact, many of the plates for the protected transgenics had no colonies on plates. This data also provides evidence that the qPCR is not just amplifying DNA of dead *Xf* cells in the protected transgenic plants.

## CONCLUSIONS

In the past year, we successfully demonstrated resistance against PD in the susceptible grape rootstock cv. Freedom by two anti-apoptotic transgenes (PR1 and MT) and one 350 bp DNA sequence associated with a nematode up-regulated gene designated p23. All three cDNAs were recovered anonymously from the plant-based cDNA screen and all have functional links with conserved domains to anti-apoptotic orthologs in the animal kingdom (1,18). We further demonstrated that expression of these three sequences, not only protected the transgenic plants against PD symptoms and plant death but maintained the population of *Xf* at four orders of magnitude or greater below the level observed in untransformed plants that died within two months ( $10^8$  bacteria per gram of stem tissue) following controlled inoculations in the greenhouse. The key point is that altered expression of the anti-apoptotic transgenes does not kill the bacteria but does restrain the titer in the asymptomatic transgenic plants from a lethal level of  $10^8$  to a level of  $10^4$  to  $10^2$  cells/gm stem tissue in the most resistant lines; the  $10^4$  titer is equivalent to that which we measured in the asymptomatic host *V. californica* 12 months after inoculation. Interestingly, the  $10^4$  cells/gm stem tissue titer level in the asymptomatic transgenic plants and *V. californica* is equivalent to that observed by Dr. Lindow in his *rpfF* transformed plants that also are asymptomatic suggesting that susceptible grape plants can tolerate a bacterial population at the  $10^4$  without showing PD symptoms. Hence, the current experiments indicate that the effect of the anti-PCD genes suppresses symptom expression but does not exert a direct inhibiting effect on the bacteria. The symptom suppressive genes do not act as antibiotics and do not affect the natural endophytic ecology of the bacteria in the xylem. In essence, an endophyte gone bad has been returned to the state of a benign endophyte.

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# OPTIMIZING GRAPE ROOTSTOCK PRODUCTION AND EXPORT OF INHIBITORS OF *XYLELLA FASTIDIOSA* POLYGALACTURONASE ACTIVITY

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**Reporting Period:** The results reported here are from work conducted August 15, 2008 to October 13, 2008.

## ABSTRACT

In response to the strategy recommended by the Advisory Board, to express plant genes for particularly effective polygalacturonase inhibiting proteins (PGIPs) or other inhibitors of *Xylella fastidiosa* (*Xf*) polygalacturonase (PG) in transgenic grape rootstocks, this approach was adopted to enhance grapevine Pierce's disease (PD) resistance. This proposal describes integrated studies aimed at the eventual deployment of that strategy. To ease the path to commercialization, PIPRA investigators will examine relevant Intellectual Property and regulatory issues associated with the use of this strategy. A reliable source of recombinant *Xf* PG will be developed and the PG will be used to screen diverse PGIPs for their ability to effectively inhibit the *Xf* PG enzyme. Grape rootstock lines will be transformed with the most effective PGIPs and signal and target sequences that maximize PGIP expression in the rootstock and its export to the non-transgenic scions. At the conclusion of the project, the capacity of the non-transgenic vines grafted on the transgenic rootstock to resist PD and produce high quality grapes will be tested.

## INTRODUCTION

*Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease (PD) in grapevines, has been observed in infected portions of vines. Several lines of evidence support the hypothesis that *Xf* uses cell wall-degrading enzymes (CWDEs) to digest the polysaccharides of plant pit membranes separating the elements of the water-conducting vessel system of plants (Thorne et al., 2006). *Xf* CWDEs breakdown and thereby increase the porosity of these primary cell wall barriers, allowing the systemic expansion of the pathogen. The genome of *Xf* contains genes putatively encoding a polygalacturonase (*Xf*PG) and several  $\beta$ -1,4-endo-glucanases (EGase), CWDEs that digest cell wall pectin and xyloglucan polymers, respectively. These CWDEs are good candidates as factors that facilitate *Xf* systemic movement and PD development. To demonstrate this, Roper et al. (2007) developed a PG-deficient strain of *Xf* and showed that the mutant bacterial strain was unable to cause PD symptoms, thus identifying the pathogen's PG as a PD virulence factor. Labavitch et al. (2006) reported that introduction of PG and EGase into explanted stems of uninfected grapevines caused breakage of the cell wall of the PM and, subsequently (Labavitch, 2007), demonstrated that substrates for these enzymes, pectins and xyloglucans, are present in grapevine PMs.

PG-inhibiting proteins (PGIPs) produced by plants limit damage caused by fungal pathogens (*B. cinerea*, the gray mold pathogen) as well as by insects (*Lygus hesperus*, the western tarnished plant bug) (Powell et al., 2000; Shackel et al., 2005). PGIPs have been shown to be selective inhibitors of PGs produced by some fungal pathogens and insects, but were reported to be ineffective in inhibiting bacterial PGs (Cervone et al., 1990). However, Agüero et al. (2005) by introducing a pear fruit PGIP gene (Stotz et al., 1993; Powell et al., 2000) into transformed grapevines demonstrated that transgenic vines expressing the pear PGIP exhibit decreased susceptibility to both fungal (*B. cinerea*) and bacterial (*Xf*) pathogens. This result implied that the pear PGIP provided protection against PD by inhibiting the *Xf*PG virulence factor, and in vitro assays using purified, recombinant *Xf*PG expressed in *E. coli*, Roper (2006) demonstrated that *Xf*PG was inhibited by the pear PGIP (Labavitch, 2006). In addition, Agüero et al. (2005) demonstrated that transgenic pear PGIP could be transported across a graft junction of genetically engineered grapevines into the aerial portions of wild-type scions.

The overall goal of the project is to develop transgenic grape rootstock lines that optimally express PGIPs that most effectively inhibit *Xf*PG. The project is designed to identify PGIPs that most effectively inhibit *Xf*PG and to optimally express that PGIP in grape. The optimization of expression includes the use of transformation components with defined Intellectual Property (IP) and regulatory characteristics, as well as sequences that result in the maximal expression of the PGIPs in rootstocks and the efficient transport of PGIP proteins through the graft junctions to inhibit *Xf*PG produced by the pathogen in scions.

## OBJECTIVES

1. Define a path for commercialization of a PD control strategy using PGIPs, focusing on IP and regulatory issues associated with the use of PGIPs in grape rootstocks.
2. Identify plant PGIPs that maximally inhibit *Xf* PG.
3. Assemble transcription regulatory elements, *Xf*-inducible promoters and signal sequences that maximize PGIP expression in and transport from roots.
4. Create PGIP-expressing rootstocks and evaluate their PD resistance.

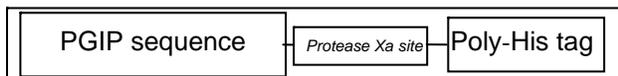
## RESULTS AND DISCUSSION

Research Objectives for Year 1-

A. Use existing pear PGIP-expressing grapes, test PD susceptibility of normal scions grafted to PGIP-expressing and -exporting roots.

Agüero et al. (2005) described the use of transgenic 'Thompson Seedless (TS)' and 'Chardonnay (Ch)' grapevines expressing the pear fruit PGIP in experiments that showed that (1) high level PGIP expression in grape tissues slows the development of PD symptoms in needle-inoculated vines and (2) PGIP expressed by a transgenic rootstock is transported via the xylem through the graft junction and into the stems of untransformed TS and Ch scions. The inoculation tests were performed on non-grafted transgenic vines, thus both root and shoot tissues would have been expressing the pear PGIP-encoding transgene (PcPGIP). However, to date, we have not shown that PGIP expressed in and translocated from roots into non-transgenic shoots can provide PD protection. Initial tests of this idea will use grafted portions of the transgenic TS and Ch vines that were generated by Dr. Agüero.

We (Greve and Labavitch) have maintained several of these transgenic grape lines and have increased the number of plants available vegetatively. In addition, we have confirmed that they still transport PGIP in the xylem sap. Zac Chestnut, a graduate student funded through this project, also has analyzed the plants by PCR to confirm PcPGIP expression. We are now planning our grafting strategy in consultation with Dr. Andy Walker. Over the Fall and Winter, we will generate plants with pear PGIP-expressing lines as rootstocks and non-transgenic lines as scions. These will be managed in the greenhouse over the Winter and the expression and transport of pear PGIP will be confirmed in early Spring. In Spring, we will do inoculations of these plants and comparable plants with non-transgenic roots and scions and follow the development of disease symptoms and, near the end of the incubation of these inoculated plants, use destructive sampling to determine the extent to which the *Xf* population has spread in the vine. We will use both the virulent "Fetzer" *Xf* strain and the "Fetzer" strain whose single PG-encoding gene was knocked out (Roper et al., 2007). Dr. Roper used the PG knock-out line to demonstrate that the pathogen's PG is a PD virulence factor.



**Figure 1.** Schematic diagram of constructs for PGIPs and PGIP-like proteins linked to the cleavable poly-His tag and expressed in *Arabidopsis*.

B. Express PGIPs in *Arabidopsis* and test for optimal inhibition of *Xf* PG.

Our strategy is to identify plant PGIPs that are maximally effective in inhibition of the *Xf* PG and identifying that optimal PGIP is another objective of the first year's work. *Arabidopsis* lines have been transformed through the floral dip technique using *Agrobacterium*-mediated transformation vectors (Clough and Bent, 1998). Transformation vectors were assembled using pCAMBIA vectors (<http://www.cambia.org.au/daisy/cambia/585.html>) that are free from intellectual property restrictions. We have successfully expressed sequences encoding five fruit PGIPs (one from pear and four from tomato) using the CAMBIA vector p1301, linking the PGIPs to the poly-His tag with an intervening protease Xa cleavage site (**Figure 1**). To prepare the transformation vectors, we removed the GUS coding region from p1301 and replaced that sequence with the PGIP full-length coding sequences including the signal peptide sequences for extracellular targeting. We have self-crossed these lines and obtained homozygous progeny identified by resistance to the selectable marker and by PCR for the transgenic sequences. We are evaluating whether the PGIPs we have expressed in *Arabidopsis* using the modified p1301 and the CaMV 35S promoter are active and have appropriate inhibition specificities, and we will continue to include the intervening protease Xa cleavage site so that, if it is necessary for obtaining active inhibiting protein, the poly-His tag used for affinity purification of the expressed PGIPs can be removed by protease Xa after purification. We also have identified three *M. truncatula* PGIP-like sequences and are in the midst of preparing *Arabidopsis* transformation vectors for their expression. For this proposal, we would like to add to the collection 5-6 other PGIP-like sequences that we have identified based on phylogenetic comparisons and charge comparisons of PGIPs (**Tables 1 & 2; Figure 2**).

The total charge on proteins is determined by pH and the sequences of each protein. The total charge of the proteins may serve as a general guide towards predicting whether PGs and PGIPs interact and therefore whether a specific PGIP is likely to

inhibit specific PGs. Certainly, the specific local chemistry is most important, but the total charge may serve as a deal breaker, so to speak, of the possibility that a PG interacts with a PGIP. Dan King (Taylor Univ.) is beginning to examine the *Xf*PG protein, as it is quite unusual. The charge of the *Xf*PG is unusually positive (+22). The only PGs King has come across with such positive charges are putative plant PGs, such as a grape PG (AAK81876). Interestingly, the grape PGIP is particularly positive as well (+19). Regardless of local chemistries, it would be hard to imagine the *Xf*PG and the grape PGIP proteins having a strong interaction for each other. From another point of view, the pear PGIP has shown some ability to inhibit the *Xf*PG, and the pear PGIP has a particularly small charge (+9). **Table 1** shows some examples of the total charges of PGs and PGIPs at six pH values.

**Table 1.** Total Protein Charge vs. pH of selected PGs and PGIPs

pH	PG									PGIP								
	<i>Xf</i> PG	<i>F. moniforme</i> PG	<i>A. niger</i> PGC	<i>A. niger</i> PGB	<i>A. niger</i> PGA	<i>A. niger</i> PG2	<i>A. niger</i> PG1	Tomato PG	Grape PG	Grape	Pear	Bean2	Bean1	Tomato	Arabidopsis 1	Arabidopsis 2	Apple	Kiwi
3.50	40.99	17.90	4.79	27.25	3.88	21.94	10.46	30.88	32.19	31.51	23.07	22.74	24.74	30.82	28.52	36.37	24.22	25.21
4.00	31.30	11.25	-9.53	18.45	-11.40	12.86	-2.13	25.51	27.26	24.98	16.07	17.62	19.62	24.43	21.86	29.49	17.42	18.85
4.50	22.24	5.44	-23.56	9.92	-26.43	4.08	-14.38	20.03	22.4	18.63	9.27	12.95	14.95	18.01	15.23	22.56	10.88	13.31
5.00	16.39	2.07	-32.08	4.67	-35.56	-1.35	-21.80	16.26	19.23	14.54	5.03	10.17	12.17	13.8	10.96	18.02	6.793	10.18
5.50	11.90	-0.13	-36.17	1.86	-39.77	-4.36	-25.41	13.54	17.15	11.84	2.64	8.55	10.55	11.14	8.405	15.22	4.344	8.333
6.00	6.79	-2.36	-38.46	-0.20	-41.90	-6.69	-27.53	10.68	15.14	9.21	0.73	7.13	9.129	8.733	6.236	12.75	2.216	6.637

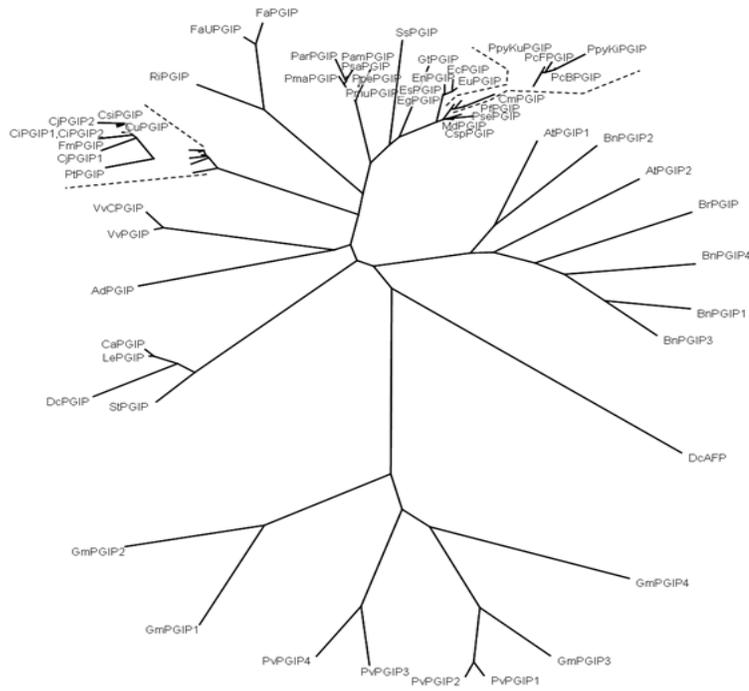
This total charge analysis has suggested to us that by examining PGIPs that have been identified in other plants, we can select PGIPs that are likely to be good candidates for inhibiting *Xf*PG and express these PGIPs in *Arabidopsis* for evaluation. To accomplish this, we have identified 52 non-redundant PGIPs in GenBank (**Table 2**) and we have evaluated their sequence similarities (**Figure 2**).

**Table 2.** List of PGIP sources.

Common name	Species	Protein <sup>a</sup>	Accession Number <sup>b</sup>
Common bean, pinto bean	<i>Phaseolus vulgaris</i> cv. Pinto	PvPGIP1	AJ864506
Common bean, pinto bean	<i>Phaseolus vulgaris</i> cv. Pinto	PvPGIP2	AJ864507
Common bean, pinto bean	<i>Phaseolus vulgaris</i> cv. Pinto	PvPGIP3	AJ864508
Common bean, pinto bean	<i>Phaseolus vulgaris</i> cv. Pinto	PvPGIP4	AJ864509
Soybean	<i>Glycine max</i> cv. Williams 82	GmPGIP1	AJ972660
Soybean	<i>Glycine max</i> cv. Williams 82	GmPGIP2	AJ972661
Soybean	<i>Glycine max</i> cv. Williams 82	GmPGIP3	AJ972662
Soybean	<i>Glycine max</i> cv. Williams 82	GmPGIP4	AJ972663
Ume (Japanese apricot)	<i>Prunus mume</i> cv. Dali	PmuPGIP	DQ364056
Chinese plum, Japanese plum	<i>Prunus salicina</i> cv. xiaohuangli	PsaPGIP	DQ364055
Common pepper	<i>Capsicum annuum</i> cv. arka abhir	CaPGIP	AM181174
Chinese cabbage	<i>Brassica rapa</i> subsp. <i>pekinensis</i>	BrPGIP	AY964100
Granny Smith apple	<i>Malus x domestica</i> cv. Granny Smith	MdPGIP	DQ185063
Peach	<i>Prunus persica</i>	PpePGIP	AY903218
Wild plum, American plum	<i>Prunus americana</i>	PamPGIP	AY883418
Flemish Beauty pear	<i>Pyrus communis</i> cv. Flemish Beauty	PcFPGIP	AY333105
Asian pear	<i>Pyrus pyrifolia</i> cv. Kinchaku	PpyKuPGIP	AY333103
Potato	<i>Solanum tuberosum</i> cv. Istrinskii	StPGIP	AY662681
Wild carrot	<i>Daucus carota</i>	DcPGIP	AY081214
False Spiraea	<i>Sorbaria sorbifolia</i>	SsPGIP	AF196947
Chinese Firethorn	<i>Pyracantha fortuneana</i>	PfPGIP	AF196929
Taiwanese Photinia	<i>Photinia serratifolia</i>	PsePGIP	AF196907
Oneseed Hawthorne	<i>Crataegus monogyna</i>	CmPGIP	AF196881
Mahaleb cherry	<i>Prunus mahaleb</i>	PmaPGIP	AF263465
Bartlett pear	<i>Pyrus communis</i> cv. Bartlett	PcBPGIP	L09264
Thale cress	<i>Arabidopsis thaliana</i> (Col.)	AtPGIP1	NM_120769
Thale cress	<i>Arabidopsis thaliana</i> (Col.)	AtPGIP2	NM_120770
Bowman's root	<i>Gillenia trifoliata</i>	GtPGIP	AF196915
Flowering Quince	<i>Chaenomeles speciosa</i>	CspPGIP	AF196871
Rape	<i>Brassica napus</i> cv. DH12075	BnPGIP1	AF529692
Rape	<i>Brassica napus</i> cv. DH12075	BnPGIP2	AF529694
Rape	<i>Brassica napus</i> cv. DH12075	BnPGIP3	AF531456
Rape	<i>Brassica napus</i> cv. DH12075	BnPGIP4	AF531457
Apricot	<i>Prunus armeniaca</i> cv. Marille Bauer	ParPGIP	AF020785
Cherry tomato	<i>Solanum lycopersicum</i> cv. VFNT	LePGIP	L26529
Kiwi	<i>Actinidia deliciosa</i> cv. Hayward	AdPGIP	Z49063
Iyokan	<i>Citrus iyo</i>	CiPGIP1	AB016205
Iyokan	<i>Citrus iyo</i>	CiPGIP2	AB016206
Rough lemon	<i>Citrus jambhiri</i>	CjPGIP1	AB013397
Rough lemon	<i>Citrus jambhiri</i>	CjPGIP2	AB015198
Sweet orange	<i>Citrus sinensis</i> cv. Hamlin	CsiPGIP	Y08618
Mikan	<i>Citrus unshiu</i>	CuPGIP	AB016204
Carrot	<i>Daucus carota</i> cv. Autumn King	DcAFP	AF055480
Red River Gum	<i>Eucalyptus camaldulensis</i>	EcPGIP	AF159168
Rose gum (Flooded gum)	<i>Eucalyptus grandis</i>	EgPGIP	AF159167
Shining gum	<i>Eucalyptus nitens</i>	EnPGIP	AF159171
Sydney Blue Gum	<i>Eucalyptus saligna</i>	EsPGIP	AF159170
Timor White Gum	<i>Eucalyptus urophylla</i>	EuPGIP	AF159169
Kumquat	<i>Fortunella margarita</i>	FmPGIP	AB020529
Trifoliolate orange	<i>Poncirus trifoliata</i>	PtPGIP	AB020528
Asian pear	<i>Pyrus pyrifolia</i> cv. Kikusui	PpyKiPGIP	AB021791
Red raspberry	<i>Rubus idaeus</i> cv. Autumn Bliss	RiPGIP	AJ620336

<sup>a</sup>Protein names given to match abbreviations used in **Figure 2**.

