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Section 1: Vector Biology and Ecology

NON-CULTURE DEPENDENT SURVEY OF THE MICROBIOTA OF THE GLASSY-WINGED SHARPSHOOTER USING 454 PYROSEQUENCING

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ABSTRACT

The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is an invasive pest that has spread across the southern and western United States. It is highly polyphagous, feeding on at least 100 species in 31 families (Hoddle et al., 2003; Turner and Pollard, 1959), and a voracious feeder, having been known to consume up to 100 times its weight in xylem fluid daily. This insect is a vector of the phytopathogen *Xylella fastidiosa* (*Xf*), which is the causative agent of Pierce's disease (PD) in grapevines. In order to evaluate the microbial flora associated with GWSS hemolymph, alimentary canal excretions and whole insect bodies were subjected to 16S pyrosequencing using the bTEFAP methodology and the resulting sequences (370-520 bp) were compared to a curated high quality 16S database derived from NCBI's GenBank. Species from the genera *Wolbachia*, *Delftia* (formerly *Pseudomonas*), *Pectobacterium*, *Moraxella*, *Serratia*, *Bacillus* and many others were detected and a comprehensive picture of the microbiome associated with GWSS was established. Some of the bacteria identified in this report are initial discoveries and having a breadth of knowledge as to the microbial flora of this insect pest can serve as a reservoir of information for developing biological control strategies. One method for biological control can be the genetic engineering of a particular bacterium to deliver certain molecules known to affect the life stage development of an insect. Another method, could be isolating a bacterium that competes with *Xf*, and re-delivering it to wild populations in excess of their natural bacterial load. Within this study, we have identified the types of bacteria which may be ubiquitous among GWSS providing us with targets to begin to investigate these future directions.

LAYPERSON SUMMARY

The glassy-winged sharpshooter is an insect pest that spreads the bacterium *Xylella fastidiosa*, the causal agent of Pierce's disease in the grapevine. Both wine and table grape production is affected by this disease and it has become a major financial burden on the industry. Bacterial DNA can be used to screen for such pathogens in insect populations and having knowledge of the bacterial pathogens of the insect can be used to develop a biological control strategy. Hemolymph, alimentary canal excretions and whole insect tissue were subjected to DNA sequencing and the resulting sequences were matched to groups of bacteria using NCBI's GenBank database. This study shows that bacteria such as *Wolbachia*, *Delftia*, *Pectobacterium*, *Moraxella*, *Serratia*, and *Bacillus* spp may be useful targets to develop biological control strategies.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) is a highly mobile pest and transmits the xylem-limited bacterium *Xylella fastidiosa (Xf)*. This bacterium can cause disease in many economically important plants including the grapevine, peach, and citrus and has become a major limiting factor in their mass production. The bacterium can also cause disease in ornamentals such as the oak, elm and sycamore. Once a plant has become infected with *Xf*, it becomes a reservoir for bacterium and can be easily spread from plant to plant by the near-continuous feeding of GWSS. Although pesticides are available for the control of the insect, resistance and harm to non-target insects is an issue of concern. Naturally occurring forms of control have been successful and should be further pursued as a complimentary strategy for insect and disease control.

Many insect taxa have obligate endosymbionts that supplement nutrition in exchange for vertical or horizontal transfer among individuals (Moran et al 2005, Buchner 1965). This mutualism has allowed insects to occupy or thrive in otherwise hostile niches. The GWSS, a xylem feeder, is known to host several bacterial species including *Baumannia cicadellinicola* and *Sulcia muelleri* (Wu et al 2006). The *B. cicadellinicola* genome is devoted to the biosynthesis of vitamins and cofactors but lacks most amino acid biosynthetic pathways, whereas *S. muelleri* apparently produces most of the amino acids needed for the host. DGGE has been used to find other symbiotic bacteria including *Wolbachia*, *Bacillus*, *Pseudomonas*, *Pedobacter*, *Methylobacterium*, and *Curtobacterium flaccumfaciens* which the authors suggest could be used as forms of symbiotic control (Lacava et al 2007). Curly et al (2007) identified bacteria closely related to *Stenotrophomonas* and *Acinetobacter* in hemolymph samples.

OBJECTIVES

1. Identify major groups of bacteria in the hemolymph, alimentary canal and whole insect.
2. Identify species of bacteria for possible transgenesis and biological control.

RESULTS AND DISCUSSION

Using 16S pyrosequencing based upon the bTEFAP methodology (Dowd et al., 2008a; Dowd et al., 2008b) optimized for the Titanium pyrosequencing platform (Roche, Indianapolis, IN) , we were able to identify 17 orders (**Figures 1-3**), 28 families and at least 38 genera (**Figures 4-6**) of bacteria. Sequences were taken from separately prepared extracts of hemolymph, alimentary canal excretions and macerated whole insects suspended in 1X PBS. The sequences were approximately 500 bp (370-520 bp) and were compared to NCBI's basic local alignment search tool (BLAST) for homologies. Some of the shorter sequences aligned to multiple genera and were placed in a separate category called "Other" because it was not clear which identification was appropriate.

The hemolymph extracts (**Figures 1 and 4**) contained over 1000 sequences aligning with the order Enterobacteriales although no *Enterobacter* were found at the genus level. Up to 27 sequences from Burkholderiales were found in the hemolymph but no *Burkholderia*, *Bordetella* or *Oxalobacter* related sequences were found at the genus level. A single sequence aligning with Rhizobiales and two sequences aligning with *Clostridium* were also found in the hemolyph.

The alimentary canal excretions (**Figures 2 and 5**) contained Enterobacteriales related sequences. This coupled with the Enterobacteriales found in the hemolymph may be a sign of cross-contamination with respect to preparation of samples. The hemolymph vessels and the alimentary canal lie close to one another in the body of the insect and may have been punctured in some trials. Thirteen sequences from *Bacillus*, 52 *Moraxella* (an opportunistic cattle and human pathogen) and 72 Serratia (a human pathogen and lab-colony limiting agent) were recovered as well.

Whole insect macerations (**Figures 3 and 6**) contained 74 sequences related to *Wolbachia*, a well-known intracellular insect pathogen that has been characterized in previous studies. This symbiont has been shown to be obligate in many arthropods and nematode species (Mavingui et al 2005) and may be a target for limiting populations of GWSS. Sequences related to *Cardiobacterium* spp. were also recovered in large numbers from all but hemolymph samples. It is not clear why this particular bacterium is present.

Sequences of *Pectobacterium* were recovered from all extracts of the glassy-winged sharpshooter. This bacterium is known to cause soft rot and black leg in potato plants through its arsenal of extracellular pectinases (Chan et al 2009). This identification is believed to be the first report of this phytopathogen in GWSS.

Although not clearly understood at this moment, sequences relating to *Delftia* sp were only recovered from the hemolymph extracts. *Delftia* sp are ubiquitous, rod-shaped, gram-negative bacteria (Hai et al 2007) that are able to degrade di-n-butylphthalate (DBP), an industrial pollutant and phthalate derivative, as a sole source of carbon and energy (Neelakanteshwar et al 2006).

Fifty two (52) sequences associated with *Moraxella* spp. were recovered from the alimentary canal excretions. This bacterium is a polymorphous gram-negative opportunistic pathogen of both humans and cattle and is known to cause conjunctivitis in both animals. It also causes ear, nose and throat infections and is known to be transmitted by flies (Ala'Aldeen 2007).

Although many different types of bacterial taxa were recovered from the extracts of GWSS it is important to recall that these sequences are small relative to even the whole 16S gene. While these 500 bp sequences are sufficient to identify bacteria to the order and perhaps even family level, their predictive ability can be less absolute at the genus and species level. However, this data can be used to design primers to "walk" down the gene and may be more adept at resolving the species level identifications. In addition, because it is estimated that less that 10% of all bacteria have ever been fully identified this study has shown that many novel genera may be associated with the GWSS microbiome. Further study of the microflora of GWSS will be needed to identify possible targets of paratransgenesis or obligate symbiont knockdown.

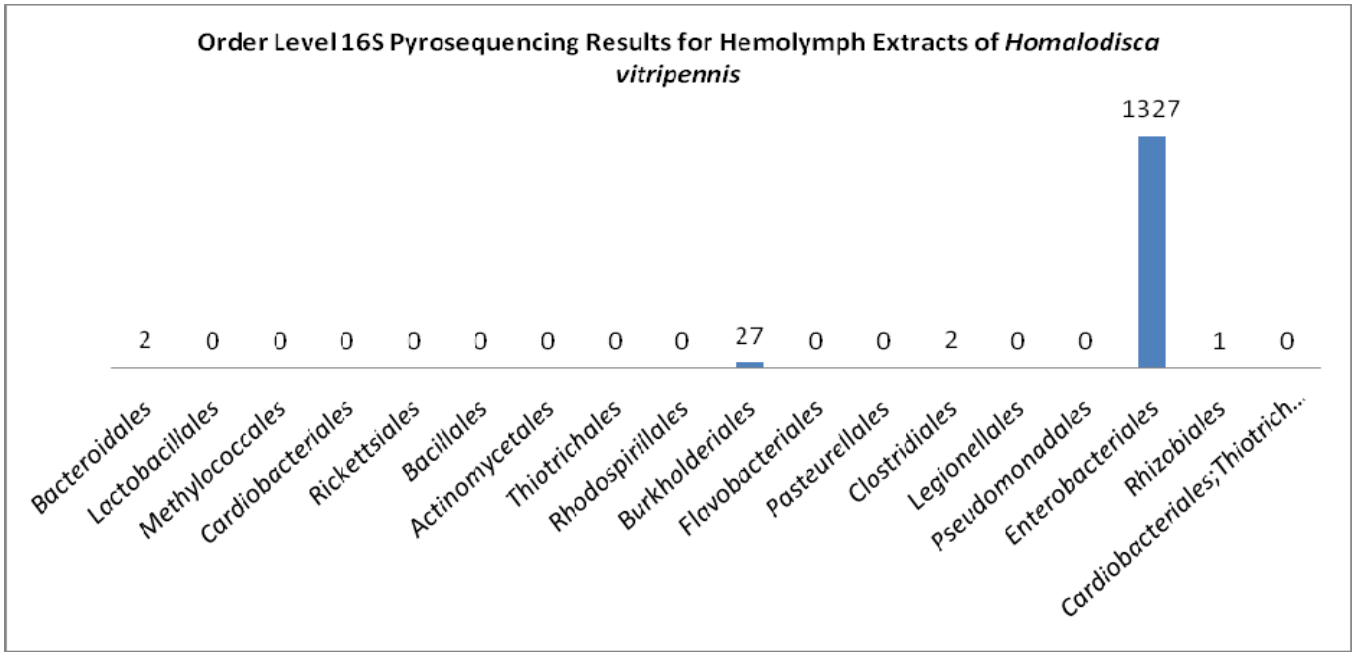

Figure 1. Order level sequencing results for the hemolymph of the GWSS. Larger numbers of sequences related to Enterobacteriales were recovered as well as Bacteroidales and Burkholderiales.