<sup>b</sup>GenBank nucleotide accession numbers.



**Figure 2.** Unrooted phylogenetic tree of PGIPs.

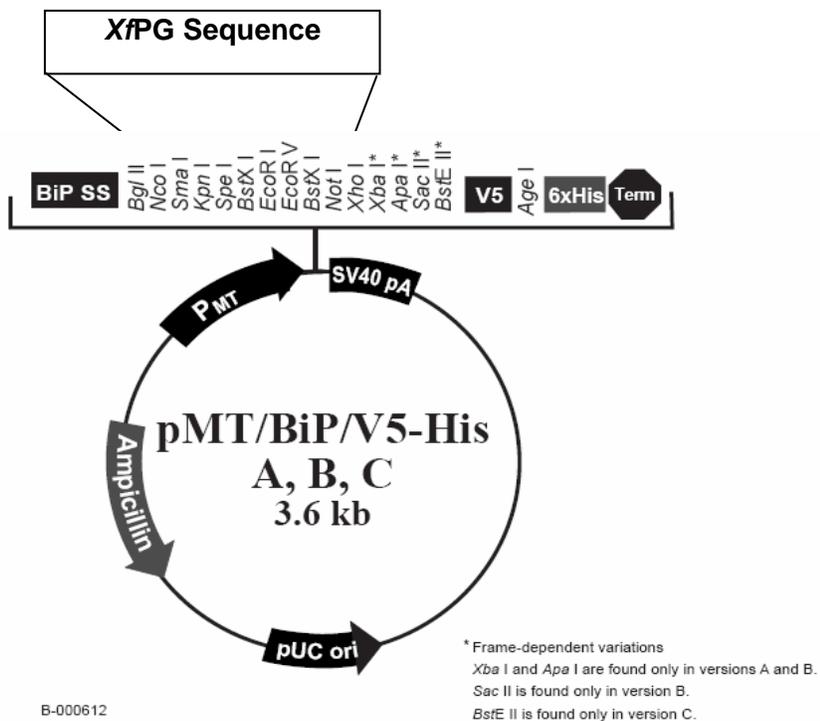
to characterize the expressed recombinant *Xf*PG. Once enzyme activity is confirmed, expression will be optimized by varying the concentration and time of exposure to the inducer followed by purification with nickel affinity chromatography.

**D. Evaluate IP and licensing status of the plant expression construct components for the PGIP-based rootstock strategy.**

For this project, we proposed to develop a PGIP plant transformation construct that optimally confers tolerance to PD AND that has maximum legal freedom-to-operate (FTO) with respect to the underlying intellectual property, thus providing the maximum potential to gain regulatory approval. The results of a preliminary IP audit of the original PGIP expression construct (see Agüero et al. 2005) revealed the DNA module contained a number of proprietary components including: the CaMV35S promoter and the nptII (kanamycin) selection marker patented by Monsanto Company, the GUS marker gene patented by CAMBIA, and the pear PGIP gene patented by the University of California. Licensing these proprietary technologies, particularly from the private sector, may prove to be a hurdle for commercial deployment. Furthermore, this analysis also noted the presence of the GUS-marker gene and His tags, which although necessary during the proof-of-concept phase, may compromise downstream regulatory approval of commercial transgenic plants. To advance this project beyond

**C. Optimally express *Xf* PG, using recombinant protein expression systems**

Since previous *E. coli* PG over-expression studies resulted in significant amounts of protein but little activity (Roper, 2006), we (with co-PI Booth at Texas State U. - San Marcos) will utilize a *Drosophila* expression system. For expression in *Drosophila*, we probably want two versions of the *Xf*PG, if possible; one with the antibody epitope and one without (in case the epitope causes trouble). The *Xf*PG gene will be ligated into pMTBiP/V5-HisA (Figure 3) and transformed into *Drosophila* Schneider 2 cells using protocols provided by Invitrogen and modified by Prof. Booth. Existing cloned *Xf*PG will be reamplified by PCR to create an NcoI site at the N terminus and probably an XhoI site at the C terminus. The sequence of the *Xf*PG will be checked and the C terminus of the inserted *Xf*PG will be modified by PCR to produce an in-frame fusion to C terminal additions (V5 (the epitope for detection) + 6xHis (for affinity purification with Ni)) without an intervening stop codon. A second expression construct will also be prepared that includes the *Xf*PG stop codon before the C terminal additions in case they interfere with protein folding. More detailed assays of active preparations (Gross, 1982; Roper et al., 2007) will be done at UC Davis



**Figure 3.** Schematic diagram of the pMTBiP vector used to transform *Drosophila* cells for *Xf*PG expression

the “proof of concept” stage, the Public Intellectual Property Resource for Agriculture (PIPRA) is conducting an in-depth analysis of all component technologies that will be integrated into the PGIP gene construct as well as the enabling technologies required to transfer the PGIP construct into a grape rootstock. This analysis also will assess the likelihood that components of the PGIP gene construct will be able to gain regulatory approval for commercialization.

## CONCLUSIONS

1. Because of the limited time that the funds have been available for this project (2 months), this report documents only the activities accomplished in that time.
2. XfPG protein has an unusually high positive charge at the pH expected in plant tissue.
3. The sequences for currently available PGIPs have been collected and compared so selections of additional PGIPs to be expressed in *Arabidopsis* represent the diversity of PGIP sequences.
4. The total charge of the PGIP proteins can be used as an indicator of the likelihood of interaction with and inhibition of the XfPG.

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## FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

# THE PIT MEMBRANE BARRIER TO *XYLELLA FASTIDIOSA* MOVEMENT IN GRAPEVINES: BIOCHEMICAL AND PHYSIOLOGICAL ANALYSIS

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**Reporting Period:** The results reported here are from work conducted November 2007 to October 13, 2008.

## ABSTRACT

The overall goal of the work in this project is to characterize the role of the pit membranes (PMs) of grapevine xylem vessels in limiting the systemic movement of *Xylella fastidiosa* (*Xf*). Work carried out in the project in previous years has made use of monoclonal antibodies that recognize specific cell wall polysaccharides (pectins with varying degrees of methyl esterification and xyloglucan [XyG]) and this work has revealed the presence of these polysaccharides in grapevine pit membranes. The demonstration that these polysaccharides are present is consistent with earlier observations indicating that polygalacturonase (PG) and endo- $\beta$ -1,4-glucanase (EGase) are used by the pathogen to digest pit membranes as its population expands and spreads systemically because these enzymes would be expected to digest pectins and XyG. We now report on tests showing that PG and EGase introduction into grape stem explants is sufficient to open the PMs so that *Xf* can freely traverse the stem explants containing several internodes. In addition, several groups, including our own, have reported that tyloses, induced barriers to pathogen movement in the grapevine xylem system, also are important in limiting Pierce's disease (PD) spread but are also an important cause of the breakdown in water movement through infected vines. Other studies have suggested that ethylene synthesis by grapevines may be an important factor in PD development and tylose formation. In this report we describe an experiment that may pave the way for testing the extent to which ethylene produced by an infected vine is responsible for the full development of PD symptoms.

## INTRODUCTION

For several years, Labavitch and the listed collaborators have been testing a model proposed to describe the development of Pierce's disease (PD) in grapevines (Labavitch et al., 2001, 2002; Labavitch and Matthews, 2003; Labavitch et al., 2004, 2005; Pérez-Donoso, 2006; Pérez-Donoso et al., 2006). Findings reported in the last four PD Symposia indicate that PG and EGase enzymes, likely produced by *Xf* resident in xylem water-conducting cells (also Roper et al. 2007) are important contributors to the escape of the pathogen from the vessels into which it has been introduced by GWSS, thus initiating its systemic spread through the vine and the subsequent development of PD symptoms. However, observations made only in the past year have suggested that seasonal changes in normal grapevine development may also contribute to the systemic spread of *Xf*, beginning in late Spring. These observations may be linked to those made by Rost, Matthews et al. (Thorne et al., 2006) suggesting that relatively long xylem conduits, likely to be of primary xylem origin, may allow relatively long distance passage (i.e., the length of two-three internodes) of *Xf* into grape leaves. While this pathway is not likely to facilitate long distance systemic spread of the pathogen through stems, it may facilitate rapid movement from stems into which *Xf* has been introduced, into leaves where disease symptoms then become evident. Work in this project will examine aspects of these reports, with a strong focus on factors that might affect the integrity of the pit membranes in grapevine xylem water conduits. In this report, we report on work that may have identified a way to test the role of ethylene produced by *Xf*-infected vines in the development of tyloses, vessel blockages that are likely to be more permanent barriers of *Xf* movement in grapevines which also are barriers that reduce water movement and, thus, may play an important role in the vine decline that accompanies PD.

## OBJECTIVES

1. To characterize the biochemical action of *Xf* EGase, *in vitro* and *in planta* and determine if it is inhibited by plant proteins that have been identified as xyloglucan-specific endoglucanase (EGase)-inhibiting proteins.
2. To examine the full range of effects on grapevine pit membrane porosity that result from introduction of cell wall-degrading polygalacturonase (PG) and EGase.
3. To repeat our 2005 observations of a late Spring, dramatic increase in the porosity of grapevine pit membranes.

## RESULTS AND DISCUSSION

*Objective 2.* In previous PD Research Symposia (Labavitch et al., 2006, 2007), we reported observations suggesting that the *Xf* PG and EGase play important roles in digestion of PMs so that the pathogen can spread through infected grapevines *via* the xylem. However, we had not shown that the combined actions of the two pathogen enzymes did, in fact, open a pathway that *Xf* could use to move through PMs. We have now used our grapevine xylem flushing system (Labavitch, 2006) to introduce PG and EGase to the lower (proximal) end of explanted stems and then followed enzyme introduction with cells of the *Xf* 'Fetzer' strain. Then the stem was continuously flushed with water and fractions of the water eluted from the distal stem end were collected. These fractions were then assayed for *Xf* presence by PCR. This experiment was replicated and in each case, PCR revealed the pathogen's presence in collected fractions. Thus, the PG and EGase open up PMs so that they no longer block pathogen movement.

*A continuing objective.* Remaining from another project that has ended and also to the overall interest in barriers that serve to limit pathogen spread that is a theme of this current project was an experiment to determine whether the production of ethylene plays a role in PD symptom development in *Xf*-inoculated grapevines. A key to performing that experiment has been the need to have a way to block the grapevine's responses to ethylene. Earlier, inconclusive tests were based on spray applications of the ethylene receptor-blocking compound 1-methylcyclopropene (1-MCP). These did not suppress the vine's ethylene response. However, earlier this year we obtained a new, sprayable formulation of 1-MCP. Postdoctoral researcher (now Asst. Prof.) Qiang Sun had reported that grapevines respond to Winter pruning by producing tyloses in vessels near to the pruning cuts (within two-three cm of the cuts), thereby blocking the vessels and showed that this was a response to the ethylene made by the cut grapevine stem tissues (Sun et al., 2006, 2007). We therefore carried out a test of the ability of the new 1-MCP spray to block pruning-induced tylose formation. Sets of 'Chardonnay' vines were used for the test. One set of six vines (the test vines) was sprayed with a solution of the new 1-MCP formulation at a concentration calculated to provide a 1-MCP concentration of 200 ppm. The other set of vines (control vines) was not treated. On the following day, the test vines were again sprayed with 1-MCP and all control and test vines were pruned. Dr. Sun's study (Sun et al., 2006) had reported extensive tylose formation in pruned vines within one week of pruning. Therefore, seven days after pruning, the terminal three cm of each of the pruned stems in the control and test vines was removed and fixed for histochemical examination of tylose development. Sections from the distal five mm of these stem explants were cut, stained with toluidine blue, and examined with the light microscope (Shackel and Labavitch, 2006). These observations indicated that there was extensive tylose development near the tips of pruned stems that had not been treated with 1-MCP and that the 1-MCP treatment had dramatically reduced tylose formation.

This result *per se* demonstrates that grapevine responses to ethylene that affect vessel function can be inhibited. The pruning-induced tylose formation is a response to ethylene produced by wounded grapevines. However, we presume that the inhibitor will also influence a vine's response to infection-promoted ethylene. Thus, we are now in a position to test the possible role of inoculation/infection-induced ethylene production in PD symptom development. This test will be carried out in the Spring/Summer, 2009.

## CONCLUSIONS

1. The introduction of pure PG and EGase, two enzymes produced by *Xf* within the grapevine xylem system, into grapevine stem explants will introduce a pathway through the vessels (presumably *via* their pit membranes) that permits free passage of the pathogen.
2. 1-MCP sprays can block grapevine responses to vine-produced ethylene, paving the way to studies that test the role of ethylene in PD symptom development.

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#### **FUNDING AGENCIES**

Funding for this project was provided by the CDFR Pierce's Disease and Glassy-winged Sharpshooter Board.

**DO CELL WALL STRUCTURES LIMIT *XYLELLA FASTIDIOSA* DISTRIBUTION IN INOCULATED,  
PIERCE'S DISEASE SUSCEPTIBLE AND RESISTANT GRAPEVINES?**

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**Reporting Period:** The results reported here are from work conducted August 15, 2008 to October 13, 2008.

**ABSTRACT**

The development of Pierce's disease (PD) in grapevines depends, at least in part, on the ability of the *Xylella fastidiosa* (*Xf*) pathogen to spread from the point of infection and ultimately develop a population that is systemic in the infected plant. This systemic spread of the pathogen is limited by the pit membranes (PMs) that separate one xylem water conduit from its neighbors and, perhaps, by the production of tyloses and polysaccharide-rich gels that are produced in and block xylem cells following infection. The work in this proposal will describe the polysaccharides of PMs, tylose cell walls and gels by using immunohistochemical tools and determine whether situations in which grapevine infection with *Xf* does not result in PD are situations in which PM integrity is not disrupted by the pathogen so that pathogen spread is limited by intact PMs and/or production of tyloses or gels.

**INTRODUCTION**

The introduction of *Xylella fastidiosa* (*Xf*) to grapevine xylem tissues often results in Pierce's disease (PD) and, ultimately, to vine death. Several studies over the past five years have indicated that the expansion of the locally introduced, relatively small population of *Xf* cells throughout the vine, creating a systemic infection, is the cause of vine death (Krivanek and Walker, 2005; Labavitch, 2007; Lin, 2005; Lindow, 2006a, b, 2007a, b; Rost and Matthews, 2007). The individual elements of the water-conducting tubes in xylem are separated from one another by the so-called pit PMs, primary cell wall "filters" whose meshwork is too small to permit *Xf* passage (Labavitch et al., 2004). Thus, it has been generally believed that the pathogen uses cell wall-degrading enzymes (CWDEs) to digest the polysaccharides of the PMs, opening the primary cell wall barrier and permitting the systemic expansion of the pathogen population.

The genome of *Xf* contains genes encoding polygalacturonase (PG) and a few  $\beta$ -1,4-endo-glucanases (EGase), CWDEs that digest cell wall pectin and xyloglucan polymers, respectively. Such enzymes are good candidates for pathogen factors that facilitate *Xf* systemic movement and PD development. This supposition has been supported by several studies performed over the past several years. Roper et al. (2007) reported the generation of a PG-deficient strain of *Xf* and showed that it was unable to cause PD symptoms, thus identifying the pathogen's PG as a PD virulence factor. Labavitch et al. (2006) reported that introduction of PG and EGase into explanted stems of uninfected grapevines caused the breakage of the PM cell wall network and, subsequently (Labavitch, 2007), that substrates for these enzymes, pectins and xyloglucans, are present in grapevine PMs.

Research in the laboratory of the PI on the present proposal has shown that PG-inhibiting proteins (PGIPs) limit the development of PD in grapevines (Agüero et al., 2005). Research in Cooperator Steve Lindow's program has focused on the role of a diffusible signal factor produced by *Xf* in controlling the pathogen's expression of virulence functions that affect whether the pathogen spreads systemically in grapevines and causes PD **or does not** (Lindow, 2007a, b). Cooperator Andy Walker and his colleagues have identified a grapevine quantitative trait loci (QTL) that contains the PD resistance (*PdR1*) locus (Walker and Riaz, 2007) that eventually will be deployed in grapevine genotypes that will have enhanced resistance to PD. Walker, Lindow and Cooperator Hong Lin (Lin, 2007), have all made use of natural variations in the PD resistance/susceptibility of different grape germplasm in order to understand the factors that influence *Xf* movement in grapevines and, therefore, PD development. It is reasonable to assume that differential PD susceptibility of grape genotypes is determined by (1) genetic variation in PM barriers to pathogen movement; differences in porosity, polysaccharide composition or susceptibility to *Xf*'s CWDEs or/and (2) the post-infection deployment of tyloses and gels, factors that could restrict the pathogen to the few vessels into which it has been introduced.

While the production of gels and tyloses in response to infection has been examined in several programs (e.g., Lin, 2005; Stevenson et al., 2004), this has not been done using techniques that can specifically identify the polysaccharides that make

up the gels and tylose walls. The utility of immunohistochemical techniques in identifying the polysaccharides of grapevine PMs has recently been demonstrated by Co-PI Sun (Labavitch, 2007). These techniques may contribute to an understanding of the differences in xylem water-conducting cell structures that have been thought by many to hold the key to grapevine resistance to PD. This proposal will use these techniques in several systems where differential resistance to PD have been shown in order to obtain the detailed structural and spatial information that may help explain why some grapevine genotypes are resistant to PD while others are not. It is important to note that while the research in this program is likely to enhance our understanding of grapevine PD resistance it will not lead immediately to new approaches to PD control.

## OBJECTIVES

1. Determine if the development of xylem obstructions (tyloses and pectin-rich gels) and the polysaccharide structure and integrity of pit membranes are affected by *Xf* inoculation of grapevines transformed to express the PGIP from pear and other plant species in rootstocks and in scions.
2. Determine whether there are differences in pit membrane porosity or polysaccharide structure between resistant and susceptible grapevines. To what extent are these PM characteristics and the production of tyloses and gels modified by introduction of *Xf* to PD-resistant and -susceptible genotypes?
3. Determine the extent to which changes in pathogen virulence resulting from altered production of diffusible signal factor (DSF) correlate with the appearance of tyloses, gels and damaged PMs in inoculated vines.
4. Determine whether the impacts of inoculation on PM integrity and the production of vascular system occlusions identified in tested greenhouse-cultured vines also occur in infected vines growing in the field.

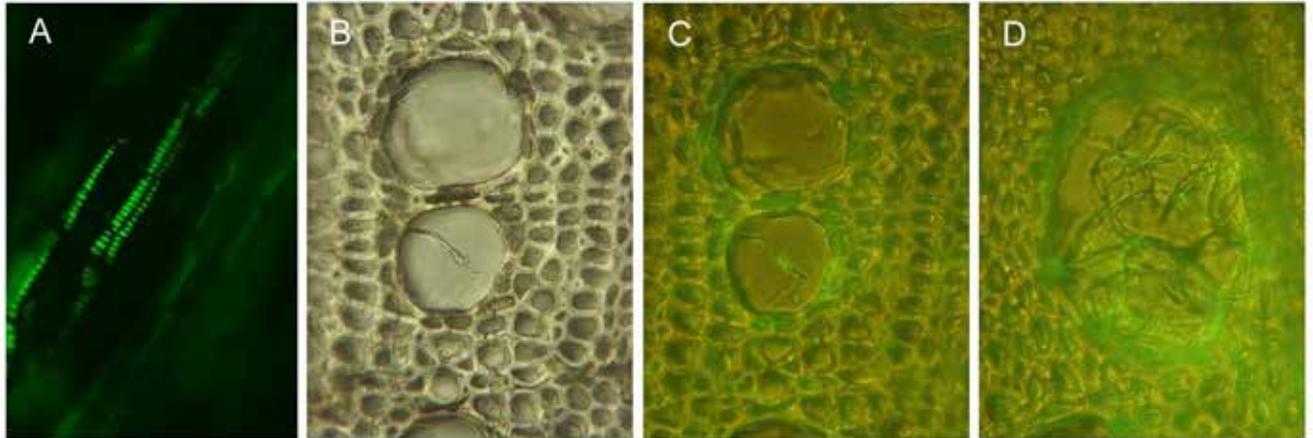
## RESULTS AND DISCUSSION

The primary work on this project will be performed at the University of Wisconsin-Stevens Point, where Co-PI Dr. Qiang Sun is an Asst. Professor. The project is currently funded only for the first of the two years of work that were proposed in January, 2008. Unfortunately, because Dr. Sun did not have a history of funding from the CDFA at his university, he could not be "advanced" research support from his Office of Research, thus his full effort at Wisconsin could not be started until the sub-contract could be established with UC Davis and UCD could not establish that until funding was available from the CDFA (mid-August). Nevertheless we have made considerable progress, primarily in developing the techniques required for addressing our four objectives in several grape genetic backgrounds. We have shown that PMs of these different grapes contain homogalacturonans, the target cell wall substrate for the *Xf* PG "PD virulence factor," setting the stage for the detailed studies identified under Objective 1. We have also begun describing variations in the gross vascular system architectures of the different grape germplasms.