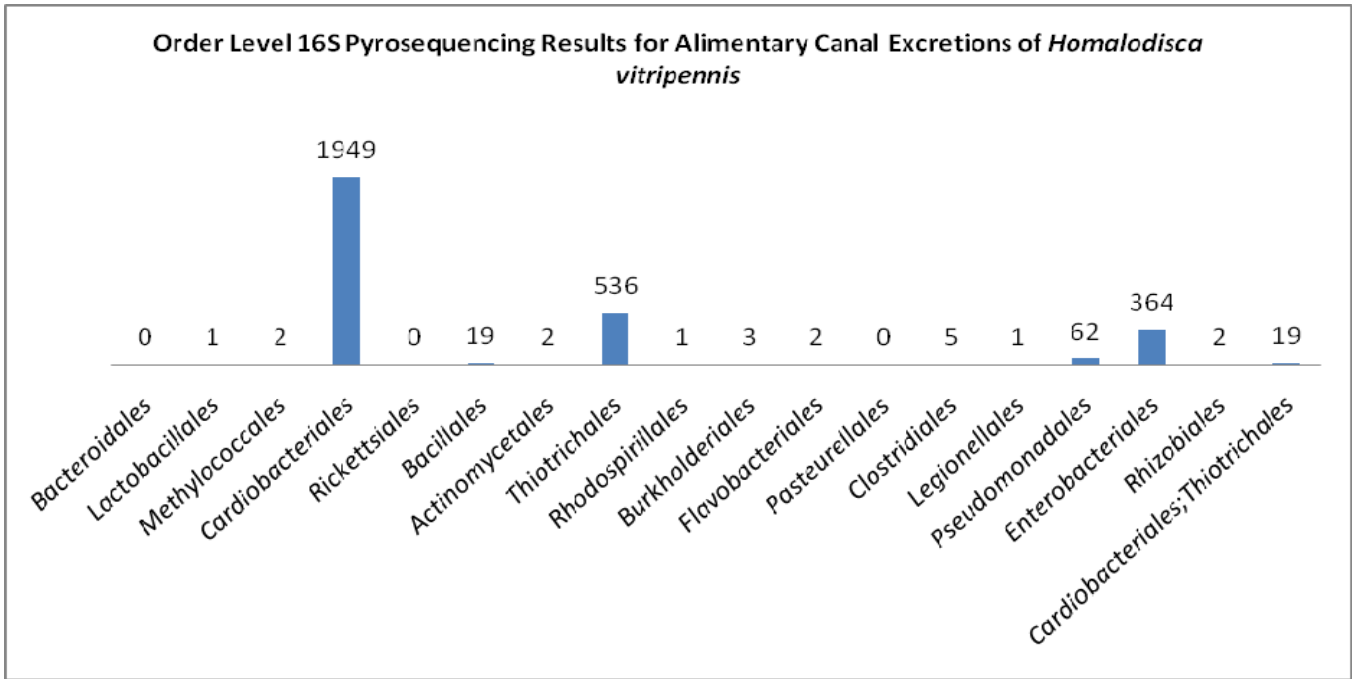

Figure 2. Order level sequencing results for the alimentary canal excretions of the GWSS. Sequences related to Cardiobacteriales, Thiotrichales, Enterobacteriales and others were recovered.

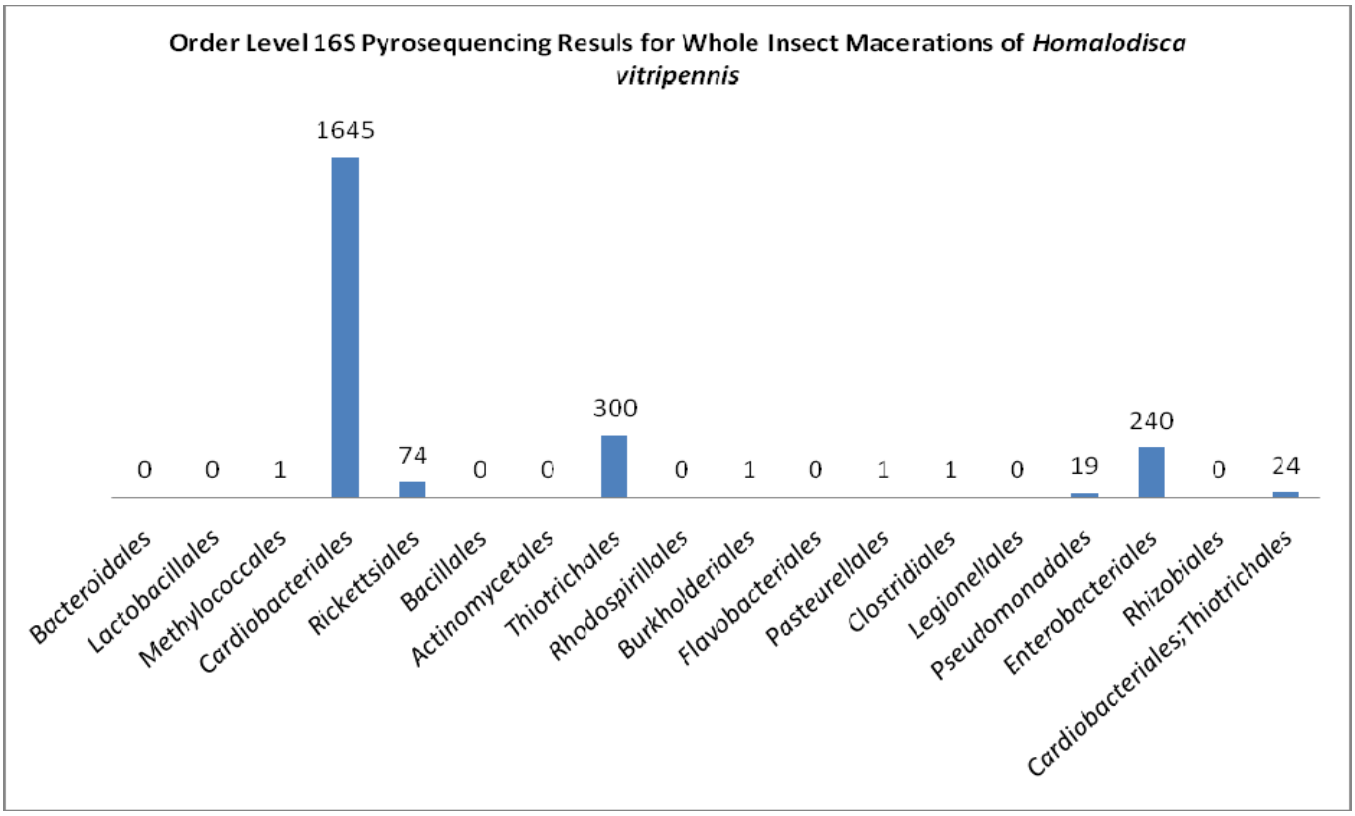

Figure 3. Order level sequencing results for the whole insect macerations of the GWSS. Sequences related to Cardiobacteriales, Thiotrichales, Enterobacteriales and others were recovered.

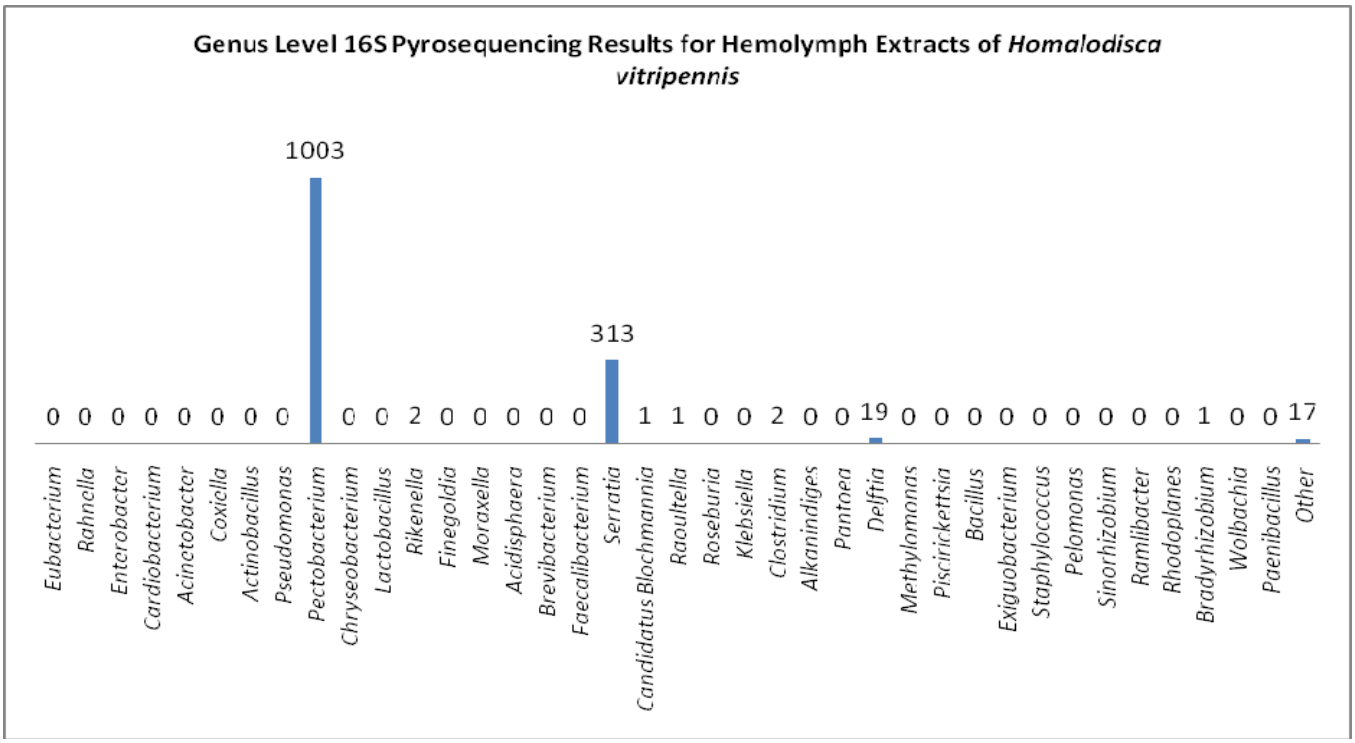

Figure 4. Genus level sequencing results for the hemolymph of the GWSS. Large numbers of *Pectobacterium* and *Serratia* were recovered. Some *Delftia* (formerly *Pseudomonas*) and other non-specific identifications were made. Note that no sequences from *Wolbachia*, an intracellular symbiont, were recovered.