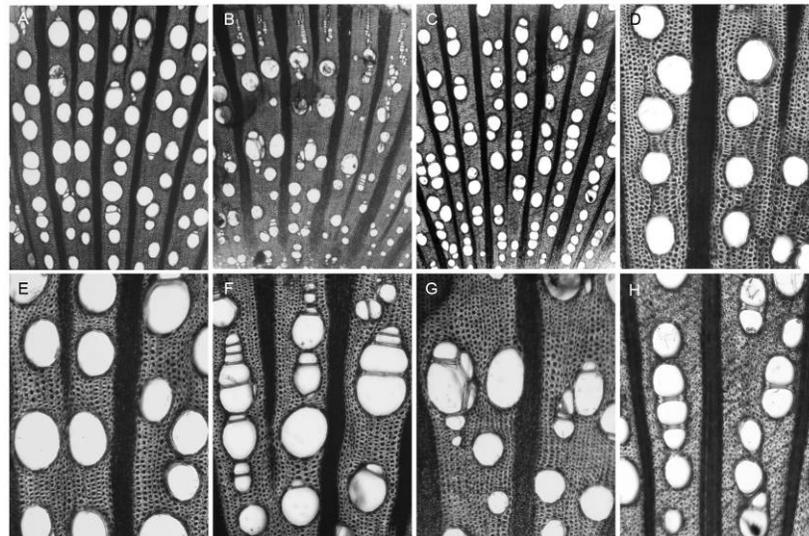
The following five grape species/cultivars of different susceptibilities to PD have been selected for our experiment: *Vitis vinifera* cv. Chardonnay (susceptible), *V. vinifera* cv. Riesling (less susceptible), *Muscadinia rotundifolia* (highly tolerant), 89-0908 and 89-0917 (both are selected from *V. arizonica* x *vinifera* and are resistant to PD). We have succeeded in using two monoclonal cell wall antibodies (JIM 5 and JIM 7) to distinguish pectin wall components of the living parenchyma cells adjacent to water-conducting vessels of the secondary xylem tissue (wood) in Chardonnay and Riesling (**Figure 1**). Our results indicate that homogalacturonans (HGs) with different levels of methyl esterification are present in PMs between vessels as well as between vessel and axial parenchyma cells (**Figure 1A-C**). Cell walls of developing tyloses (**Figure 1D**) and inner secondary wall of xylem fibers (**Figures 1C and D**) also contained the HGs. In our next step, the protocols established will be used to identify HGs in the other grape species/cultivars, particularly in PMs that are thought to be the barriers that should limit systemic spread of *Xf* in grapevines. Some other wall antibodies (CCRC-M1 and 2F4 etc.) will also be tested to detect other possible cell wall components (pectins and xyloglucans) in all the five grape groups. Our ultimate goal is to use these tools for localized cell wall component visualization to determine whether the post-inoculation integrity of pit membranes differs between PD-susceptible, -tolerant and -resistant grape germplasm (Objectives 1 to 3).

Understanding vessel morphology is essential to elucidate any possible differences in susceptibility of these grape groups, thus we have also made some anatomical analyses of secondary xylem. Our results indicate that there are major differences among these groups in the arrangement, density and diameter of vessels. In Riesling (**Figure 2A and E**) and Chardonnay (**Figure 2D**), vessels are relatively evenly distributed in xylem, are mostly solitary and have less difference to one another in size. Vessel density is also close in these two cultivars (34.6/mm<sup>2</sup> in Chardonnay and 30.7/mm<sup>2</sup> in Riesling). However, vessel diameters in Chardonnay (68.2 µm) are generally smaller than those in Riesling (84.7 µm). In 89-0917, vessels are not uniformly distributed in xylem tissue with a density of 42.8/mm<sup>2</sup>. They are usually solitary or in multiples of 3-5 cells. Solitary vessels are usually larger while most vessels in multiples are much smaller. Vessels in 89-0908 are more or less evenly spread through the secondary xylem and usually form radial chains of 3-6 cells. Vessels have an average diameter of 66.4 µm, but individual vessels show large size differences. In *Muscadinia rotundifolia*, vessels usually forms radial chains of 2-5 cells and individual vessel sizes (56.5 µm diameter, at average) vary less than in some other groups. The vessel density is highest (53.1/mm<sup>2</sup>) among the five groups. Morphological analysis of pits and pit membranes on lateral vessel walls has also been made (**Figure 3**). Two types of pits (intervessel pits and vessel-parenchyma pits) are common in all the five grape groups (**Figure 3A**). As for vessel parenchyma pits, PMs are intact in all groups (**Figure 3E**), except for 89-0917 in which PMs that are broken in a relatively regular pattern are common. No other obvious differences have been found in the

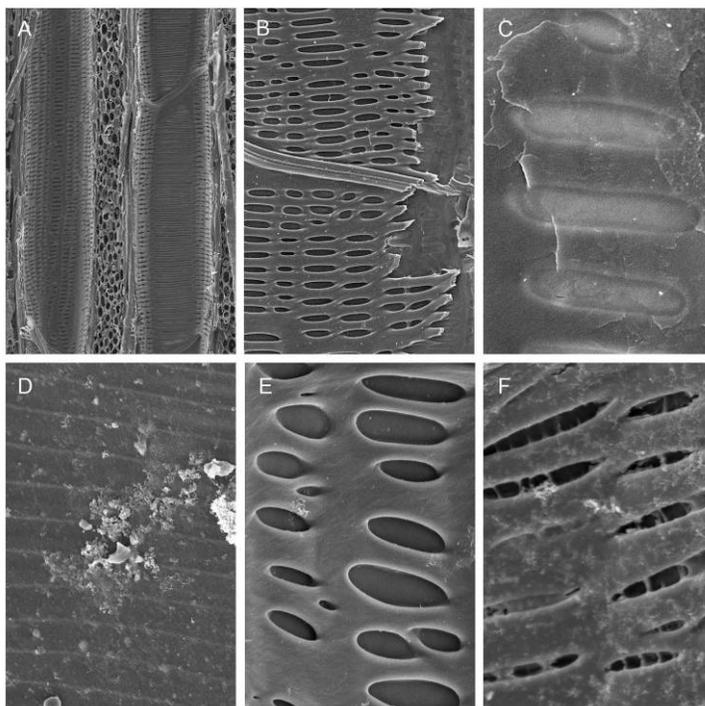
structures and distributional patterns of pits on vessel walls (**Figure 3B-D**). Further investigation is still needed to clarify any interconnection between these anatomical characteristics and susceptibility. As for vessel-parenchyma pits, PMs are intact in all the groups (**Figure 3E**) except 89-0917 in which PMs that are broken in a relatively regular pattern are common.



**Figure 1.** Cell wall composition revealed by JIM 5 (A) and JIM 7 (B-D) in Chardonnay, a susceptible cultivar. JIM 5 and JIM 7 can be used to distinguish weakly methyl esterified homogalacturonans (Me-esterified HG) and heavily Me-esterified HGs, respectively. A. Green fluorescence from the rows of pit membranes (PMs) between a vessel and axial parenchyma cells indicates the presence of weakly Me-esterified HGs. B. Image of xylem tissue under transmission illumination, showing vessels, parenchyma cells surrounding vessels, and fiber cells. C. Image of xylem tissue under both transmission light and fluorescent light. Green fluorescence is emitted from parenchyma cell walls, PMs between vessel and parenchyma cells and fiber inner wall layers, indicating the presence of heavily Me-esterified HGs in these locations. D. Transverse section of a vessel containing tyloses, showing HG presence in tylose cell walls.



**Figure 2.** Differences in the distribution, arrangement and sizes of vessels among grapes of different PD susceptibilities. A and E. Riesling. Vessels are larger in diameter than other grape groups and mostly solitary, occasionally in groups of up to 3 vessels. B and G. 89-0917 grape. Vessels are usually in multiples of 3 – 5 and individual vessels differ in size. C and H. *Muscadinia rotundifolia*. Vessels of similar size are usually in radial chains of 3-5 cells. D. Chardonnay. Vessel arrangement is similar to Riesling. F. 89-0908 grape. Radial chains of 3-6 vessels are common and vessels differ in size.



**Figure 3.** Pits and pit membranes in lateral vessel walls in grapes of three different susceptibilities. A-C. *Muscadinia rotundifolia* (highly resistant grape species). A. Two types of pits are present on vessel lateral walls: vessel-axial parenchyma pits (the vessel on the left) and intervessel pits (the vessel on the right). B. Vessel-axial parenchyma pits. Vessel secondary walls have been partially peeled on the right. C. Pit membranes between vessel and axial parenchyma cells are in a ladder-like arrangement (scalariform) along the vessel axis. D and E. Riesling (less susceptible *vinifera* cultivar), showing that scalariform intervessel pit membranes are arranged tightly. E. Bordered vessel-axial parenchyma pits, showing intact pit membranes. F. Bordered vessel-axial parenchyma pits in tolerant 89-0917 grape (*V. vinifera* x *arizonica*, tolerant grape). Many pit membranes are broken in a more or less regular way.

## CONCLUSIONS

1. Because of the limited time that the funds have been available for this project, this report documents only a small portion of the work planned for year 1.
2. Immunohistochemical studies indicate the presence of simple homogalacturonan pectins in the cell wall fabric of pit membranes from PD-susceptible, -tolerant and -resistant grape grapevines.
3. Xylem vessel diameters and distribution patterns of vessels within the secondary xylem tissues of PD-susceptible, -tolerant and -resistant grape grapevines are described.

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# BACTERIAL POPULATIONS IN GRAPEVINES APPARENTLY RESISTANT TO PIERCE'S DISEASE OF GRAPEVINE

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

## ABSTRACT

Specific strains of the bacterium *Xylella fastidiosa* are economically important plant pathogens and cause scorch diseases in a variety of plants. One of these strains causes a scorch disease known as Pierce's disease (PD) of grapevine. This disease has caused significant disruption to the wine industry centered in the Temecula, California region; at the height of the most recent PD epidemic in the late 1990's, 25% of the grapevines in this area were lost before emergency quarantine and control measures could be instituted. Under these circumstances, the 2006 discovery of a population of apparently PD-resistant grapevines in the area was of particular interest. The vines were all located in a single vineyard, which had total PD-related losses of approximately 10%, while a neighboring vineyard suffered a nearly 100% loss of the same variety. In addition, a similar phenomenon was observed in a grapevine population located on the Agricultural Operations grounds at the University of California, Riverside. While the cause of this apparent resistance is unknown, one possible explanation for this resistance is that it is being conferred by bacteria present in resistant vines but not in susceptible vines. In order to test this hypothesis, cane samples from both the apparently susceptible populations and the apparently resistant populations were surface sterilized and plated onto standard microbiological media. Any observed bacterial growth was diluted into standard liquid media and then streaked out in order to obtain pure cultures, which were identified using 16S sequencing. Current results show that multiple *Paenibacillus* species are present more often in asymptomatic plants than in symptomatic plants at both locations.

## INTRODUCTION

Specific strains of the bacterium *Xylella fastidiosa* (*Xf*) cause disease in almonds, grapevines, and a variety of other economically important plants (Davis 1978, 1980 and Purcell 1999). *Xf* is spread by the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, formerly known as *H. coagulata* (Redak et al. 2004 and Takiya et al. 2006).

In grapevines, one strain of this bacterium is the cause of Pierce's disease (PD). Since the preferred host of GWSS is citrus, vineyards close to a citrus grove are at increased risk for the development of PD (Perring et al. 2001). In addition, Chardonnay vines are known to be more susceptible to PD than other varieties (UC IPM). The Weaver vineyard is planted with Chardonnay vines and is immediately across from two citrus groves, meaning that it is at high risk of developing PD. However, while adjacent Chardonnay vineyards suffered catastrophic crop failure, the Weaver vineyard had a PD-related loss of far less, approximately 10%. This observation was of special interest since many of the plants in this vineyard were old enough to have survived the initial PD epidemic that occurred after the GWSS was accidentally introduced into California. The Agricultural Operations vineyard at the University of California, Riverside contains both symptomatic and asymptomatic Chardonnay vines in close proximity. These vines are younger than the ones at the Weaver Vineyard.

One possible explanation for this phenomenon is that it is being conferred by bacterial endophytes that live inside the apparently resistant plants but not in the more susceptible plants. The endophytic bacterium, *Curtobacterium flaccumfaciens*, has already been shown to confer resistance to *Xf* in sweet orange plants (Lacava et al. 2004).

To test this hypothesis, cane samples from asymptomatic and symptomatic grapevines at both locations were surface-sterilized and then plated on microbiological media. The genus of any resulting bacterial growth was then identified using 16S gene sequencing. The 16S gene has been widely used to classify unknown organisms (Turner 1997). Because this gene evolves very slowly, it is most useful for classifying organisms at the genus level, but not at the species or subspecies level (Weisburg et al. 1991). Even so, it is widespread practice to include a species name when identifying bacteria based on this sequence. These designations can be considered putative in nature.

## OBJECTIVES

The primary goal of this research was and continues to be to test the initial hypothesis through isolating bacterial endophytes from symptomatic and asymptomatic grapevines at both locations and using 16S analysis to identify them.

## RESULTS AND DISCUSSION

A comparison of the endophytes isolated from the vines at both locations showed that members of the genus *Paenibacillus* occurred more frequently in asymptomatic vines than in symptomatic vines (**Table 1**) (Parker 2008). This observation was

of special interest since it is already known that *Paenibacillus polymyxa* can confer resistance to the bacterial plant pathogen *Erwinia carotovora* in gnotobiotic *Arabidopsis thaliana* plants (Timmusk and Wagner 1999). It is possible that *Paenibacillus* could be playing a similar role inside the asymptomatic grapevines, since one of the *Paenibacillus* isolates tested in the laboratory was found to retard the growth of the PD strain of *Xf* in both co-culture and in grapevines (A. Arora, personal communication). In addition, it has recently been shown that other members of *Paenibacillus* can reduce the growth of *Xf* or even clear it altogether on microbiological media (Kirkpatrick and Wilhelm 2007).

In addition, the presence of certain endophytes within the plants (most notably *Bacillus*) appeared to be dependent on the time of year the plants were sampled (Parker 2008). In the Weaver vineyard, *Bacillus* was most commonly isolated in May. However, in the Agricultural Operations vineyard, *Bacillus* was most frequently in May and October (see **Tables 2 and 3**). The reasons for this are not yet clear.

**Table 1.**

Code	Genus	Bit Score	E-value	Symptomatic Plant	Date Collected
45V16 1F C1	<i>Achromobacter</i>	2039	0	No	4-May-06
45V16 2D C1	<i>Bacillus</i>	863	0	No	4-May-06
45V16 2E C1	<i>Bacillus</i>	2771	0	No	4-May-06
46V16 2D C1	<i>Bacillus</i>	500	7.00E-138	No	4-May-06
46V19 1C C7	<i>Bacillus</i>	2454	0	No	27-Jul-06
46V19 2D C1	<i>Bacillus</i>	2736	0	No	4-May-06
46V19 2F C1	<i>Bacillus</i>	2605	0	No	4-May-06
47V1 1A C1	<i>Paenibacillus</i>	2789	0	No	4-May-06
47V1 1B C1	<i>Bacillus</i>	1844	0	No	4-May-06
47V1 1C C2	<i>Bacillus</i>	2389	0	No	18-May-06
47V1 1E C1	<i>Bacillus</i>	1162	0	No	4-May-06
47V1 1F C1	<i>Bacillus</i>	2365	0	No	4-May-06
47V1 2B C1	<i>Paenibacillus</i>	293	1.00E-75	No	4-May-06
47V1 2C C3	<i>Bacillus</i>	2692	0	No	31-May-06
47V3 1B C1	<i>Bacillus</i>	2351	0	No	4-May-06
47V3 1C C2	<i>Bacillus</i>	1015	0	No	18-May-06
47V3 1E C12	<i>Bacillus</i>	401	2.00E-108	No	5-Oct-06
47V3 2D C13	<i>Bacillus</i>	1009	0	No	19-Oct-06
47V3 O C3	<i>Planococcus</i>	2609	0	No	31-May-06
47V8 1A C9	<i>Bacillus</i>	2561	0	Yes	24-Aug-06
47V8 R6	<i>Bacillus</i>	2627	0	Yes	4-May-06
48V10 1B C1	<i>Bacillus</i>	2591	0	No	4-May-06
48V10 1B C2	<i>Bacillus</i>	2407	0	No	18-May-06
48V15 1C C3	<i>Bacillus</i>	1084	0	No	31-May-06
48V15 1D C2	<i>Bacillus</i>	2605	0	No	18-May-06
48V15 2A C7	<i>Bacillus</i>	910	0	No	27-Jul-06
48V19 2F C2	<i>Bacillus</i>	2379	0	No	18-May-06
49V9 1A C2	<i>Bacillus</i>	979	0	No	18-May-06
49V9 1C C2	<i>Bacillus</i>	2533	0	No	18-May-06
49V9 1C C3	<i>Bacillus</i>	2670	0	No	31-May-06
49V9 1D C3	<i>Bacterium</i>	880	0	No	31-May-06
49V9 1F C1	<i>Bacillus</i>	1203	0	No	4-May-06
49V9 1F C2	<i>Bacillus</i>	2660	0	No	18-May-06
49V9 1F C7	<i>Bacillus</i>	1154	0	No	27-Jul-06
49V9 2B C1	<i>Bacillus</i>	2577	0	No	4-May-06
49V9 2C C1	<i>Bacillus</i>	650	0	No	4-May-06

**Table 2.** Presence of *Bacillus* in Grapevines in the Weaver Vineyard, by Month.

	May	June	July	August	September	October	November	December
Plant								
45V16	X							
46V16	X							
46V19	X		X					
47V1	X							
47V3	X					X		
47V8				X				
48V10	X							
48V15	X		X					
48V19	X							
49V9	X							

**Table 3.** Presence of *Bacillus* in Grapevines in Agricultural Operations, by Month.

	May	June	July	August	September	October	November	December
Plant								
A-4	X					X		
B-3	X		X					
C-1	X		X			X		
D-7						X		
E-1								
F-15						X		
G-6	X					X		
H-11	X			X				
I-6						X		
J-9	X			X		X		

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# **XYLELLA FASTIDIOSA TRANSMISSION BY GLASSY-WINGED SHARPSHOOTERS AND SMOKETREE SHARPSHOOTERS FROM ALTERNATE HOSTS TO GRAPEVINES**

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

## **ABSTRACT**

The goal of this project is to evaluate the importance of many common weed, agricultural, and cover crop plants that are found in close proximity to vineyards as sources of *Xylella fastidiosa* (*Xf*) from which glassy-winged sharpshooter (GWSS) and smoketree (STSS) sharpshooters can acquire and transmit *Xf* into grapevines. In our studies, we were unable to recover *Xf* from bell pepper, cotton, sunflower, horseweed, annual fescue 'Zorro', birdsfoot trefoil, or sudangrass plants. *Xf* was successfully isolated from needle-inoculated alfalfa, basil, lima bean, tomato, goosefoot, Spanish broom, tree tobacco, annual ryegrass, black mustard, Blando brome, New Zealand white clover, Hykon rose clover, cowpea, fava bean, field pea, meadow barley, and California red oats. We have determined that STSS can transmit *Xf* between fava bean plants, between alfalfa plants, and from alfalfa to grapevines. We were unable to detect successful transmission from fava bean-to-grapevine, tomato-to-tomato, tomato-to-grapevine, cowpea-to-cowpea, or cowpea-to-grapevine. All 36 STSS died on goosefoot before the end of the 48-hr acquisition access period, indicating that goosefoot is a poor host for STSS. Goosefoot also appears to be a poor host for *Xf*. We have confirmed successful transmission of *Xf* by GWSS for cowpea-to-cowpea and tomato-to-tomato tests (**Table 3**). GWSS transmission of *Xf* from cowpea-to-grapevine or tomato-to-grapevine could not be detected.

## **INTRODUCTION**

Over 140 plants are known to host Pierce's disease (PD) strains of *Xylella fastidiosa* (*Xf*) (Costa et al. 2004, Freitag 1951, Raju et al. 1980, 1983, Shapland et al. 2006, Wistrom and Purcell 2005, <http://www.cnr.berkeley.edu/xylella/temp/hosts.htm>). Many of these plants are found in close proximity to vineyards, and some are even used as cover crops in vineyards (Statewide IPM Program 2007). While considerable research has identified *Xf* hosts, little work has been done to determine if sharpshooters can acquire the bacteria from these hosts and transmit it to grapevines. If this does not occur, then the alternate host is of little consequence in PD epidemiology. Conversely, plants that contribute inoculum for sharpshooter acquisition and transmission to grape should be removed if growers wish to reduce primary spread into their vineyards.

To successfully implement a program to remove pathogen sources, we first must identify those sources. The introduction into California of GWSS, an insect with a broad host range, theoretically increases the probability of disease spread from these alternate host plants to grape. For this to occur, GWSS must feed on the infected plant in such a way to acquire *Xf* from plant, and successfully transmit the acquired pathogen to grapevines. While studies have shown mechanical and insect transmission to wide variety of alternate hosts (Freitag 1951, Purcell and Saunders 1999), they have demonstrated transmission from only a handful of alternate hosts to grapevines (Hill and Purcell 1995, 1997). We are unaware of research published on transmission of *Xf*, PD strain, from alternate hosts into grapevines using GWSS or STSS, a native California sharpshooter also found in grape growing regions, as the vector.

## **OBJECTIVES**

Using GWSS and STSS vectors:

1. Evaluate the acquisition and transmission of *Xf* to grapevines from agricultural crop plants known to be PD hosts that are grown in the vicinity of vineyards.
2. Evaluate the acquisition and transmission of *Xf* to grapevines from weed plants known to be PD hosts that are grown in the vicinity of vineyards.
3. Evaluate the acquisition and transmission of *Xf* to grapevines from vineyard cover crop plants.

## **RESULTS AND DISCUSSION**

Twenty-four of 42 proposed plant species have been needle-inoculated with *Xf* (**Table 1**). *Xf* does not appear to be able to survive in bell pepper, cotton, sunflower, horseweed, annual fescue 'Zorro,' birdsfoot trefoil, or sudangrass plants. A few positives were detected at two weeks post-inoculation with ELISA for bell pepper, cotton, and horseweed, but no plants tested positive by ELISA at four-weeks, nor were they positive by culture, indicating a possible transient infection or detection of dead *Xf* cells by early ELISA. Cultures were clean and negative for *Xf*. Sunflower tested positive by ELISA for all 20 plants, but the cultures were clean and negative. However, the sunflower also died very quickly, which may explain why it was not detected by culture. We will repeat needle inoculations for this plant species this winter. Final tests are pending for birdsfoot trefoil and annual fescue before we can comfortably say they are not hosts for *Xf*.

*Xf* was successfully isolated from needle-inoculated alfalfa, basil, lima bean, tomato, goosefoot, Spanish broom, tree tobacco, annual ryegrass, black mustard, Blando brome, New Zealand white clover, Hykon rose clover, cowpea, fava bean, field pea, meadow barley, and California red oats. We did not isolate *Xf* from basil until 16 weeks post-inoculation. All ELISA tests for Basil were positive, including for the negative controls, indicating that the commercial kit for *Xf* from Agdia, Inc., is not suitable for testing this plant species. The cultures for the negative controls were always negative, including at 16-weeks post-inoculation when the other cultures were obtained.

**Table 1.** ELISA and culture results for plant species needle-inoculated with *Xf*.

Type	Common Name	Scientific Name	ELISA +	Culture +	<i>Xf</i> Recovered?
Agriculture Crops	Alfalfa	<i>Medicago sativa</i>	20/20	14/20	Yes
	Basil	<i>Ocimum basilicum</i>	20/20*	10/20	Yes
	Bell Pepper	<i>Capsicum annuum</i>	5/20**	0/20	No
	Cotton, Upland	<i>Gossypium hirsutum</i>	2/15**	0/15	No
	Lima Bean, Fordhook 242	<i>Phaseolus lunatus</i>	2/18	1/18	Yes
	Tomato, Rutgers	<i>Solanum lycopersicum</i>	15/39	8/38	Yes
Weeds	Common Sunflower (commercial variety)	<i>Helianthus annuus</i>	20/20*	0/20	No
	Goosefoot	<i>Chenopodium sp.</i>	7/40***	5/33	Yes
	Horseweed	<i>Conyza Canadensis</i>	2/20**	0/20	No
	Spanish Broom	<i>Spartium junceum</i>	17/20	17/20	Yes
	Tree Tobacco	<i>Nicotiana sp.</i>	12/20**	2/20	Yes
Cover Crops	Annual Ryegrass	<i>Festuca sp.</i>	6/20	6/20	Yes
	Annual Fescue, Zorro	<i>Lolium multiflorum</i>	0/20	0/20	Final test pending
	Black Mustard	<i>Brassica nigra</i>	17/20	13/20	Yes
	Blando Brome	<i>Bromus hordeaceus</i>	16/20	13/20	Yes
	Birdsfoot Trefoil	<i>Lotus spp.</i>	10/20	0/20	Final test pending
	Clover, New Zealand White	<i>Trifolium repens</i>	15/20	2/20	Yes
	Clover, Hykon Rose	<i>Trifolium hirtum</i>	16/20	10/20	Yes
	Cowpea, California Blackeye	<i>Vigna unguiculata</i>	22/40	16/35	Yes
	Fava Bean, Windsor	<i>Vicia faba</i>	30/40	7/20****	Yes
	Field Pea, Miranda	<i>Pisum sativum</i>	14/39	3/11	Yes
	Meadow Barley	<i>Hordeum brachyantherum</i>	9/20	4/20	Yes
	Oat, California Red	<i>Avena sativa</i>	12/20	2/20	Yes
Sudangrass	<i>Sorghum bicolor var. sudanense</i>	0/20	0/20	No	

\* False positives.

\*\* Most or all positives in 2-week ELISA test; possible transient infection or dead cells detected.

\*\*\* Very slow-growing *Xf*, detected well after 4-weeks.

\*\*\*\* Fava bean contains many other microorganisms that contaminate and probably obscure positive culture results. Also, fava bean occasionally produces false positives by ELISA.

Insect transmissions have been done for both vectors on alfalfa, cowpea, fava bean, tomato, basil, and goosefoot (**Tables 2 and 3**). We confirmed successful transmission of *Xf* by STSS for fava bean-to-fava bean, alfalfa-to-alfalfa, and alfalfa-to-grapevine (**Table 2**). Therefore, STSS can transmit *Xf* between fava bean plants, between alfalfa plants, and from alfalfa to grapevines. We were unable to detect successful transmission from fava bean-to-grapevine, tomato-to-tomato, tomato-to-grapevine, cowpea-to-cowpea, or cowpea-to-grapevine. All 36 STSS died on goosefoot before the end of the 48-hr acquisition access period (AAP), indicating that goosefoot is a poor host for STSS. Goosefoot also appears to be a poor host for *Xf*, as few cultures were obtained from needle-inoculated plants, and all were extremely slow growing, except for one. Transmission tests using STSS on basil are underway at the time of this writing and results are not yet available.

We have confirmed successful transmission of *Xf* by GWSS via culture for cowpea-to-cowpea and tomato-to-tomato tests (**Table 3**). GWSS transmission of *Xf* from cowpea-to-grapevine or tomato-to-grapevine could not be detected. Transmission tests for GWSS on alfalfa are underway and the results are pending. Only four of 24 GWSS survived the 48-hr acquisition

access period (AAP) on goosefoot. The surviving four insects were placed on a clean grapevine test plant, although they appeared to be in the process of dying. Results for the single goosefoot-to-grapevine test will be available by the December meeting. Goosefoot appears to be a poor host for *Xf*, STSS, and GWSS, and is probably insignificant in PD epidemiology. Transmission tests for basil using GWSS have been completed, but results are not available at the time of this writing.

We had established clean, captive-reared GWSS and STSS for experiments. However, they were decimated by an infestation of the parasitoid wasp, *Gonatocerus ashmeadi* early this year. We determined the source of infestation, corrected it, and have been rebuilding the colonies since Spring 2008 by regularly collecting adults from the field for oviposition in captivity. Eggs produced are collected from field colonies and hatched in the laboratory, and then transferred to clean plants. We currently have a few hundred captive-reared nymphs of each species, and several mating pairs and gravid females still producing eggs. We resumed insect transmission experiments in early September 2008, and we are in the process of conducting transmission tests as the current captive-reared nymphs reach maturity. In the meanwhile, we have continued to grow, needle-inoculate, and evaluate potential PD hosts from the list.

**Table 2.** STSS transmission results for cowpea, fava bean, tomato, and alfalfa.

Transmission Test	ELISA +	Culture +	Successful Transmission?
Cowpea-to-Cowpea	5/5	0/5	Not detected
Cowpea-to-Grapevine	2/5	0/5	Not detected
Cowpea Group Grapevine-to-Grapevine Controls	3/6	0/6	Not detected
Fava Bean-to-Fava Bean	1/5	1/5	Yes
Fava Bean-to-Grapevine	4/5	0/5	Not detected
Tomato-to-Tomato	1/5	0/5	Not detected
Tomato-to-Grapevine	3/5	0/5	Not detected
Fava Bean/Tomato Group Grapevine-to-Grapevine Controls	2/4	0/4	Not detected
Alfalfa-to-Alfalfa	3/5	2/5	Yes
Alfalfa-to-Grapevine	2/5	2/5	Yes
Alfalfa Group Grapevine-to-Grapevine Controls	1/5	1/5	Yes
Goosefoot (none survived AAP)	0/0	0/0	No
Basil-to-Basil	Pending	Pending	Pending
Basil-to-Grapevine	Pending	Pending	Pending
Basil Group Grapevine-to-Grapevine Controls	Pending	Pending	Pending

**Table 3.** GWSS transmission results for cowpea, fava bean, tomato, goosefoot, and basil.

Transmission Test	ELISA +	Culture +	Successful Transmission?
Cowpea-to-Cowpea	4/5	2/5	Yes
Cowpea-to-Grapevine	3/5	0/5	Not detected
Cowpea Group Grapevine-to-Grapevine Controls	3/5	0/5	Not detected
Fava Bean-to-Fava Bean	2/5	0/5	Not detected
Fava Bean-to-Grapevine	1/5	0/5	Not detected
Tomato-to-Tomato	3/5	1/5	Yes
Tomato-to-Grapevine	2/5	0/5	Not detected
Fava Bean/Tomato Group Grapevine-to-Grapevine Controls	2/10	0/10	Not detected
Basil-to-Basil	Pending	Pending	Pending
Basil-to-Grapevine	Pending	Pending	Pending
Basil Group Grapevine-to-Grapevine Controls	Pending	Pending	Pending
Goosefoot-to-Grapevine	Pending	Pending	Pending
Alfalfa-to-Alfalfa	Pending	Pending	Pending
Alfalfa-to-Grapevine	Pending	Pending	Pending
Alfalfa Group Grapevine-to-Grapevine Controls	Pending	Pending	Pending

## CONCLUSIONS

This project addresses the 2006 Scientific Summit category of “*Understanding transmission of the disease,*” and relates directly to the acquisition and transmission of *Xf* by GWSS. It also has relevance to several of the recommendations developed by the National Academy of Science, National Research Council (2004). First and foremost, the definition of the Category 1 research option is that it “holds a reasonable promise of generating successful tools for management of PD/GWSS, either in the short term or the long term.” By determining the plants that truly contribute to primary spread by sharpshooters, we can give growers another strategy (i.e. removing those plants) in an effort to reduce bacterial inoculum

around their vineyards. This proposal also meets the general criteria defined in the NRC report in recommendation 2.2, of “contributing to PD/GWSS management and its sustainability,” and it applies specifically to recommendation 3.9 of examining plants “for effective transmission rates from host to grape.”

Bell pepper, cotton, horseweed, and sudangrass did not sustain infection after needle-inoculation with *Xf*, indicating that these plants are very unlikely to harbor *Xf* infection in the field. This is especially good news about horseweed since it is an extremely common weed in vineyards and is reported as resistant to herbicides. It appears that *Xf* is also unable to infect annual fescue or birdsfoot trefoil via needle-inoculation, although results from the 16-week (final) test are still pending. Sunflower has been reported as a host by other researchers and we were surprised that we did not obtain any *Xf* isolates from it. We used a commercial variety, which we grow for our insect colonies, while obtaining seed from wild sunflower. We will test the wild sunflower along with the commercial variety again.

We obtained *Xf* isolates from alfalfa, basil, lima bean, tomato, goosefoot, Spanish broom, tree tobacco, annual ryegrass, black mustard, Blando brome, New Zealand white clover, Hykon rose clover, cowpea, fava bean, field pea, meadow barley, and California red oats. We recovered *Xf* from at least 50% of test plants for alfalfa, basil, Spanish broom, black mustard, Blando brome, and Hykon rose clover, indicating that these are likely hosts for *Xf* in the field. Although we obtained some isolates from lima bean, tree tobacco, annual ryegrass, New Zealand white clover, field pea, meadow barley, and California red oats, further testing is required to gain a better understanding of their potential as alternative hosts for *Xf* in the field, since needle-inoculation is a severe and unnatural form of infection that is unlikely to happen in the field. As in the case of the goosefoot, we found that we could obtain isolates from a needle-inoculated plant, but that it was a poor host overall for PD and both vectors tested. Therefore, goosefoot is unlikely to serve as a source or reservoir of *Xf* in the field. If these plants have natural defenses against acquiring or sustaining a *Xf* infection when needle-inoculated with millions of bacteria, it is likely that an infection by a vector transmitting far fewer bacterial cells would not be sustained either. However, there are insect-pathogen-plant interactions involved that must be tested before such a conclusion can be made definitively. Further studies mimicking more natural acquisition and transmission using insects should be done for a more complete understanding of the roles each plant and vector species might play in the field.

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# BREEDING PIERCE'S DISEASE RESISTANT TABLE AND RAISIN GRAPES AND THE DEVELOPMENT OF MARKERS FOR ADDITIONAL SOURCES OF RESISTANCE

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

## ABSTRACT

Twenty-two seedless x seedless crosses to develop additional BC2 and BC3 *V. arizonica* and BC1 SEUS BD5-117 families were made in 2008. Powdery mildew resistance was included in five of these crosses. These crosses produced 5,148 berries, 8,824 ovules and 1,861 embryos. Nine seeded BC1 crosses based on *V. arizonica* and SEUS PD resistance sources were made, resulting in 1,393 seed. Two BC2 and 12 BC3 families (*V. arizonica* source of resistance) consisting of 1,191 individuals were screened at the seedling stage in the greenhouse with SSR markers for resistance. A total of 363 were resistant and planted in the field. Greenhouse screening was completed on 57 selections and 20 of the 21 resistant individuals were from *V. arizonica*. Twelve resistant selections have been planted in the field at Weslaco, Texas to determine their field resistance. An additional 89 plants and 692 embryos have been produced to increase the size of the C33-30 x BD5-117 family for molecular marker development. A total of 105 SSR primers are polymorphic between the parents and screening of the first 154 individuals to develop a framework map has started. Greenhouse testing of 75 individuals was completed with 21 being resistant. An additional 49 are currently in greenhouse tests.

## INTRODUCTION

Pierce's disease (PD) has existed in California since the late 1800's when it caused an epidemic in Anaheim. A number of vectors for PD already exist in California, and they account for the spread and occurrence of the disease. The introduction of the glassy-winged sharpshooter to California in the 1990's significantly increased the spread and damage caused by PD. Other vectors exist outside California and are always a threat. All of California's commercially grown table and raisin grape cultivars are susceptible to PD. An effective way to combat PD and its vectors is to develop PD resistant cultivars so that PD epidemics or new vectors can be easily dealt with. PD resistance exists in a number of *Vitis* species and in *Muscadinia*. PD resistance has been introgressed into grape cultivars in the southeastern United States, but fruit quality is inferior to *V. vinifera* table and raisin grape cultivars grown in California. Greenhouse screening techniques have been improved to expedite the selection of resistant individuals (Krivanek et al. 2005, Krivanek and Walker 2005). Molecular markers have also been identified that make selection of PD resistant individuals from *V. arizonica* in these families even quicker (Krivanek et al. 2006). The USDA, ARS grape breeding program at Parlier, CA has developed elite table and raisin grape cultivars and germplasm with high fruit quality. Embryo rescue procedures for culturing seedless grapes are being used to help introgress the seedless trait with PD resistance quickly (Emershad et al. 1989). This collaborative research gives the unique opportunity to develop high quality PD resistant table and raisin grape cultivars for the California grape industry.

## OBJECTIVES

1. Develop PD resistant table and raisin grape germplasm/cultivars with fruit quality equivalent to standards of present day cultivars.
2. Develop molecular markers for *Xf*/PD resistance in a family (SEUS) other than those from *V. arizonica*.

## RESULTS

### Objective 1

This year the seedless embryo culture crosses concentrated on using *V. arizonica* and SEUS (BD5-117) sources of resistance. Twenty-two crosses were made and produced 5,184 berries, 8,824 ovules and 1,861 embryos (21% embryos/ovules) (**Table 1**). Five of the seedless crosses combined *V. arizonica* PD resistance with powdery mildew resistance. Nine seeded crosses from *V. arizonica* and SEUS sources of resistance were made (**Table 2**). Fruit has been harvested and 1,393 seeds extracted for germination in January.

Two BC2 and twelve BC3 families (89-0908 *V. arizonica* source of resistance) produced in 2007 and growing in the greenhouse as small plants this spring were tested for molecular markers associated with the PdR1 locus on chromosome 14 (**Table 3**). A total of 1,191 individuals were tested with SSR markers and 1,121 showed markers on both sides of the PdR1 region as expected. Eighty-seven percent had either resistant or susceptible bands, similar to 2007 results, which makes selection for resistance effective. A total of 363 individuals (32% of those showing markers) were resistant and planted to the field from the greenhouse. The susceptible individuals were discarded making use of field space much more efficient. Greenhouse testing of 57 selected individuals from BC2 *V. arizonica* and SEUS populations was completed and 21 identified as resistant. All resistant individuals were from *V. arizonica* except one from SEUS, showing the high level of resistance

passed on by *V. arizonica*. Another 70 selections are in greenhouse tests that will be completed this fall. Greenhouse testing is absolutely necessary to make the final decision about resistance of individual selections. Because the highest levels of resistance are being obtained from *V. arizonica* and BD5-117, we have and will continue to emphasize their use over other sources of resistance in the breeding program. Fruit samples have been taken from four populations observed last year to continue comparing fruit quality between resistant and susceptible seedlings to verify that fruit quality is segregating independently from PD resistance. Raisins are being produced from the resistant *V. arizonica* with small aborted seeds. Evaluation of over 2,000 fruiting seedlings in the field is being conducted this year to determine those with fruit quality good enough to consider as parents or potential cultivars. Twelve advanced selections that were rated as resistant in the greenhouse have been planted in a replicated plot at the USDA ARS research station, Weslaco, Texas. This is an area with high levels of PD and glassy-winged sharpshooters and is being used to determine field resistance of these selections.