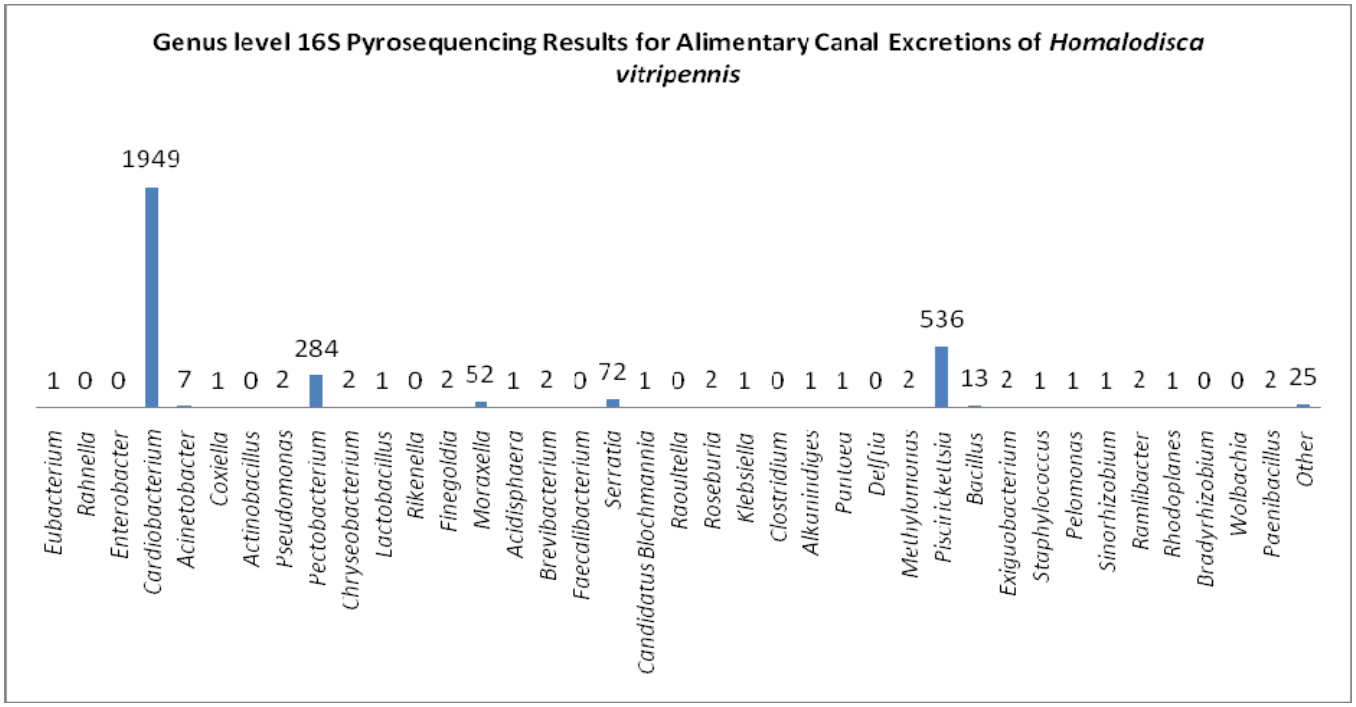

Figure 5. Genus level sequencing results for alimentary canal excretions of the GWSS. Many sequences of *Cardiobacterium, Pectobacterium, Piscirickettsia* and *Serratia* were recovered. Other non-specific identifications were made. Note that no sequences from *Wolbachia*, an intracellular symbiont, were recovered.

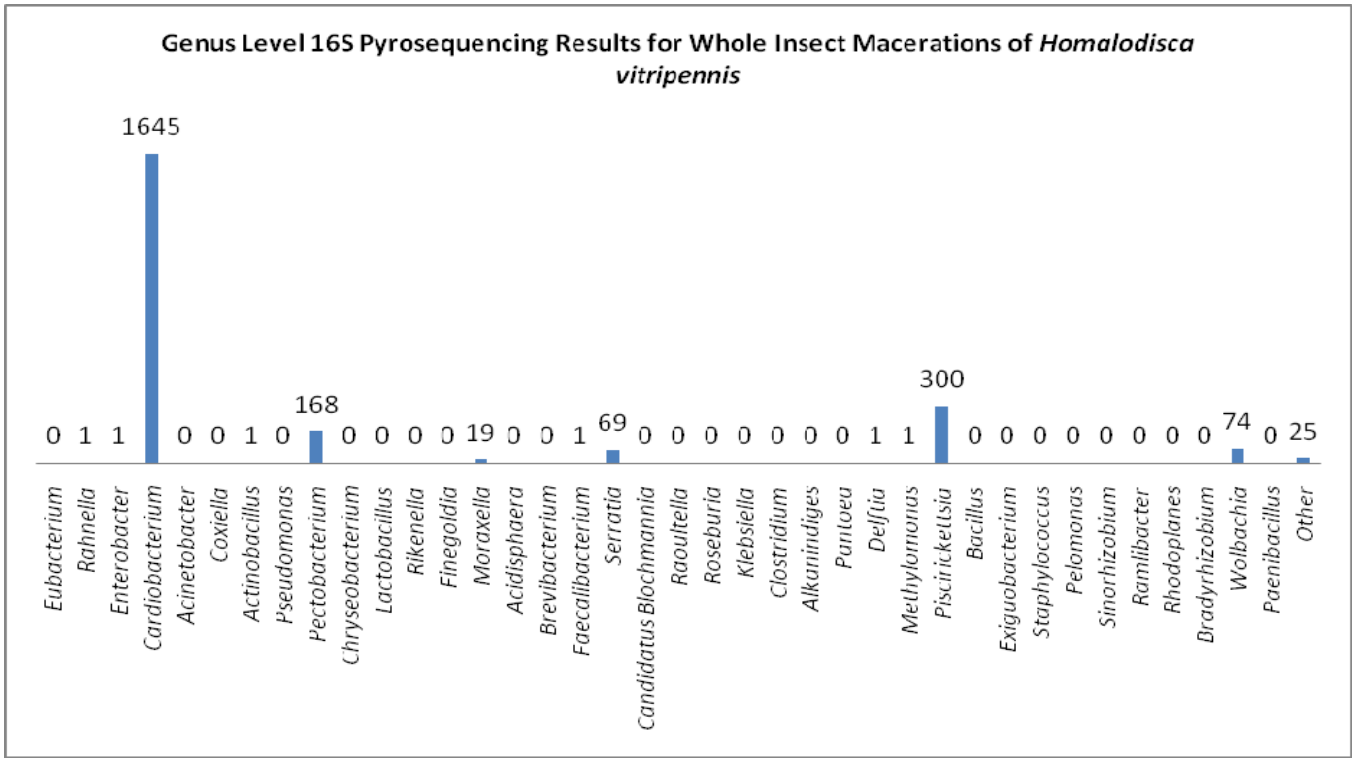

Figure 6. Genus level sequencing results for whole insect macerations of the GWSS. Many sequences of *Cardiobacterium, Pectobacterium, Piscirickettsia* and *Serratia* were recovered. Other non-specific identifications were made. Note that sequences from *Wolbachia*, an intracellular symbiont, were only recovered from whole tissue extracts.

CONCLUSIONS

Studies on the tsetse fly (*Glossina* spp.) have shown that transinfection by a genetically transformed strain of endosymbiont and expression of foreign gene products can be used to block transmission of a disease causing agent (Aksoy et al 2008). Paratransgenesis in the glassy-winged sharpshooter has not yet been extensively studied but one trial (Bextine et al 2004) did succeed in delivering *Alcaligenes xylosoxidans denitrificans*, expressing a red fluorescent protein to GWSS using a novel

feeding strategy. The bacterium was able to occupy the same area in the foregut normally associated with *Xf* infection. Using this system as a template and the results from the 16S pyrosequencing, we can now pursue multiple avenues of paratransgenesis.

The presence of *Delftia* sp exclusively in the hemolymph of GWSS provides an exciting possibility for this bacterium as a potential tool. A study by Wang et al (2008) showed that a new formulation of media was successful in culturing *Delftia tsuruhatensis* to a level 4.7 times higher than with un-optimized media. Other culture media are also available.

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IDENTIFICATION AND WHOLE EXTRACTION OF *HOMOLADISCA COAGULATA-VIRUS01 (HoCV-01* FROM TEXAS GLASSY-WINGED SHARPSHOOTER POPULATIONS

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ABSTRACT
The glassy-winged sharpshooter (GWSS) is an invasive pest and important vector of *Xylella fastidiosa (Xf)*, a xylem-limiting bacteria that causes Pierce's disease (PD) in grapes as well as other agricultural diseases. The primary method of managing the spread of *Xf* is controlling its insect vector populations. Methods such as chemical control are not target specific and lead to problems such as residue contamination, injury to non-target organisms, and insecticide resistance. Identifying agents that can impact GWSS populations is the goal of a biological control strategy. In this study, we have identified and extracted whole GWSS *Virus 01 (HoCV-01)* from populations of GWSS collected in Texas. *HoCV-01* is a novel virus that harbors pathogenic potential with regard to GWSS. Future plans for *HoCV-01* include reintroduction into GWSS populations through feeding. Increased amounts of *HoCV-01* ingestion may lead to weakened populations of GWSS that are more susceptible to control methods such as insecticides. This would decrease the amount of insecticide needed to produce a desired mortality rate in insect populations.

LAYPERSON SUMMARY
The glassy-winged sharpshooter (GWSS) is the most economically important insect with respect to the spread of *Xylella fastidiosa (Xf),* the causal agent of Pierce's disease (PD). Therefore control of this insect is of paramount importance to the management of the disease. While insecticides have been used successfully to reduce the economic impact of this disease system, alternate methods of population insect control are needed to continue management in the future. Biological control offers alternatives to chemical control that can be effective in negatively impacting insect population without harmful environmental effects or concern for insecticide resistance. In this work, we molecularly describe a virus that shows promise as a tool for biological control. While this virus does not cause significant acute mortality, it may reduce the fitness of insects to a point where other control methods would be more effective. We suggest that viral infection will make insects more sensitive to insecticide treatment, resulting in lower LD50 rates for achievement of significant control. This means that lower levels of insecticide can be used effectively.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) 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 several agricultural industries in North America and is associated with many plant diseases such as Pierce's disease (PD), and oleander leaf scorch. PD of grapevine has become a well recognized *Xylella*-related disease; the vector profile 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 of PD depends heavily on the ability to control its vectors, especially GWSS.

Methods of vector manipulation such as chemical control with the use of insecticides are not target specific and lead to problems such as residue contamination, injury to non-target organisms, and insecticide resistance. The search for more benevolent pest management strategies has led to the use of biocontrol agents such as fungi and parasitoids. However, by utilizing viruses that currently reside in GWSS populations,a viral bio-control that is even more precise may be developed (Hunnicutt et al., 2006).

HoCV-01 is a member of the genus *Cripavirus* and family *Dicistroviridae (*Hunnicutt et al., 2006). It is a novel virus that harbors pathogenic potential with regard to GWSS. The focus of this study was the identification and extraction of whole *HoCV-01* found in populations of GWSS collected in Texas. Once identification was complete, the genome was sequenced and checked for variation which may produce an increase in virulence. Following sequencing, whole *HoCV-01* was extracted in order to reintroduce it into GWSS populations.

OBJECTIVES
1. Identify *HoCV-01* in populations of GWSS collected in Texas.
2. Sequence viral capsid protein and check for variation between strain found in Texas and strain found in California.
3. Extract and purify whole *HoCV-01* with intent to reintroduce into uninfected populations of GWSS.

MATERIALS AND METHODS

RNA Extraction. GWSS bodies were collected in microcentrifuge tubes and homogenized. GWSS RNA was separated from the solution and purified using a Qiagen RNeasy kit (Qiagen™, Germantown, MD).

RT-PCR & Gel Electrophoresis. Each 1µL GWSS RNA sample was combined with 10µL 2X Reaction Mix (Invitrogen Molecular Probes™, Eugene, OR), 0.4µL forward primer, 0.4µL reverse primer, 0.4µL Platinum® Taq DNA Polymerase (Invitrogen Molecular Probes™, Eugene, OR), and 8µL DEPC H_2O in solution. Samples were then subjected to a Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). 2µL of each GWSS DNA sample was subjected to gel electrophoresis using 2µL ladder, 2µL loading dye per sample, and a 1% agarose gel containing 100mL TAE buffer and 1g agarose gel. Gels were subjected to 100V and 400A for 50 min. and checked under Ultraviolet light in a Bio Doc-It Imaging System (Cole-Parmer™, Hanwell, London).

Sequencing PCR & Ethanol Precipitation. Sequencing was done on sight in the Bextine Molecular Biology Laboratory at The University of Texas at Tyler. An amount of 2µL of each GWSS DNA sample was combined with 2µL Nano-pure H_2O, 2µL primer (only the forward or the reverse primer were used in this step), 4µL GenomeLab™ DTCS Quick Start Mix (GenomeLab™, Fullerton, CA), and taken through a Sequencing Polymerase Chain Reaction, or Sequencing PCR in an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). Each sample was then combined with 2µL 3M NaOAc, 2µL 100nM EDTA, 1µL 20mg/mL Glycogen, vortexed thoroughly, and subjected to an ethanol precipitation. During the ethanol precipitation, sample DNA was purified for sequencing with separate washes of ice cold 95% and 70% ethanol. The resulting pellet of purified DNA was mixed with 40µL Sample Loading Solution, vortexed, and transferred to a sequencing plate.