**Table 1.** 2008 table and raisin grape PD resistant seedless crosses and the number of ovules and embryos produced.

Female	Male	Type	No. Emas- culations	No. berries Opened	No. Ovules	No. Embryos
89-0908 <i>V. rupestris</i> x <i>V. arizonica</i>						
05-5502-05	A 85- 40	Table BC3	2,691	452	650	147
Scarlet Royal	05-5502-42	Table BC3	3,860	92	127	19
04-5554-8	A63-85	Raisin BC3	7 bags <sup>a</sup>	296	650	173
05-5555-108	A56-92	Raisin BC3	2,054	306	525	114
05-5551-49	Selma Pete	Raisin BC3	3,897	487	556	49
05-5502-05	Y315-400	PM Table BC3	2,529	443	795	72
Y314-360	05-5502-05	PM Table BC3	2,310	59	54	11
04-5554-19	Y313-137	PM Raisin BC3	800	94	136	26
04-5554-8	Y313-137	PM Raisin BC3	7 bags	440	882	230
05-5551-108	Y313-137	PM Raisin BC3	2,435	129	154	8
<b>Total</b>			<b>20,576</b>	<b>2,798</b>	<b>4,529</b>	<b>849(18.7%)</b>
B43-17 <i>V. rupestris</i> x <i>V. arizonica</i>						
04-5002-42	Y129-161	Table BC1	2,902	264	675	189
04-5051-14	A56-92	Raisin BC1	4,168	345	700	177
04-5051-4	A63-85	Raisin BC3	6 bags	333	434	159
<b>Total</b>			<b>7,070</b>	<b>942</b>	<b>1,809</b>	<b>525(29.9%)</b>
SEUS source of resistance BD5-117						
C33-30	BD5-117	Genetic family	10 bags	1,847	2,350	692
01-5026-20	B28-128	Table BC1	7 bags	165	350	31
01-5026-28	B28-126	Table BC1	7 bags	285	328	73
01-5026-31	C62-109	Table BC1	7 bags	149	350	126
01-5026-20	01-5026-8	Table F1 sib	7 bags	152	207	7
01-5026-20	01-5026-33	Table F1 sib	7 bags	206	375	52
01-5026-10	A56-66	Raisin BC1	2,602	188	350	91
01-5026-20	A56-92	Raisin BC1	7 bags	140	351	51
01-5026-20	A51-55	Raisin BC1	7 bags	16	11	0
01-5026-10	Y313-137	PM Raisin BC1	2,683	143	164	56
<b>Total</b>			<b>5,285</b>	<b>3,291</b>	<b>4,836</b>	<b>1,179(24.4%)</b>

<sup>a</sup>Parents with female flowers were not emasculated, only bagged and pollinated.

**Table 2.** 2008 table and raisin grape PD resistant seeded x seedless crosses and the number of seeds produced.

Female	Male	Type	No. Emasculations	No. Seeds
89-0908 <i>V. rupestris</i> x <i>V. arizonica</i>				
05-5501-26	C45-64	Table BC3	3 bags <sup>a</sup>	41
05-5501-26	A40-93	Table BC3	3 bags	12
04-5554-1	A56-94	Raisin BC3	800	385
05-5501-26	Y315-400	PM Raisin BC3	3 bags	29
05-5501-26	Y314-360	PM Raisin BC3	3 bags	10
B43-17 <i>V. rupestris</i> x <i>V. arizonica</i>				
04-5001-11	C58-37	Table BC1	3 bags	334
04-5001-8	C61-123	Table BC1	5 bags	191
SEUS BD5-117 source of resistance				
01-5026-11	C45-64	Table BC1	3 bags	15
01-5026-21	A50-33	Raisin BC1	3 bags	376
<b>Total</b>			<b>800 + 26 bags</b>	<b>1,393</b>

<sup>a</sup>Parents with female flowers were not emasculated, only bagged and pollinated.

**Table 3.** Determination of seedling resistance based on molecular markers for 89-0908 BC2 families made in 2007.

Family	Type Cross	No. Resistant <sup>a</sup>	No. Susceptible <sup>b</sup>	No. Recombinants	No data <sup>c</sup>	Total
07-5001	Table BC3	1	78	39	2	120
07-5002	Table BC3	0	15	4	0	19
07-5003	Table BC3	3	58	5	0	66
07-5006	Table BC3	0	1	0	0	1
07-5051	Raisin BC3	13	23	1	1	38
07-5052	Raisin BC3	72	90	14	10	186
07-5054	Raisin BC3	5	20	2	1	28
07-5055	Raisin BC3	1	7	1	2	11
07-5056	Raisin BC3	3	2	4	0	9
07-5057	Raisin BC3	14	17	5	1	37
07-5058	Raisin BC3	27	49	8	5	89
07-5059	Raisin BC2	111	108	25	12	256
07-5060	Raisin BC2	60	95	21	2	178
07-5061	Raisin BC3	53	48	18	34	153
<b>Total</b>		<b>363</b>	<b>611</b>	<b>147</b>	<b>70</b>	<b>1,191</b>

<sup>a</sup>Resistant = marker on both sides of *PdR1* region.

<sup>b</sup>Susceptible = no *PdR1* markers.

<sup>c</sup>No data = genotypes that amplified with one marker, off types and that failed with both markers

**Table 4.** Results of greenhouse test for determination of PD reaction.

Population	Resistance Source	Total sent	Testing Compete		Test pending No. tested	Total Tested	No. to repropagate <sup>a</sup>
			No. tested	No. resistant			
BD5-117 map	BD5-117	154	75	21	49	124	30
Arizonica	PdR1	113	28	20	70	98	24
Other PD	SEUS	65	29	1	16	45	20
<b>Total</b>		<b>332</b>	<b>112</b>	<b>42</b>	<b>135</b>	<b>267</b>	<b>74</b>

<sup>a</sup> Not all selections had enough replications in greenhouse for definitive results.

## Objective 2

The PD resistant grape selection BD5-117 from Florida was hybridized with the seedless table grape selection C33-30 and 154 individuals have been produced that are fruiting. BD5-117 has given the highest number of resistant offspring of any of the SEUS resistant selections to date and makes an excellent choice for study for molecular markers. The fruit of these seedlings is being evaluated for berry size, fruit quality and seed/aborted seed size. Cuttings of all 154 were made for

evaluation of PD resistance in the greenhouse. Testing is complete on 75 individuals, with 21 being resistant. An additional 49 are currently being tested and 37 need to be repropagated for testing (**Table 4**). Additional plants are being made to increase the family size to at least 500 individuals. In 2006, 65 plants were produced. In 2007, an additional 89 plants were produced and planted in the field. This year 1,847 berries were produced for this cross from which 2,350 ovules were cultured and 692 embryos produced. In 2008 SSR primers that have shown polymorphism are being tested on all 154 individuals to develop a framework map. A total of 105S polymorphic markers have been identified to date.

## CONCLUSIONS

Families for the development of PD resistant seedless table and raisin grape cultivars continue to be produced. Emphasis was placed on making additional *V. arizonica* BC3 crosses (93% *V. vinifera*) and BC1 crosses of BD5-117. The use of molecular markers has simplified and sped up the identification of PD resistant individuals from *V. arizonica*. Seedless table and raisin grape selections with PD resistance and improved fruit quality have been made in both BC2 *V. arizonica* and F1 BD5-117 families. One hundred five polymorphic SSR primers have been identified in the BD5-117 family in the search for molecular markers from sources of resistance other than *V. arizonica*. SSR primers are now being tested on all 154 individuals from the BD5-117 family to develop a framework map. The development of PD resistant table and raisin grape cultivars will make it possible to keep these grape industries viable in PD infested areas. Molecular markers will greatly aid the selection of PD resistant individuals from SEUS populations.

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## FUNDING AGENCIES

Funding for this project was provided by the University of California Pierce's Disease Grant Program, and the Consolidated Central Valley Table Grape Pest and Disease Control District.

## ASSESSMENT OF THE IMPORTANCE OF ALFALFA TO THE EPIDEMIOLOGY OF XYLELLAE DISEASES IN THE SAN JOAQUIN VALLEY OF CALIFORNIA

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**Reporting Period:** The results reported here are from work conducted October 2005 to October 2008.

### ABSTRACT

The role of alfalfa in the epidemiology of xylellae diseases in the San Joaquin Valley of California was assessed. Alfalfa was investigated as it is a known host of *Xylella fastidiosa* (*Xf*) and often harbors large populations of a native vector, *Draeculacephala minerva*. Laboratory inoculation of 14 cultivars of alfalfa indicated that all cultivars tested were suitable hosts. The persistence of infections in alfalfa was followed in four cultivars over one year. For plants held outdoors, detection of *Xf* via PCR declined during the winter and increased again during the summer, suggesting that cool winter temperatures decreased titers of *Xf*. Sampling of alfalfa fields seasonally found that incidence of *Xf* in alfalfa was low with only six positive samples detected out of >4,000 screened. All positive samples were collected in summer agreeing with seasonal trends in *Xf* detection observed in controlled studies. Abundance of *D. minerva* in alfalfa was high, although the highest numbers were caught on traps located on weedy field margins. Preference of *D. minerva* for weeds in alfalfa fields would limit the spread of *Xf* in alfalfa. The results indicate that alfalfa can serve as a source of vectors, but its role as an inoculum source is unclear. Future work should determine the incidence of *Xf* in weeds commonly found in alfalfa fields that are preferred feeding hosts of *D. minerva*.

### INTRODUCTION

Pierce's disease of grape and almond leaf scorch disease has been chronic problems in California's San Joaquin Valley. In the San Joaquin Valley of California, the green sharpshooter (*Draeculacephala minerva*) is thought to be the most important vector. Green sharpshooters are often abundant in alfalfa fields and alfalfa is a known host of *Xylella fastidiosa* (*Xf*). Alfalfa is thought to play an important role in the epidemiology of xylellae diseases because alfalfa is often planted in proximity to almond and grape and clusters of diseased almond trees and grape vines are often observed on orchard or vineyard edges which border alfalfa. Due to the large acreage planted with alfalfa in the San Joaquin Valley, its potential to serve as a host of *Xf*, and its propensity to harbor vectors, we initiated studies to assess the role of alfalfa in the epidemiology of xylellae diseases.

### OBJECTIVES

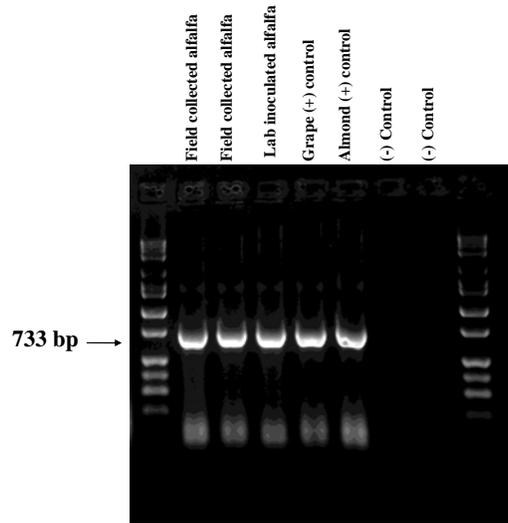
1. Estimate *Xf* incidence in forage alfalfa planted adjacent to grape and/or almond.
2. Characterize the seasonal abundance and dispersal of green sharpshooters present within and emigrating from alfalfa.
3. Determine the relative susceptibility of selected alfalfa cultivars to infection by *Xf*.

### RESULTS

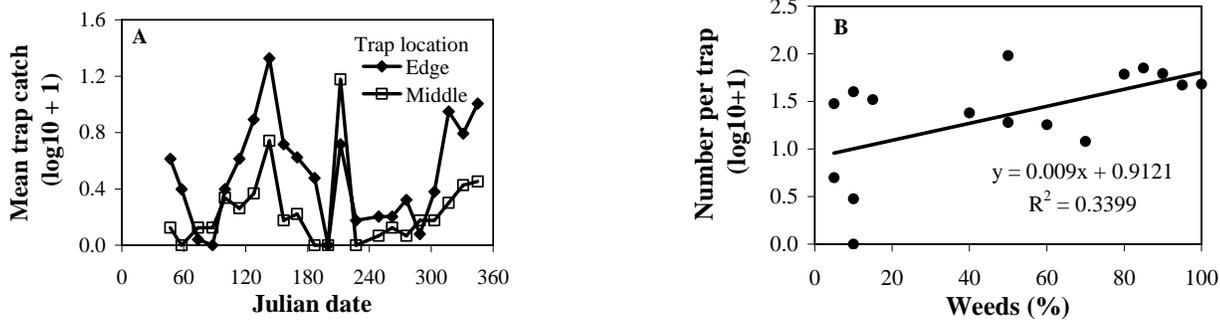
*Objective 1. Estimate Xf incidence in forage alfalfa planted adjacent to grape and/or almond.* We sampled alfalfa fields in Fresno, Tulare, and Kern counties seasonally (winter, spring, summer & fall) to estimate incidence of *Xf* starting in summer of 2005 to present. To date, >4,000 samples have been screened for the presence of *Xf* using conventional PCR (Minsavage et al. 1994). Of those samples, six have been confirmed positive. Two positives came from a collection in Fresno County during the summer of 2005 (**Figure 1**). The other four positives came from another collection in Fresno County during the summer of 2007.

**Objective 2. Characterize the seasonal abundance and dispersal of green sharpshooters present within and emigrating from alfalfa.** The abundance and spatial distribution of the green sharpshooter was monitored in alfalfa fields in Fresno, Kern, and Tulare counties throughout 2006, 2007, and 2008. Four transects of yellow sticky traps were placed in each field. Traps were counted and replaced biweekly. Preliminary analysis of the distribution of green sharpshooter within alfalfa fields indicates some important trends. First, green sharpshooters were more abundant on field edges than in the middle of fields (**Figure 2A**). Similarly, the number of insects caught per trap was often associated with the percentage of ground cover that was weeds (**Figure 2B**). Together, this indicates that *D. minerva* adults prefer weeds that are found along field margins.

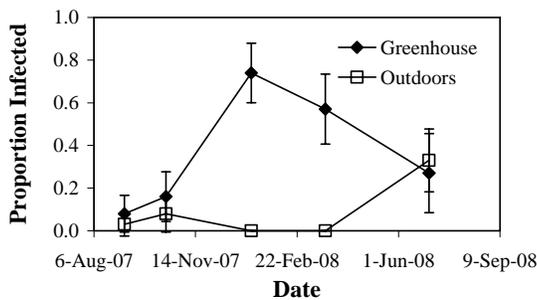
**Objective 3. Determine the relative susceptibility of selected alfalfa cultivars to infection by *Xf*.** Fourteen alfalfa cultivars were screened to determine their relative susceptibility to infection by four different *Xf* strains (Temecula, Dixon, M12, and M23). Plants were screened for infection using conventional PCR methods 12 weeks after inoculation. *Xf* was detected in at least three out of 24 plants for each cultivar and the percentage of plants infected averaged across the four strains varied from 13 to 48%.



**Figure 1.** Examples of alfalfa positives detected using RST 31/33.



**Figure 2.** Examples from preliminary analysis of trap catch data. A) Mean number of *D. minerva* caught on sticky traps located on the edge of an alfalfa field versus the middle of an alfalfa field in Fresno County during 2007. B) Association of the number of *D. minerva* caught per trap between January 8, 2007 and August 3, 2007 and the percentage of ground cover surrounding a trap that was weeds for an alfalfa field in Kern County.



**Figure 3.** Incidence of *Xf* in needle-inoculated plants held in the greenhouse versus outdoors. All plants were inoculated on the same date in July of 2007.

For 5 cultivars (CUF 101, Moapa 69, WL 530 HQ, WL 625 HQ, and WL 342 HQ) a more detailed experiment was conducted to determine the seasonal fate of *Xf* in alfalfa. Approximately 20 plants of each cultivar were needle inoculated in July of 2007. Half of the plants were held outdoors in a screen cage and the other half were held indoors in a greenhouse. Plants were screened for the presence of *Xf* regularly using standard PCR methods. Screening of samples in October of 2007 indicated no differences between plants held outdoors versus those held in the greenhouse (**Figure 3**). However, by January of 2008 all samples from plants held outdoors were negative for *Xf* whereas most samples from plants held in the greenhouse were positive (**Figure 3**). This suggests that cool winter temperatures reduced the titer of *Xf* in plants held outdoors. By July of 2008, the incidence of *Xf* was the same for both sets of plants suggesting that cool winter temperatures did not

eliminate infections from plants held outdoors, but simply reduced *Xf* titers to levels that were not detectable via PCR. These results indicate important seasonal fluctuations in *Xf* titer.

## **CONCLUSIONS**

All alfalfa cultivars tested were suitable hosts for *Xf* and green sharpshooters were abundant in alfalfa fields (**Figure 2**). Incidence of *Xf* in field collections averaged over all sites and dates was low (six out of >4,000 samples tested) and all *Xf* positive samples were collected during the summer. Monitoring of needle inoculated plants held throughout the year suggest that *Xf* titers decline during the winter (**Figure 3**), supporting the observation that *Xf* positive alfalfa samples were collected only in the summer. Trapping of *D. minerva* in alfalfa fields indicates that they prefer weedy field margins and likely feed preferentially on weeds versus alfalfa. If true, this would limit the spread of *Xf* in alfalfa. The results suggest that alfalfa can serve as a source of vectors, but that the role of alfalfa as an inoculum source is unclear. Future work should focus on examining the incidence of *Xf* in weeds commonly found in alfalfa fields that are preferred feeding hosts of *D. minerva*.

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## **FUNDING AGENCIES**

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# MAP-BASED IDENTIFICATION AND POSITIONAL CLONING OF *XYLELLA FASTIDIOSA* RESISTANCE GENES FROM KNOWN SOURCES OF PIERCE'S DISEASE RESISTANCE IN GRAPE

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

## ABSTRACT

This report presents updated results of refined mapping of the Pierce's disease (PD) resistance locus, *PdR1*, in the 04190 (397 plants) and 9621 population (433 plants) (both with resistance from *V. arizonica* b43-17). The *PdR1* locus is 0.38 and 0.97 cM from flanking markers in 9621 population and 0.39 and 0.23 cM in the 04190 population. Positioning of resistance locus has slightly changed, but marker-assisted screening and physical mapping efforts have not been affected. Between July 2007 and July 2008 two BAC libraries, each with one restriction enzyme (*Hind* III and *Mbo* I), were completed and we initiated the screening of the *Hind* III BAC library with flanking markers and identified 24 positive BAC clones. BAC end sequencing was carried out on 14 of BAC clones and complete sequencing of two clones (H23P13 and H64M16) that represented two haplotypes (either allele) of b43-17 was initiated. With the availability of new greenhouse screen data from both 9621 and 04190 populations and utilization of new SSR markers from chromosome 14 to refine marker data, the placement of *PdR1* locus was slightly shifted. The new flanking markers are VVCh14-56 and VVCh14-70, instead of VVCh14-56 and VVCh14-10. We then began using the Pinot Noir genome sequence to develop homologous markers that could be used for BAC library screening, and developed a set of nine markers (without SSR repeats) for BAC library screening. We are in process of isolating the BAC clones that represent VVCh14-70 end of the genomic sequence.

## INTRODUCTION

Previous reports have described the mapping of resistance to *Xylella fastidiosa* (*Xf*) based on b43-17 in four (9621, 04191, 04190, and 04373) populations (Douceff et al. 2004; Riaz et al. 2006). A complete report on the origin of the "89 group" seedlings, two of which, D8909-15 and F8909-17, are the parents of the 9621 population has been published (Riaz et al. 2007). The genetic mapping of the Pierce's disease resistance locus, *PdR1*, in three populations (04190, 04373 and 9621 populations) with emphasis on chromosome 14 and an explanation of segregation distortion mechanism was also completed (Riaz et al. 2008). A manuscript describing marker-assisted screening for breeding table and wine grape cultivars is in final revision (Riaz et al. 2009) and a report on the multigenic resistance from *V. arizonica* b42-26 is in preparation. We have recently refined the position of *PdR1a* and *PdR1b* in the 9621 and 04190 populations by increasing the number of recombinants that were generated from the ongoing wine and table grape breeding program and the addition of new markers. Greenhouse screening of 64 genotypes in the 04373 population showed that all plants are resistant verifying homozygous resistance in b43-17. We also completed the development of a BAC library from the resistant b43-17 and started BAC library screening with tightly flanking markers. Mapping populations were developed from b40-14 and b42-26 background to allow mapping of PD resistance from these two additional sources. Greenhouse screening of selected F1 genotypes containing b40-14 as a male parent verify b40-14 is homozygous resistant.