DNA Sequencing. DNA samples were sequenced using a CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA). Prior to loading the samples into the sequencer, each sample was combined with one drop of Mineral Oil. Data was analyzed using Bioedit® Sequence Alignment Editor (Ibis Biosciences, Carlsbad, CA).

Whole Virus Extraction. Infected GWSS bodies were placed in a mortar and pestle and homogenized in 100mL of phosphate buffer containing 0.02mg DETCA. The homogenate was then transferred to 50mL centrifuge tubes and centrifuged at 1600rpm for 20 minutes in an Eppendorf 5804R Centrifuge (Eppendorf , Hamburg, Germany). The resulting supernatant was split into two ultra-centrifuge tubes, combined with more phosphate buffer with DETCA, vortexed, and ultra-centrifuged at 22,000rpm for 16 hours in a Sorvall® RC-5B Refrigerated Superspeed Centrifuge (DuPont Instruments, Wilmington, DE). Following ultra-centrifugation, the supernatant was discarded, and the pellet was dissolved with 5mL phosphate buffer with 0.4% Na-deoxycholic acid and 4% Brij 52. The resulting solution was centrifuged and 1600rpm for 15 minutes, passed through a 0.45µm filter, and collected into large Eppindorf tubes. The unrefined *HoCV-01* solution was placed in a dialysis membrane, placed in a large beaker containing a stir-bar and ddH_20, and placed in a refrigerator at 4°C. The ddH_20 was changed out ever five-six hours until a white precipitate could be seen in the dialysis membrane. The purified *HoCV-01* solution was collected into micro-centrifuge tubes and stored at -80°C.

RESULTS AND DISCUSSION

HoCV-01 was detected in GWSS populations collected in Texas. Sequence comparison of the Texas strain of *HoCV-01* against the sequenced California strain (Hunnicutt et al., 2006) shows some variation. The percent similarity between the strains is **98.8%.**

Base pair 828, Cytosine in the California strain, is a Thymine in the consensus strain (**Figure 1**). This changes the amino acid translation from Serine (polar side chain) into Leucine (non-polar side chain). Also, due to a Guanine insertion in the consensus strain at base pair 904 (possibly a deletion in the California strain), variation downstream in the amino acid chain was observed (**Figure 1**).

The presence of variation between the Texas *HoCV-01* sequence and the California *HoCV-01* sequence is a possible indication that the Texas strain may exhibit increased virulence. The Guanine insertion at base pair 904 caused variation in all downstream amino acid translation. This could lead to changes in protein folding and ultimately changes in protein function. Altered protein functions may cause an increase in virulence in the Texas *HoCV-01* strain.