This report details three genetic maps (9621, 04190, and 04373), the initial screening of the 07744 and 04191 populations, the development of new markers from the Pinot Noir genome sequence, and most importantly the development, characterization and screening of the b43-17 BAC library. The initial physical mapping in conjunction with the genetic map is also reported and will lead to understanding how these resistance genes function.

## OBJECTIVES

1. Completely characterize and refine the *Xf* resistance locus on chromosome 14 by genetic mapping in four populations 04190 (*V. vinifera* F2-7 x F8909-08), 9621 (D8909-15 x F8909-17), 04191 (F2-7 x F8909-17), and 04373 (*V. vinifera* F2-35 x *V. arizonica* b43-17).
2. Study the inheritance of PD resistance from other genetic sources (b42-26 and b40-14).
3. Develop a BAC library for the homozygous resistant genotype b43-17 (parent of F8909-08 and F8909-17) and screen the library with closely linked markers.
4. Complete the physical mapping of *PdR1a* and *PdR1b* and initiate the sequencing of BAC clones that carry *PdR1a* gene candidates.

## RESULTS AND DISCUSSION

*Objective 1.* As mentioned in the previous report, the resistant genotypes F8909-17 and F8909-08 inherited different sister chromatids (haplotypes) from the homozygous resistant parent b43-17. It was noted that F8909-08 has a 50 cM region in which marker segregation is distorted and the same markers are distorted in b43-17 indicating a region with suppressed

recombination. However, the same markers on the F8909-17 map were not distorted in this region (Riaz et al. 2008). This report presents an updated map of the 9621 population developed with 433 genotypes and additional markers. The genetic position of the *PdR1a* resistance locus is slightly shifted and it is between marker VVCh14-56 and VVCh14-70 (**Figure 1**). **Table 1** shows the key recombinants from this population. We also marker screened a total of 458 additional plants from the 9621 population with linked markers (VVIP26 and VMC2a5) and a subset of 49 recombinant plants were selected to greenhouse screen, which are key to fine-scale positioning of *PdR1*. Tightly linked markers were added to the set of recombinant plants and three key recombinants were detected. Greenhouse screen results will be available next year. The F8909-17 resistance source was also used in breeding PD resistant grapes and 24 recombinant plants were selected from five different crosses (180 plants) and are being greenhouse screened which result in updates of these genetic maps.

Previously, the 04190 population consisted of 361 progeny and *PdR1b* mapped between markers VvCh14-02 and UDV095/VvCh14-10 within a 0.4 cM distance. We have completed screening and mapping of 36 additional plants from this population. The position of *PdR1* moved from between VvCh14-02 and UDV095/VvCh14-10 to VvCh14-02 and VvCh14-28/VVCh14-29/VVCh14-30. These new markers were developed from the Pinot Noir genomic region that corresponded to the VMCNg3h8 clone sequence (**Table 2**). Using the cloned VMCNg3h8 sequence, we obtained a 99Kb contig and new markers were developed. In the previous published map, VMCNg3h8 was not polymorphic for the 04190 population and that genomic region was not represented. The new markers were added to the base population of 397 plants and map was updated (**Figure 1**). The greenhouse screen was repeated for key recombinants, which also helped refine the data. In addition, marker analysis discovered 23 recombinants from 15 different crosses (1000 plants) that contain F8909-08. These recombinant plants are in the process of being greenhouse screened. Data on these recombinants is critical for fine scale mapping, so greenhouse screens are repeated to rule out all possible mistakes.

A *V. vinifera* F2-35 x F8909-17 cross generated a fourth mapping population, 04191, of 153 progeny. This population provides genotypes with a 50% *vinifera* background for breeding and more recombinant plants for genetic mapping. It also provides a population where resistance from F8909-17 can be examined without possible confounding effects from D8909-15 (since D8909-15 has a multigenic resistance from b42-26). We added markers that are tightly linked to *PdR1* to this set, categorized resistant, recombinant and susceptible genotypes based on marker information, and selected recombinant genotypes based on flanking markers. This population will be critical for the identification of any minor genes that might contribute to resistance. Therefore, we are expanding the framework genetic mapping to all 19 chromosomes. For this purpose, we are initiating greenhouse screen of all 153 plants. The plants were propagated and results will be available by March 2009.

*Objective 2.* Thus far we have used three resistance sources (b43-17, b40-14 and b42-26 – **Table 3**). It is easier to breed with single locus traits as is the identification of genes using map-based positional cloning. Resistance from b43-17 is inherited as a single gene while resistance from b42-26 and its offspring D8909-15 is quantitatively inherited perhaps by multiple genes on multiple chromosomes. We initiated genetic mapping in the F1 population from the b42-26 background (05347 – **Table 1**). Greenhouse screening of a subset found 48 genotypes were resistant and 13 were susceptible. A total of 337 markers were tested on small a parental data set. Results found a high level of homozygosity for b42-26 (only 113 markers were polymorphic); 184 markers were homozygous for the male parent b42-26, 40 markers did not amplify. We completed 70 markers on a set of 64 genotypes and the remaining polymorphic markers are in process to develop a framework map. This set of 64 genotypes is not an adequate number for this mapping project, and this cross was repeated in Spring 2008 to produce at least 188 plants in the core population.

Previous results determined that *V. arizonica* b40-14 is a promising homozygous resistant genotype. We screened 45 genotypes from an F1 cross of *V. rupestris* x b40-14 and all were resistant except three genotypes with intermediate results. In Spring 2007, these resistant F1 genotypes were crossed to other susceptible and resistant genotypes to verify the single dominant gene mode of inheritance (07744 and 07386 – **Table 1**). We completed DNA extractions from 122 seedlings from 07744 and 105 seedlings for 07386. Marker testing is in the process to create a framework map of the 19 chromosomes, and polymorphic markers will be added to the 07744 population. Greenhouse screening results from these plants will be completed by March 2009. Initially framework genetic maps in F1 and BC1 populations will be developed utilizing 96 to 188 genotypes. Once the resistance locus and QTLs are localized, markers will be added to saturate the linkage groups where the resistance loci reside.

*Objective 3 and 4.* Two BAC libraries (each with a different restriction enzymes) from the homozygous resistant b43-17 were developed. Library screening was carried out twice with two markers (VVCh14-10 and VVCh14-56), both tightly linked to *PdR1*. This identified 24 positive clones– four of the positive clones were positive with both markers: H23-P13, H34-B5 and H64-M16 and H45-J22. The inclusion of new marker and greenhouse screen information moved the *PdR1* locus between markers VVCh14-56/VVCh14-02 and VVCh14-70 (**Figure 1 and Table 2**), which required the BAC library be screened to find the clones in the genomic region at the end of VVCh14-70 marker. The 14 positive BAC clones that were selected with flanking marker were amplified with marker VVCh14-56, which is polymorphic (with two alleles) for b43-17 and can be used to distinguish and group clones. In an attempt to develop more markers, we utilized the 695Kb region from the Pinot noir genome sequence that covers the marker VVCh14-56 and VVCh14-27/VMCNg2b7.2 (**Figure 1**). It is important to note

that this region is from two different scaffolds (9 and 21). A total of 10 primers were developed that spread across 60 to 80Kb of the 695Kb sequence from Pinot noir. Nine of these markers amplified successfully (**Figure 2**). We also developed SSR markers from this region that were placed in between the screening markers (**Figure 2**). Currently the resistance locus resides between Ch14-56 and Ch14-70; a physical distance of 340Kb. Based on the genetic map from 9621 population, the physical and genetic distance correlates as 1cM is equal to about 216Kb. Based on the previous reported position of the *PdRI* locus, we initiated shotgun sequencing of the H23P13 and H64M16 clones. These clones represent two haplotypes of b43-17. A total of 173Kb region of H23P13 clone was assembled after primer walking. Given the new position of *PdRI*, we will be able to utilize the 35Kb region that spans the region from marker VVCh14-56 and beyond, and rescreen the BAC library.

## CONCLUSIONS

Results from this project have allowed us to: 1) understand the segregation of PD resistance in two different backgrounds; 2) develop a framework genetic map for *Xf* resistance; 3) select markers for effective marker-assisted selection (MAS) in grape breeding; and 4) begin development of a physical map of genomic fragments that carry the *PdRI* locus, leading to map-based positional cloning of PD resistance genes. MAS has allowed the generation of PD resistant BC3 progeny with 94% of their parentage from elite *V. vinifera* wine grapes in a dramatically shortened time period. We have also constructed a BAC library for b43-17 to isolate the *PdRIa* gene candidates. In order to expand the range of PD resistances by exploiting other resistant accessions, we are studying the inheritance of PD resistance in two other backgrounds. Resistance in *V. arizonica* b40-14 is inherited as a single gene. We are using quantitative trait loci (QTL) analysis in the 0023 and 05347 populations to study PD resistance from *V. arizonica* b42-26 whose resistance is controlled by several genes. The genetic mapping, placement of a variety of resistance genes/traits will allow MAS to broaden resistance and make it more durable. Map-based efforts will also enable us to characterize the variants of PD resistance genes.

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## FUNDING AGENCIES

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**Table 1.** The key recombinants from the 9621 population. The genotypes in bold red font are key recombinants with a recombination event between the marker and the *PdR1a* resistance locus. “0” indicates a susceptible allele and “1” indicates a resistant allele.

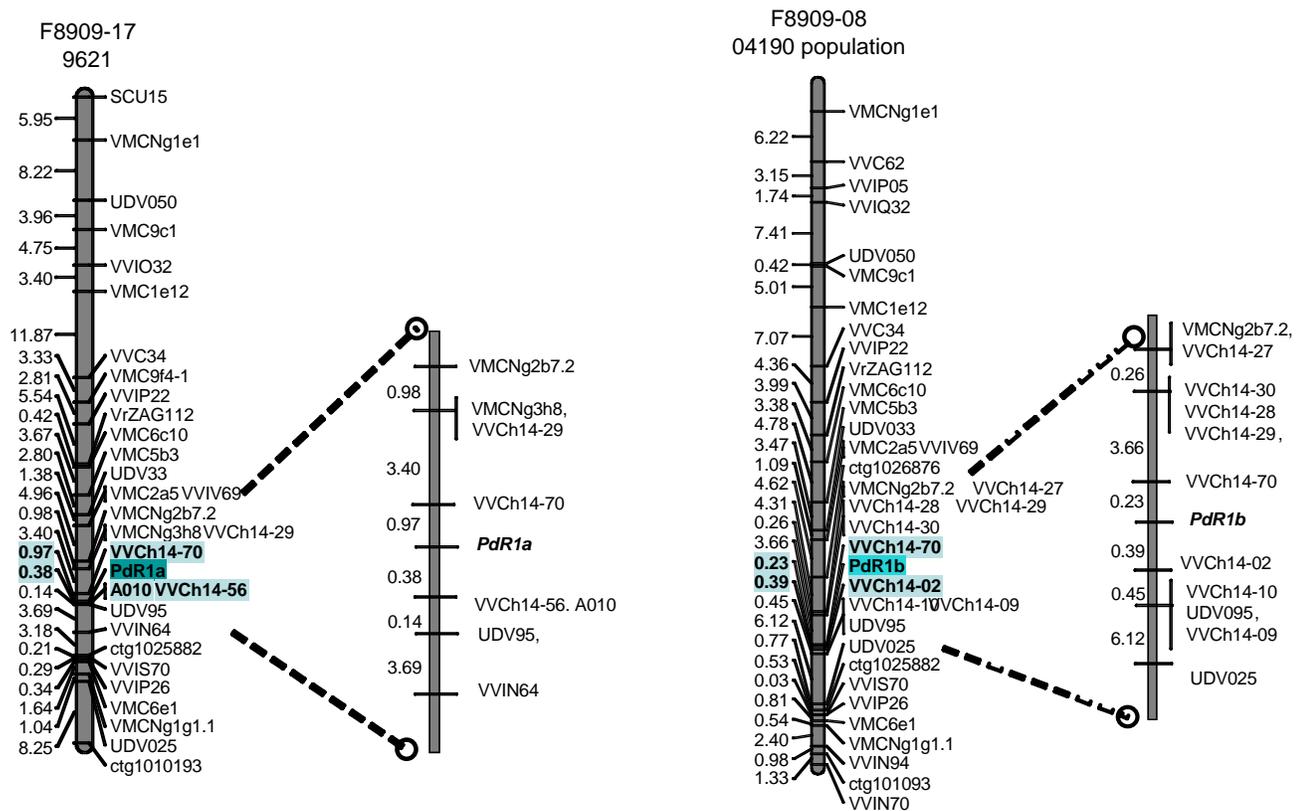
9621 Genotype	UDV095	A0101	VVCh14-56	<i>PdR1a</i>	VVCh14-70	VVCh14-29	VMCNg 3h8	VMCNg 2b7.2
-36	0	0	0	0	0	1	1	1
-45	0	0	0	0	0	1	1	1
-259	0	0	0	0	0	1	1	1
-320	0	0	0	0	0	1	1	1
-363	0	0	0	0	0	1	1	1
-376	0	0	0	0	0	1	1	1
-400	0	0	0	0	0	1	1	1
-416	0	0	0	0	0	1	1	1
-426	0	0	0	0	0	1	1	1
-470	0	0	0	0	0	1	1	1
<b>-08</b>	0	0	0	<b>0</b>	<b>1</b>	1	1	1
<b>-194</b>	0	0	0	<b>0</b>	<b>1</b>	1	1	1
<b>-554</b>	0	0	0	<b>0</b>	<b>1</b>	1	1	1
<b>-629</b>	0	0	0	<b>0</b>	<b>1</b>	1	1	1
<b>-28</b>	0	0	<b>0</b>	<b>1</b>	1	1	1	1
<b>-38</b>	0	0	<b>0</b>	<b>1</b>	1	1	1	1
-15	-	1	1	1	1	0	0	0
-23	1	1	1	1	1	0	0	0
-31	1	1	1	1	1	0	0	0
-337	1	1	1	1	1	0	0	0
-345	1	1	1	1	1	0	0	0
-360	1	1	1	1	1	0	0	0
-337	-	1	1	1	1	0	0	0
-397	1	1	1	1	1	0	0	0
-409	1	1	1	1	1	0	0	0
-428	1	1	1	1	1	0	0	0
-505	-	1	1	1	1	0	0	0
-595	1	1	1	1	1	0	0	0
-697	1	1	1	1	1	0	0	0

**Table 2.** List of new markers that were developed from Pinot noir genome sequence and were utilized on 4 different populations.

Name	PN contig id	New marker	Amp size	04190	9621	04373	04191
A010	VV78X214158.8	VVCh14-02	170	Y	Y	N	Y
		VVCh14-56			Y		Y
UDV095	VV78X004565.11	VVCh14-09	170	Y		Y	
		VVCh14-10	210	Y	N	Y	N
VMCNg2b7.2	VV78X072246.8	VVCh14-27	193	Y	Y	Y	Y
VMCNg3h8	VV78X190796.4	VVCh14-28	167	Y	Y	Y	Y
		VVCh14-29	200	Y	Y	N	Y
		VVCh14-30	206	Y	Y	N	Y
		VVCh14-70	193	Y	Y	N	Y

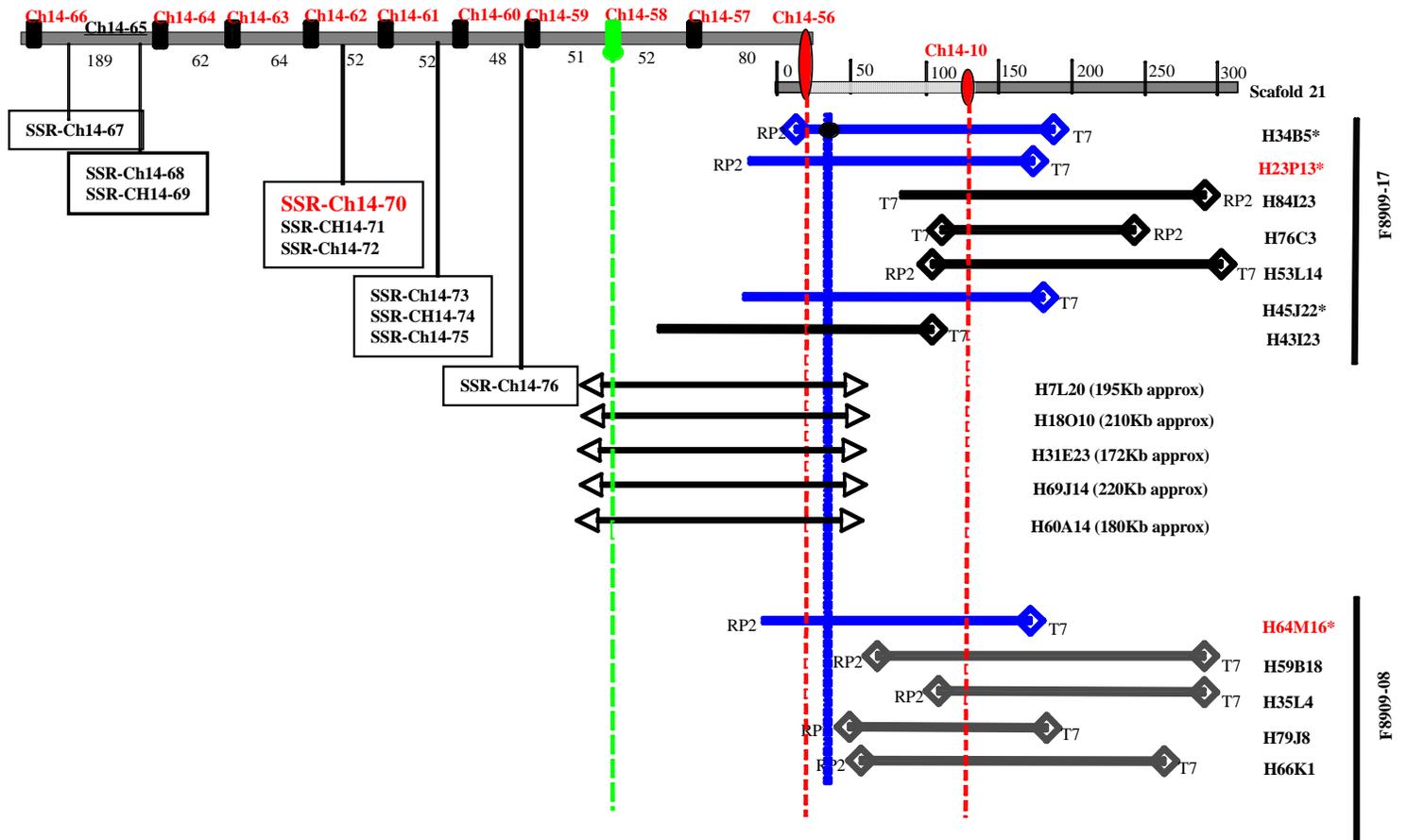
**Table 3.** Parentage and species information for populations and genotypes being used to map PD resistance.

Population / Genotype	Species / Parentage
b42-26	<i>V. arizonica/girdiana</i>
b43-17	<i>V. arizonica/candicans</i>
b40-14	<i>V. arizonica</i>
D8909-15	<i>V. rupestris</i> A. de Serres x b42-26
F8909-08 and F8909-17	<i>V. rupestris</i> A. de Serres x b43-17
F2-7 and F2-35 (females)	<i>V. vinifera</i> (Carignane x Cabernet Sauvignon)
9621	D8909-15 x F8909-17
0023	F8909-15 x <i>V. vinifera</i> B90-116
03300/5	101-14Mgt ( <i>V. riparia</i> x <i>V. rupestris</i> ) x F8909-08
04190	F2-7 x F8909-08
04191	F2-7 x F8909-17
04373	F2-35 x b43-17
05347	F2-35 x b42-26
07744	R8918-05 x <i>V. vinifera</i> Airen
07386	R8917-02 x <i>V. vinifera</i> Airen



**Figure 1.** SSR-based genetic map of chromosome 14 from the 9621 (left) and 04190 (right) populations.

Fig. 2. Detail of physical map of chromosome 14 for the region that harbor resistance to PD. Preliminary screening was carried out with Ch14-10 Ch14-56. Currently the PdR1 locus reside between Ch14-56 and Ch14-70. Five clones are also positive with screening marker Ch14-58 (in green). Only Ch14-70 marker was polymorphic and could be mapped in three populations.



## BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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

### ABSTRACT

The use of marker-assisted selection (MAS; see our companion report) and our acceleration of the seed to seed breeding cycle to three years have allowed very rapid progress towards our goal of Pierce's disease (PD) resistant winegrapes. Populations from the 2007 crosses were screened with MAS for both PD and powdery mildew (*Run1*) where appropriate and only those with the markers were planted in the field. The 2008 crosses were made to: 1) Use the *PdR1* allele from 8909-08 to broaden the *vinifera* winegrape lines at the 93.75% *vinifera* level; 2) Combine *PdR1* with the powdery mildew resistance gene *Run1* at the 90.6% *vinifera* level; 3) Combine *PdR1* with the LG13 powdery mildew resistance gene *REN1* at the 87.5% *vinifera* level; 4) Use 8909-17 based resistance with diverse *vinifera* winegrapes to produce resistant progeny at the 87.5% *vinifera* level; 5) Use the F1 progeny of the homozygous PD resistant b40-14 *V. arizonica* to produce a breeding and mapping population that is 75% *vinifera*; 6) use elite winegrapes to broaden and expand the *V. shuttleworthii* breeding lines producing progeny that are 75% and 87.5% *vinifera*; and 7) Produce rootstocks with *PdR1* and broad-based nematode resistance. Inoculations were made to selections with *PdR1* and either 87.5% and 75% *vinifera* at our Beringer, Napa County trial. Finally, small-scale wine lots were made from five 87.5% *vinifera PdR1* selections from wine grape backgrounds. Fruit evaluation and must analysis were performed on numerous other promising progeny at this level.

### INTRODUCTION

The Walker lab is uniquely poised to undertake this important breeding effort, having developed rapid screening techniques for *Xylella fastidiosa* (*Xf*) resistance (Buzkan et al. 2003, Buzkan et al. 2005, Krivanek et al. 2005a 2005b, Krivanek and Walker 2005), and in possession of unique and highly resistant *V. rupestris* x *V. arizonica* selections, as well as an extensive collection of southeastern grape hybrids, to allow the introduction of extremely high levels of *Xf* resistance into commercial grapes. They have produced plants that are 93.75% *V. vinifera*, from winegrape cultivars, with resistance from our b43-17 *V. arizonica/candicans* resistance source. There are two sources of *PdR1*, 8909-08 and 8909-17, both siblings of b43-17. These selections have been introgressed into a wide range of winegrape backgrounds over multiple generations, and resistance from southeastern United States (SEUS) species is being advanced in other lines. However, the resistance in these later lines is complex and markers have not been developed to expedite breeding.

### OBJECTIVES

1. Breed Pierce's disease (PD) resistant winegrapes through backcross techniques using high quality *V. vinifera* winegrape cultivars and *Xf* resistant selections and sources characterized from our previous efforts.
2. Continue the characterization of *Xf* resistance and winegrape quality traits (color, tannin, ripening dates, flavor, productivity, etc.) in novel germplasm sources, in our breeding populations, and in our genetic mapping populations.

### RESULTS AND DISCUSSION

**Objective 1** – The breeding cycle for the development of PD resistant grapes has been reduced to three years (seed to seed) using marker-assisted selection (MAS) with the b43-17 resistance sources and their progeny. Our goal at this point is to introgress our PD and *PdR1* resistance sources into a large number of *V. vinifera* winegrapes backgrounds. Until we get to the backcross 4 (BC4) (96.8% *V. vinifera*), there is not much point to growing very large numbers of progeny from any given cross. With the 3-year seed-to-seed cycle, we will plant BC4 progeny in 2010. **Table 1** presents the crosses made in Spring 2008 with the numbers of seeds produced. The goals of this years crosses were: 1) Use the *PdR1* allele from 8909-08 to broaden the *vinifera* winegrape lines at the 93.75% *vinifera* level; 2) Combine *PdR1* with the powdery mildew resistance gene *Run1* at the 90.6% *vinifera* level; 3) Combine *PdR1* with the LG13 powdery mildew resistance gene *REN1* at the 87.5% *vinifera* level; 4) Use 8909-17 based resistance with diverse *vinifera* winegrapes to produce resistant progeny at the 87.5% *vinifera* level; 5) Use the F1 progeny of the homozygous PD resistant b40-14 *V. arizonica* to produce a breeding and mapping population that is 75% *vinifera*; 6) Use elite winegrapes to broaden and expand the *V. shuttleworthii* breeding lines producing progeny that are 75% and 87.5% *vinifera*; and 7) Produce rootstocks with *PdR1* and broad-based nematode resistance.

To date, three groups of plants have been greenhouse screened for *Xf* resistance in 2008 (**Table 2**). Group A tests were done to verify the expression of *PdR1* from b43-17 in the 04190 (*V. vinifera* F2-7 x 8909-08) population. This group also tested advanced 87.5% *V. vinifera PdR1* carrying parents, which were used in the 2007 crosses to create 94% *V. vinifera* progeny with *PdR1*. This group also included the parents of new mapping populations: one based on single gene resistance from *V.*

*arizonica* b40-14 (R89); and the other based on multigenic resistance from *V. arizonica/girdiana* b42-26 (05347). The Group B tests examined progeny of Midsouth and BD5-117 crossed to advanced *vinifera* wine types. Both of these parents continue to produce resistant progeny, but very few and in ratios that suggest a complex inheritance; the use of BD5-117 produced seven resistant plants in population of 18 and none in an additional population of eight. The use of Midsouth and a *V. smalliana* x *vinifera* parent both resulted in one resistant plant of six progeny. All 13 progeny from a cross using Haines City had lower ELISA values than the known resistant Blanc du Bois in the greenhouse screen. Two of these progeny were used as parents in the 2008 crosses (**Table 1e**) and greenhouse tests found them to be as resistant as parents carrying *PdR1*, although Haines City does not contain *PdR1*. Eight promising rootstocks based on *PdR1* were also tested in this group and all were resistant. Group C tests focused on recombinants from our 2006 breeding populations to aid fine-scale *PdR1* mapping efforts and on the F1 progeny of b40-14 crossed to *V. vinifera* discussed below.

**Objective 2** – Although resistance from other backgrounds is complex and quantitative, which results in few resistant progeny from crosses to *vinifera* cultivars, we continue to advance a number of lines. In order to better understand the limits of other PD resistance sources, the following resistance sources are being studied:

*V. arizonica* b42-26 – *Xf* resistance in the 0023 (D8909-15 (*V. rupestris* x b42-26) x *V. vinifera* B90-116) population is strong, but is quantitatively inherited. Quantitative trait locus (QTL) analysis has identified a major QTL that accounts for about 20% of the variability (preliminary results). Previous efforts with the 0023 were focused on table grape breeding, and found that the 0023 population (F1, 1/4 b42-26) had about 30% resistant progeny. This population has a large number of weak genotypes, few females with viable seeds, and generally lacks fertility. The progeny of a cross of a resistant 0023 genotype crossed back to *vinifera* (BC1) were tested and only 7% were resistant. Greenhouse testing of 05347 (*vinifera* F2-35 x b42-26) to examine the b42-26 resistance source in a less complex background (without the confounding effect of *V. rupestris*) was completed last year. In 2007, crosses using elite *V. vinifera* wine type pollen were made to a number of females in this population and 140 genotypes were planted this spring for future evaluation. This spring the cross 05347 was repeated to expand this mapping population (**Table 1e**).

*V. arizonica* b40-14 – Over the last seven years, we have greenhouse tested 45 F1 progeny of PD susceptible *V. rupestris* Wichita Refuge crossed with PD resistant b40-14 (R89 series). Only one genotype has failed to test resistant over that time period (data not shown). In 2006, we crossed *V. vinifera* F2-35 x b40-14 and established 198 seedlings for testing. In 2007, we crossed the *V. vinifera* variety Airen onto two of the PD resistant R89 genotypes and planted a total of 163 genotypes in Spring 2008. We have initiated greenhouse screening of these two populations for initial mapping of a new *PdR* locus. From our previous R89 testing, we expect the F1 progeny of b40-14 crossed to *V. vinifera* to all be PD resistant. To date, we have completed greenhouse testing of seven genotypes. Lack of PD phenotypic symptoms on all seven and very low mean cfu/ml ELISA values for the first three give some credibility to that expectation. We are planning on using the progeny of the 06339 crosses made this year (**Table 1e**) for further mapping efforts to better characterize this very strong, and morphologically and genetically different source of PD resistance.

*V. shuttleworthii* Haines City – Based on the encouraging greenhouse screen results for this resistance source as reported above, in 2008 we made the BC1 (75% *vinifera*) and BC2 (88% *vinifera*) using a BC1 from our earlier table grape work that tested particularly well and had reasonable wine grape characteristics (**Table 1e**).

Given that low levels of *Xf* exist in resistant plants, it will be important to also have PD resistant rootstocks to graft with resistant scions and prevent them from dying on susceptible rootstocks. We completed screening of eight promising progeny from crosses of 101-14 x F8909-08. Evaluation for grafting ability and testing against phylloxera and nematodes and finally field testing will follow. In 2008, we made additional PD resistant rootstock crosses resulting in 1397 seeds (**Table 1f**).

**Field and Wine Evaluations** – The A81 series (BC1, 75% *vinifera*) 8909-08 allele type of *PdR1* is in its third year of field testing at the Beringer Yountville test site; ELISA and visual symptom results have been consistent with greenhouse assays. Selections from the 045554 (BC2, 88% *vinifera*) were grafted onto Dog Ridge (currently the only certified PD resistant rootstock) and were planted at Yountville in Spring 2007. These genotypes have been marker tested and their PD resistance status confirmed by greenhouse testing. Twelve genotypes were resistant, four were recombinants (one resistant and three susceptible in the greenhouse test). These were needle inoculated for the first time on May 22, 2008. The A81 series was inoculated at the same time for the second time. Both groups will be sampled for ELISA testing this fall.

Three of eight advanced red wine selections (U0501-12, U0502-01 and -10) containing *PdR1* that are 87.5% *vinifera* from crosses with Syrah and Chardonnay were replicated for small-scale fermentation in 2006 and wines made again this fall. Between four and 20 liters of wine from each were produced along with similar amounts of Barbara and Zinfandel as *V. vinifera* controls and Lenoir as the standard PD resistant control to standardize these very small-scale fermentations. Two additional wines were made for the first time this year from siblings of the above crosses: U0502-20 (white) and U0502-26 (red). All these selections were evaluated for their productivity, flowering and ripening dates, and berry and cluster weights. Vine, fruit and juice analyses are presented in **Tables 3a** and **3b**, and images of the leaves and fruit are in **Figure 1**. Numerous other genotypes from crosses involving elite *vinifera* wine cultivars were examined for fruit evaluation and must analysis. ETS Laboratories ([www.etslabs.com](http://www.etslabs.com)) of St. Helena kindly donated their fruit analysis and phenolics panel, which

uses a wine-like extraction to model a larger fermentation. Surprisingly, none of the U05 series analyzed contained significant levels of diglucoside anthocyanins, which are negative quality markers for hybrid wines with American grape species and which would create problems with exporting wines to the EU. Cuttings of the best of these were established in our Davis vineyard this spring so that we can get small-scale wine lots made for evaluation in 2009. A new MS student is examining the reasons for the lack of diglucoside anthocyanins in these selections to determine whether the *arizonica*-resistance sources possess these anthocyanins.

#### Powdery Mildew

Any new PD resistant variety should also be resistant to powdery mildew. We have been exploring powdery mildew resistance in a number of backgrounds including Olmo's VR (*vinifera* x *rotundifolia*) hybrids, which form the base of international efforts at characterizing *Run1*, the *rotundifolia*-based locus responsible for resistance to powdery mildew. The 2008 season field evaluations of the 2006 crosses show the markers correlating perfectly with field resistance to powdery mildew on the leaves, canes, rachis and fruit. The goal with these individuals is to cross our advanced PD resistant selections with selections from these powdery mildew resistant progeny (**Table 1b**). This spring 537 plants of crosses between genotypes with *PdR1* and other types with *Run1* were planted on 1' x 1' spacing in a nursery to screen for powdery mildew resistance. This allowed the elimination of weak plants and reduces the cost of MAS screening where we continue to see segregation distortion against the *Run1* locus in some lines. We tested 136 plants in the nursery screen for powdery mildew resistance cell of which are preparing for marker testing for both *Run1* and *PdR1* to verify the utility of MAS for the combined traits. Plants with both loci will go to the field for evaluation of fruit and horticultural characteristics. In 2008 we also made crosses to examine powdery mildew in two other backgrounds: a source of the *RENI* locus (a separate powdery mildew resistance locus on chromosome 13, from the *V. vinifera* table grape Karadzhandal (**Table 1c**) and the Chinese species *V. romanetii*. We produced 564 seeds using Karadzhandal, clusters from crosses with *V. romanetii* are being processed.

#### **CONCLUSIONS**

This project continues to breed PD resistant winegrapes with the primary focus on the *PdR1* resistance source so that progress can be expedited with MAS. Populations with *Xf* resistance from other sources are being maintained and expanded, but progress is slower with these sources. We continue to supply plant material, conduct greenhouse screens and develop new mapping populations for our companion project on fine-scale mapping of PD resistance leading to the characterization of the *PdR1* resistance locus. The first testing of small-scale wine from advanced selections with 87.5% *vinifera* from winegrapes was done in Fall 2007, and they scored remarkably well. Evaluation of the 2008 wines is pending.

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**Table 1.** 2008 crosses and numbers of seed produced.

Resistant Type	<i>Vinifera</i> Parent of Resistant Type	<i>Vinifera</i> Types used in 2007 crosses	# Seeds Produced
1a. Monterrey <i>V. arizonica/candicans</i> resistance source (F8909-08) to produce progeny with 93.75% <i>V. vinifera</i> parentage.			
U0502	Chardonnay	F2-7 (Cab x Carignane)	262
U0505	Cabernet Sauvignon	Tannat	694
1b. Monterrey <i>V. arizonica/candicans</i> resistance source (F8909-08) and <i>Run1</i> powdery mildew resistance to produce progeny with 90.6% <i>vinifera</i> parentage.			
U0502	Chardonnay	06353, e78 allele pattern	82
U0505	Cabernet Sauvignon	06717, e78 allele pattern	138
1c. Monterrey <i>V. arizonica/candicans</i> resistance source (F8909-08) and a <i>vinifera</i> PM resistance source to produce progeny with 87.5% <i>vinifera</i> parentage.			
A81-17	A38-7	Karadzhandal	564
1d. Monterrey <i>V. arizonica/candicans</i> resistance source (F8909-17 allele) to produce progeny with 87.5% <i>V. vinifera</i> parentage.			
06324	Chenin blanc	Airen, Cabernet Sauvignon, Chardonnay, Tannat	484
06372	Malaga Rosada	Clairette blanche, Tannat	946
06381	F2-7 (Cab x Carignane)	Tannat	151
1e. Other PD resistance sources: b40-14 <i>V. arizonica</i> (06339) progeny are 87.5% <i>vinifera</i> . The <i>V. shuttleworthii</i> PD resistance sources 0098-03 progeny are 87.5% <i>vinifera</i> and 04394 progeny are 75% <i>vinifera</i>			
06339	F2-35 (Cab x Carignane)	Malaga Rosada, Tannat	325
0098-03	NR	Cabernet Sauvignon, Chardonnay	324
04394	NR	Cabernet Sauvignon, Clairette blanche, F2-35, Tannat	1,130
b42-26	NR	F2-35	827
1f. Rootstock crosses to combine PD and nematode resistance.			
03300-048	06301, Wyoming Riparia, Riparia Gloire, 44-53 mgt		1,397

**Table 2.** PD resistant winegrape progeny just completed or currently in greenhouse screening for PD resistance.

Group	Genotypes	N	Inoculation Date	ELISA Date	Resistance Source(s)
A	04190, 9621, 2007 parents	150	10/18/2007	1/31/2008	b43-17 (both alleles)
B	D89, R89, 9621, 03300/5 (PD rootstocks), 03182, 03187, 04183, 04394, 2007 parents retest	157	3/20/2008	6/26/2008	b43-17, BD5-117, Midsouth, Haines City
C	2006 recombinants, 06339	29	5/20/2008	8/21 & 9/25/08	b43-17, b40-14

**Table 3a.** Phenotypic observations of reference varieties and select progeny with the *PdR1* resistance source.

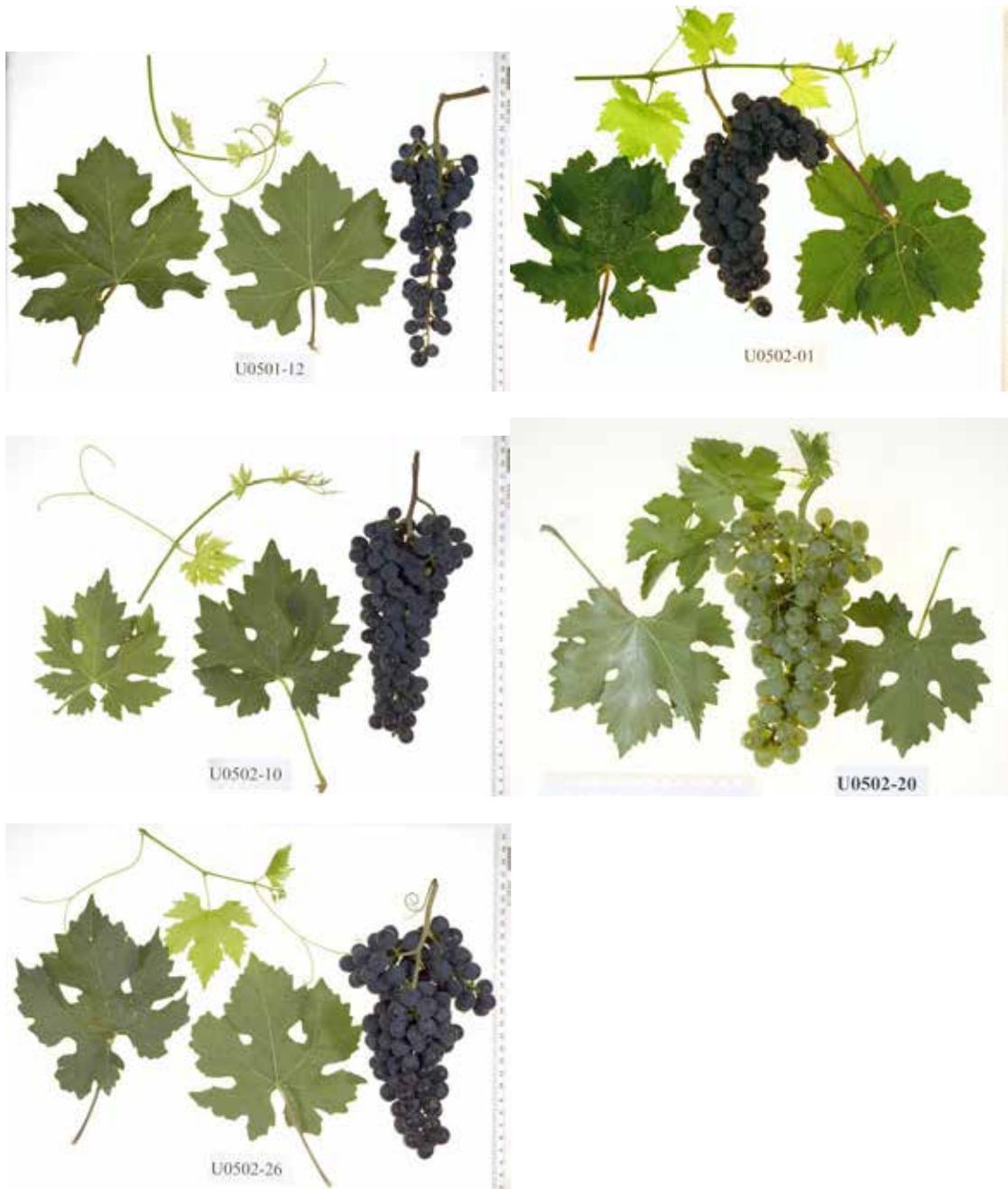
Genotype	Parentage	Percent <i>vinifera</i>	2008 Bloom Date	Berry Color	Berry Size (g)	Avg Cluster Wt. (g)	Ripening Season	Prod 1= v low 9= v high
Barbara	Historic	100%	5/5/08	B	2.4	290	Late	6
Zinfandel	Historic	100%	5/5/08	B	2.6	405	Mid	7
U0501-12	A81-138 x Syrah	87.5%	5/11/08	B	1.0	160	late	4
U0502-01	A81-138 x Chardonnay	87.5%	5/5/08	B	2.0	210	mid-late	4
U0502-10	A81-138 x Chardonnay	87.5%	5/5/08	B	1.7	275	very early	8
U0502-20	A81-138 x Chardonnay	87.5%	5/10/08	W	2.0	201	Late	8
U0502-26	A81-138 x Chardonnay	87.5%	5/9/08	B	2.1	375	mid-late	6
Lenoir	<i>V. aestivalis</i> hybrid	<50%	5/16/08	B	0.8	201	Late	6

**Table 3b.** Analytical evaluation of reference varieties and advanced selections with the *PdRI* resistance source. All analysis courtesy of ETS Laboratories, St. Helena, CA.

Genotype	L-malic acid (g/L)	°Brix	potassium (mg/L)	pH	TA (g/100mL)	YAN (mg/L (as N))	catechin (mg/L)	tannin (mg/L)	Total antho-cyanins (mg/L)
Barbara	2.83	25.0	2170	3.36	0.87	431	31	201	300
Zinfandel	2.43	23.5	1870	3.55	0.62	191	34	322	386
U0501-12	3.22	27.2	2020	3.51	0.74	3.98	48	781	1161
U0502-01	7.36	23.3	3240	3.70	0.96	567	81	364	530
U0502-10	4.36	22.3	1800	3.47	0.82	305	73	565	828
U0502-20	4.94	24.0	2600	3.62	0.90	544	-	-	-
U0502-26	5.55	24.3	2420	3.64	0.91	699	65	225	811
Lenoir	5.54	28.7	3050	3.63	0.83	230	160	405	2396

**Table 3c.** Sensory evaluation of reference varieties and advanced selections with the *PdRI* resistance source.

Genotype	Juice Hue	Juice Intensity	Juice Flavor	Skin Flavor	Skin Tannin (1=low, 4= high)	Seed Color (1=gr, 4= br)	Seed Flavor	Seed Tannin (1=high, 4= low)
Barbara	pink-brown	low	neutral, acidic	jam, berry	2	4	nutty,spicy	3
Zinfandel	orng-brown	medium	jam, hay	fruity	2	4	nutty,bitter	1
U0501-12	red	med-dark	fruity	fruit jam	2	4	neutral	2
U0502-01	pink-brown	medium	fruity-PN	sweet fruit	1	3	spicy	1
U0502-10	pk-red-orng	med-dark	slight vegetal	mildly fruity	1	4	nutty,spicy	1
U0502-20	green	medium	neutral, fruity	fruity	1	4	spicy,bitter	1
U0502-26	pink	medium	bright, spicy	fruity	2	4	nutty	3
Lenoir	red	dark	mildly fruity	fruity	1	4	nutty	4



**Figure 1.** Pictures of the 87.5% *vinifera* PD resistant wine grape selections used for small-scale winemaking at UCD in 2008.



# ***Section 6: Economics***





## THE ECONOMICS OF PIERCE'S DISEASE IN CALIFORNIA

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**Reporting Period:** The results reported here are from work conducted July 2008 to October 2008.

### ABSTRACT

The goal of this research project is to estimate the medium to long-run economic impact to growers and consumers of California's diverse agricultural crops, and to taxpayers from the establishment of the glassy-winged sharpshooter (GWSS) in California, and to estimate how different public policy responses affect the costs and benefits to growers and consumers. The costs and benefits to consumers, producers and taxpayers will be estimated using market models that take into account changes in the costs of production, total production by newly infested growers in California and all other growers, trade, and consumer demand.

### INTRODUCTION

In 1989, a pest new to California, the glassy-winged sharpshooter (GWSS), was collected in Irvine, CA. Since then the GWSS has spread throughout most of southern California and limited infestations of the GWSS are found as far north as the southern San Joaquin Valley counties of Kern and Fresno (CDFA 2008). Initially thought to mimic the feeding patterns of native California sharpshooters, by the late 1990s it became apparent that the GWSS was a more deadly vector of the bacterium *Xylella fastidiosa* (*Xf*) than native sharpshooters because of its wide host range and ability to feed on and transmit the pathogen to older grape wood. Pierce's disease (PD) has been endemic to California since the 19<sup>th</sup> century. However, because the GWSS is a more deadly vector of the bacterium, its establishment in southern counties has led to an increase in both the severity and incidence of the disease in infested regions. Initial infestations in the Temecula Valley caused large losses for growers due to vine death and the removal of vineyards. In 1999, losses to growers were estimated to be \$46 million (Brown et al. 2002).

In 2000, soil applied imidacloprid (Admire<sup>®</sup>) was granted a Section 18 emergency use permit (Jetter et al. 2001) and has since proven to be the most effective chemical treatment of GWSS (Barry Hill, CDFCA, 2008, personal communication; Jennifer Hashim-Buckey, UCCE, 2008, personal communication; Judy Leslie-Stewart, Consolidated Central Valley Table Grape Pest and Disease Control District, 2008, personal communication). In the Central Valley, the use of Admire<sup>®</sup> replaced the use of Provado<sup>®</sup>, a foliar formulation of imidacloprid that was less effective in controlling the GWSS. Consequently, one cost to the grape industry to treat GWSS is not the cost of Admire<sup>®</sup>, but the difference in cost between Admire<sup>®</sup> and Provado<sup>®</sup>. Manual controls include pulling out infected vines, or in some cases, vines that may be infected (Barry Hill, CDFCA, 2007, personal communication) in order to remove the bacterium from the vineyard before it can be transmitted by GWSS or other vectors.

Public agencies, including research universities and governmental agencies, have also been conducting research on effective techniques to manage the GWSS. With regard to the management of GWSS and PD, universities have been engaged in research involving the use of biological control agents for the GWSS and developing hybrid varieties resistant to PD. For the biological control program, a number of egg parasitoids have been imported into California and released to reduce populations of GWSS. To date, these parasitoids appear to be most effective in citrus, and in the coastal and interior regions of southern California. With regard to plant breeding, research on a new variety of PD resistant wine grapes used in the production of blended wines is promising, but is still in the testing stage (Andrew Walker, UC Davis, 2008, personal communication).

Governmental agencies have been involved in two control programs to manage and contain the GWSS. One treatment involves the control of the GWSS on citrus before it can move into vineyards and transmit the PD bacterium. This program overcomes the divide created between the citrus growers who are not typically affected by GWSS and would not typically treat for GWSS, and grape growers who are negatively affected by large populations of GWSS migrating from citrus to grapes. Currently any citrus grove within ¼ mile of a trapped vine (i.e. a trap placed in a vineyard contains a GWSS) is treated, unless the grove is located along the northern boundary of the infestation, in which case the barrier is ½ mile from a trapped vine. While some citrus growers may benefit from the control of the GWSS and other pests in their groves, chemical treatments may also disrupt IPM pest control practices, imposing additional costs on the citrus industry. All these effects are important to include in any economic analysis of PD in California.

Finally, there is a state quarantine in place to limit the spread of the GWSS into uninfested grape growing areas of California. The quarantine consists of on-site sanitation practices, inspections and surveys, and spraying plant leaves with a chemical

such a methomyl (Lannate<sup>®</sup>) to treat difficult to detect egg masses not caught by inspectors. As a result, management of PD in California includes a bundle of methods that have economic impacts on the wine, table and raisin grape, citrus, and nurseries industries. These different methods to control GWSS and PD have significantly improved the situation, and damages today are not as severe as initially anticipated. Even though better methods have been developed to manage GWSS, the costs of production for each industry have not returned to pre-GWSS infestation levels.

Due to the size of the industries affected by the control of GWSS and PD in California, even small changes in the costs of production can have a major impact on the benefits and costs to producers, consumers and taxpayers. The grape industry is a major agricultural producer in California. With average annual revenues (2004-2006) to the wine, table and raisin grape industries totaling \$3 billion, grape production is the largest fruit industry in California (USDA 2006a). When revenues from the citrus and nursery industries are combined with the revenues from the grape industry, their total revenues of \$20.8 billion make this the second largest agricultural sector in the U.S. behind corn (\$26.8 billion) and before soybeans (\$18.3 billion) (USDA 2006a; USDA 2006b; Jetter 2007).

Growers with GWSS and PD are affected economically through higher costs of production. Given the size of the grape industries in infested counties, higher costs of production will put upward pressure on market prices. With higher market prices newly infested growers are able to recoup a portion of their higher costs of production. Higher market prices will cause consumers to purchase less, however. With higher prices and lower consumption, consumers are also worse off from the establishment of GWSS. The higher prices will make growers in uninfested areas of California, and in the rest of the U.S. better off. These growers receive the higher market prices, but do not incur the higher costs of production. Additional costs accrue to taxpayers who bear the costs of the public management programs. An economic analysis needs to include all these effects.

## OBJECTIVES

1. Estimate the costs and benefits to wine grape, table grape and raisin growers, consumers and taxpayers from changes in the costs of grape production due to the establishment of the GWSS. The changes in production costs will be based on current best practices and will include chemical treatments, removal of infested vines, quarantine restrictions and public control programs.
2. Estimate the costs and benefits of public policies to manage and contain the GWSS. The public control policies include public programs to treat the GWSS in citrus to prevent its spread into grape vineyards in the spring, and the associated containment program. An additional public policy to contain the spread of GWSS and, thus, the transmission of PD, is a state quarantine on the movement of nursery, citrus and other host crops out of infested regions.
3. Estimate the optimal check-off rate for the grape industries that benefit from the treatment of the GWSS on overwintering crops. The rate will take into account the costs and benefits to the grape growers in both infested areas and areas that benefit from the containment of the GWSS within infested areas, and the costs and benefits to growers of overwintering crops. The results of the first two objectives will be used as parameters in the model that estimates check-off rates.

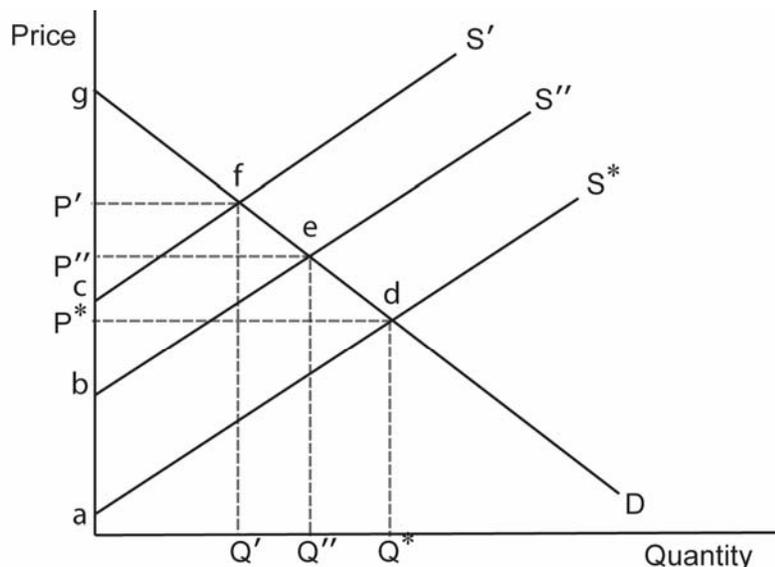
### *Analytical Approach to Measuring the Economic Effects of Pierce's Disease in California*

The increase costs of production affect newly infested producers directly because they bear the burden of paying the increased costs of production; however, consumers and producers are also affected through the market effects of the changes in the costs of production. These effects can be shown graphically. **Figure 1** presents the market effects of the increased incidence of PD due to the establishment of the GWSS on the market for grapes (here defined as wine, table and raisin grapes) and the development of effective GWSS control methods. The market contains suppliers, who are willing to supply grapes and initially represented by supply curve  $S^*$ . The supply curve is upward sloping because as prices increase growers will grow more grapes and supply more grapes to the market. The market also contains consumers who purchase grapes and are represented by the demand curve  $D$ . The curve is downward sloping because as prices decrease, consumers will want more grapes. The market is in equilibrium at point  $d$ . At point  $d$ , price is equal to  $P^*$  and the quantity demanded by consumers,  $Q^*$ , is exactly equal to the quantity supplied by producers.

At the initial equilibrium point there are some consumers who are willing to pay more than  $P^*$  and some producers who could offer their products at a market price less than  $P^*$  and still make a profit. The consumers who are willing to pay more may have more income than other consumers, or just a greater preference for grapes and grape products. The maximum amount that each consumer would be willing to pay for grapes is represented by the demand curve. The difference between what consumers are willing to pay and the actual price that they do pay is called consumer welfare. In **Figure 1**, consumer welfare is equal to area  $P^*gd$ .

The producers who could profitably accept less than the market price are producing grapes at a lower cost than other producers. The minimum amount at which each producer would supply grapes to the market is represented by the supply curve. The difference between the price at which producers would offer their goods to market and the actual price they receive is called producer welfare. In **Figure 1**, producer welfare is equal to area  $P^*ad$ .

The establishment of the GWSS in select counties in California initially causes the supply curve to shift up from  $S^*$  to  $S'$ . For supply curve  $S'$  the new equilibrium point is  $f$ . At point  $f$ , the equilibrium price is  $P'$ , and the equilibrium quantity is  $Q'$ . For example, this shift could represent the losses in the Temecula Valley as PD spread with the GWSS and diseased vines were removed.



**Figure 1.** Market effects for grapes produced in GWSS infested counties.

Over time, management of the GWSS improves and losses decrease. This causes the supply curve to shift from  $S'$  to  $S''$ . Thus, supply curve  $S''$  represents the current situation with respect to the management of GWSS and PD. For supply curve  $S''$ , the new equilibrium point is  $e$ , price is  $P''$  and market supply is  $Q''$ . For example, over time growers in the Temecula Valley learned that treating a vineyard with the Admire<sup>®</sup> formulation of imidacloprid can effectively reduce GWSS populations and the incidence of PD. While vineyards can now be replanted, the cost to produce grapes has increased above the pre-GWSS environment because growers must now incur the additional expense of applying Admire<sup>®</sup>.

For Objective 1, the losses to the different grape industries in California will be estimated assuming a shift in the supply curve from  $S$  to  $S''$ . The estimated losses to consumers and producers will be equal to area  $beda$ . For Objectives 2 and 3, the initial market equilibrium will reflect the current situation and practices in California. In **Figure 1**, this is at point  $e$ , where the demand curve,  $D$ , and supply curve,  $S''$ , intersect. It is assumed that should the public management of GWSS be discontinued, the supply curve would shift upward again. As an example, assume that the supply curve  $S''$  shifts back up to  $S'$  if the public programs are discontinued. The estimated losses to producers and consumers would then be equal to area  $cfeb$ .

The graphical analysis above illustrates the situation in which all grape production in a specific region is affected. Within that region all growers are worse off due to higher costs, but losses to some degree are minimized through higher market prices. Consumers are worse off due to higher prices, and lower consumption. With regard to the case of PD in California, growers located in regions free of the GWSS, and growers in other states where the GWSS is native, will be better off due to the establishment and spread of GWSS in select counties of California. Growers without GWSS receive higher prices, but do not incur higher management costs due to control of GWSS. Additional costs accrue to taxpayers who bear the costs of the public management programs. An economic analysis needs to include all these effects. Due to the relative newness of the establishment of the GWSS, the scenarios estimated will include a sensitivity analysis that reflects the best estimates of the range of possible effects by scientists researching and managing the GWSS.

Once all costs and benefits of the establishment of the GWSS are estimated, and the costs and benefits of the public program to treat GWSS in citrus are estimated, the check-off rates that growers would need to pay in order to take over the citrus GWSS control program will be determined. Because research and the most effective means to complete the public control program is still being conducted, there is still a vital role public agencies have in reducing the short-term effects on producers and, especially, consumers, of commodities affected by  $Xf$  and GWSS. In the long-run though, taxpayer financed control of

the GWSS will probably not continue. Even though public funding will continue for the foreseeable future, this research project will put the economic evaluation tools into place if budgetary shortfalls at the state or federal level put pressure on policy makers to downsize the public program, and the industries affected by GWSS need to respond quickly.

## RESULTS

### *Economic Effects in the Southern San Joaquin Valley*

A meeting was held with grape growers, and public agencies involved with the public control program to determine how the establishment of GWSS has affected different groups in this area. Three groups are affected by control of the GWSS in the southern San Joaquin Valley, grape growers, citrus growers and tax payers. A majority of grape growers apply imidacloprid annually to control GWSS and prevent the vine-to-vine transmission of PD. Applications are typically at the maximum rate of 14 fl oz an acre (4.6 lb ai/gal formulation) through the irrigation system. While there is a low incidence of PD in Kern and Fresno counties, the incidence can rapidly increase should GWSS not be controlled. The treatments with imidacloprid also provide some benefits to the control of variegated leafhopper and are a suppressant of the grape and vine mealybug. No quarantine costs are incurred by grape growers as mature fruit destined for the fresh market is hand harvested and field packed.

Citrus growers are affected by the public control program and quarantines against moving citrus out of infested areas. With the public control program, citrus growers are reimbursed for their treatments of GWSS. Participation in the public program is currently voluntary for the citrus grower. To control for GWSS in citrus, an application of Assail is made in the fall followed by an application of imidacloprid in the spring. Imidacloprid is applied at a rate of 32 fl oz an acre (2 lb ai/gal formulation) through the irrigation system. The control program is conducted on an area-wide basis to achieve longer-term reductions in GWSS populations. The control in citrus occurs once every three years unless monitoring indicates an increase in GWSS populations. The treatments with imidacloprid may provide minor benefits to control of other pests. Imidacloprid may help suppress nematodes, citrus peelminer and California red scale. Better control of these insects can be achieved by applying an additional amount of imidacloprid when treating for GWSS; however, the grower is responsible for those costs. The citrus industry is affected by the interior quarantine and fruit from infested areas needs to be inspected and treated before leaving a quarantine area. Quarantine treatments involve fumigation using EverGreen (pyrethrum + piperonyl butoxidor). Turbocide has also been mentioned as a material that can be used as a fumigant. Taxpayers bear the costs of the public program and the state quarantine. These costs include the payments to citrus growers, management costs of the program, and inspection and monitoring costs.

The remaining areas that will be included in this study are the southern California grape growing areas that also treat for GWSS, but where the public program is less widespread, the northern San Joaquin Valley grape growing area that is currently free of GWSS, but has a higher incidence of PD, and the major wine grape growing areas of northern California that are also currently free of GWSS. Growers in the areas free of GWSS do not incur any direct costs due to the presence of GWSS. They are also beneficiaries of the quarantine program to contain GWSS in the southern San Joaquin Valley. Thus, their benefits need to be included in the analysis of Objective 3.

## CONCLUSIONS

The containment of GWSS affects both the grape and citrus industries, especially in the southern San Joaquin Valley counties. Even though grapes are treated annually and citrus once every three years, citrus receives treatments with two pesticide applications and a greater amount of imidacloprid. Thus, even though GWSS is a minor pest of citrus, the per acre costs of control are similar to the costs being born by the grape growers. While the per acre costs are similar, because the treatments in citrus are being born by the taxpayer there are no market effects with respect to changes in market prices or production. In comparison, treatments by grape growers are partially passed through to consumers, making consumers worse off. The complete economic analysis will take all these effects into account.

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