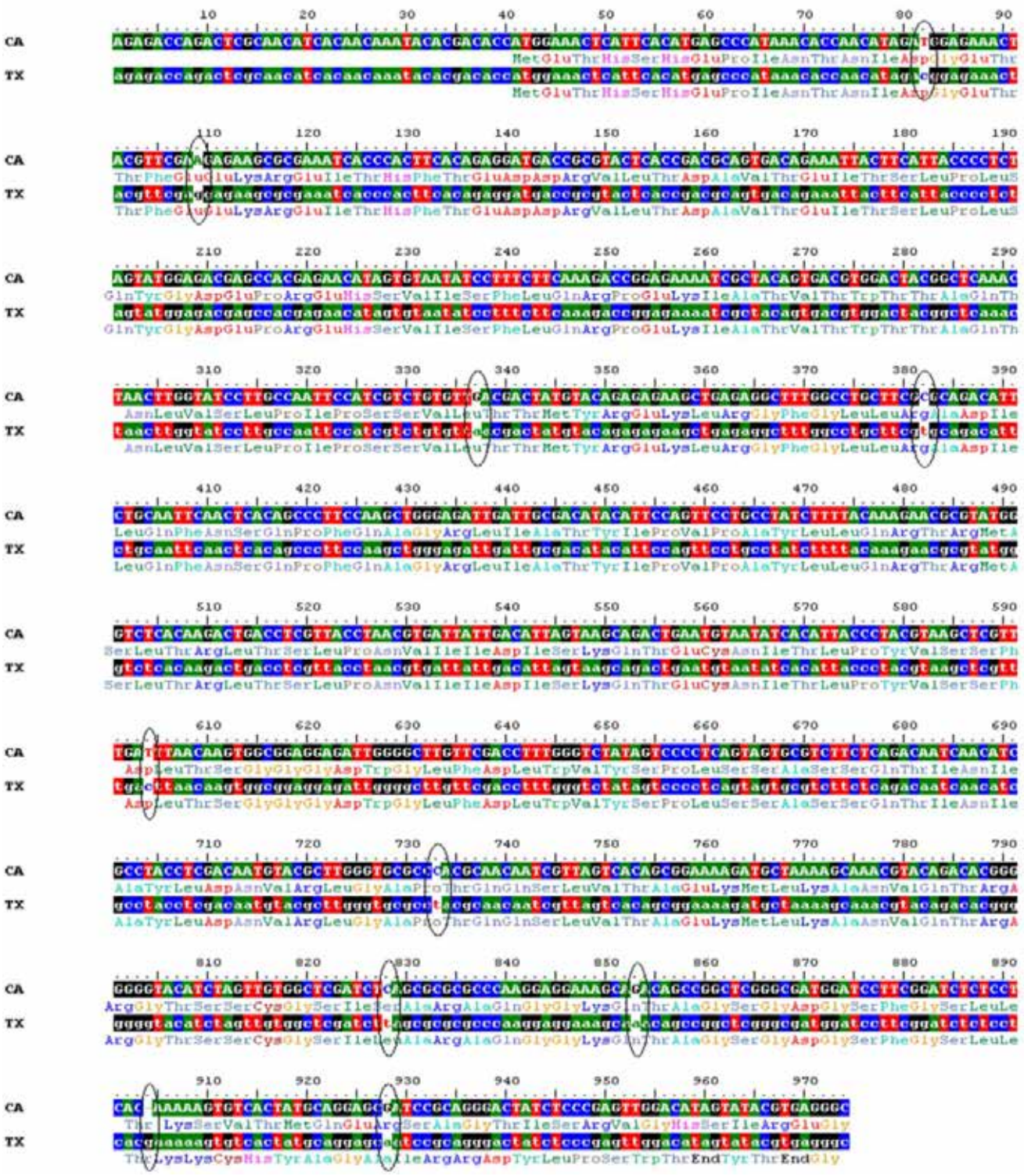

Figure 1. Sequence comparison with protein translation of the California *HoCV-01* strain and the Texas HoCV-1 strain. Note the circled areas which indicate variation between the two strains.

CONCLUSIONS

The presence of *HoCV-01* in populations of GWSS collected in Texas is crucial in developing an ideal viral bio-control and pest management strategy. The possible increase in virulence in the Texas strain is also an indication of the pathogenic potential of *HoCV-01*. This novel virus has been extracted and purified. In a following study, purified *HoCV-01* will be reintroduced to a population of uninfected GWSS in order to determine increases in population weakness or mortality that may result. The results of this experiment are crucial in further understanding the insect vector, GWSS. The management of PD depends heavily upon the ability to control its insect vectors.

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PHYLOGENETIC ANALYSIS OF HEAT SHOCK PROTEINS IN THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis* (Germar); Hemiptera: Cicadellidae) is the major vector of *Xylella fastidiosa (Xf)*, the causal agent of Pierce's disease (PD) of grapes. As genomic information becomes available more research on leafhopper stress responses are possible. Due to the importance of the GWSS in transmission and spread of *Xf*, a cDNA library was constructed from adult and fifth-instars GWSS, resulting in 5,906 expressed sequence tags (ESTs). After quality scoring, 4,445 sequences underwent assembly which produced a set of 2,123 sequences that putatively represented distinct transcripts. BLASTX analysis identified four significant homology matches to heat shock proteins, (HSP) which are the focus of this study. The overall importance and function of HSPs lie in their ability to maintain protein integrity and activity during stressful conditions, such as extreme heat, cold, drought, or other stresses. Phylogenetic analyses using these four HSP sequences provided further support of transcript by the identification of specific motifs. This study shows that highly conserved genes like HSPs are a viable alternative to ribosomal DNA in elucidating phylogenetic relationships.

LAYPERSON SUMMARY
In this study, we generated and analyzed a cDNA library, which is a representation of all the protein coding genes in an organism, of the insect pest glassy-winged sharpshooter (GWSS). We isolated four incomplete sequences from a large family of proteins called heat shock proteins (HSP). Although they are called HSP, these proteins actually perform many activities in the cell that allow for GWSS to survive stresses like extreme temperatures, pesticides, and even viral infection. This study compares the HSP sequences from GWSS to those of other insect species to help describe the relationship and history of GWSS to find ways to track changes in GWSS territory and life history.

INTRODUCTION
Organisms respond to heat shock or other environmental stress by inducing the synthesis of proteins some of which are known as heat shock proteins (HSP) (Lindquist 1986, Sorenson 2003). Infections, temperature changes, inflammation, toxins, hypoxia, starvation and even exercise can result in increased production of heat shock proteins (Sorenson 2003). HSP aid in folding, targeting and tracking of nascent proteins, promote transcription, are involved in cellular division and can be up regulated via cell signaling in addition to environmental stimuli (Feder and Hofmann 1999).

Small heat shock proteins (sHSP) have an approximate molecular weight of less than 30kDa and are molecular chaperones, maintaining proper protein structure by blocking aggregation of denaturing proteins, aiding nascent protein folding and assisting construction of quaternary structure (Fu and Chang 2004, Gu et al. 2002, Bova et al. 2002, Sobott 2002).

Among the HSP families is a group of well-conserved proteins with an approximate molecular weight of 70 kDa, known as the HSP70 family. Most species have several proteins belonging to this family. Some of these members are only expressed under stress conditions (strictly inducible), while some are present in cells under normal growth conditions (Craig 1989) and are not heat-inducible (Pelham 1986), and are known as heat shock cognates (HSC). In eukaryotes, HSP70 can work with sHSP to restore functionality to heat-denatured proteins (Lee and Vierling 2000) or co-chaperone with HSP40 to fold nascent proteins into proper tertiary structure by temporarily binding to hydrophobic domains until sequence translation is complete (Douglas et al. 1994).

The 90 kDa heat shock proteins (HSP90) is one of the most prolific proteins in eukaryotic cells, constituting 12% of cellular proteins under baseline conditions (Sreedhar 2004). Their functions and morphological evolution have been extensively studied and include signal transduction, protein folding and degradation of denatured proteins (Nadeau 1993, Jakob 1994). Increased functionality of HSP90 is acquired when associated with its co-chaperones, playing an important role in the folding of newly synthesized proteins. Apart from its co-chaperones, HSP90 binds to an array of substrate proteins, where the necessary co-chaperones varies and depends on the actual substrate (Jakob 1995). Understanding heat shock proteins in insects, especially leafhoppers, will provide insights into the biological adaptive elasticity of these important agricultural pests to stressors such as insecticides, parasitization, and temperature.

The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis* (Germar); Hemiptera: Cicadellidae), is an insect pest that occurs throughout most of the southern USA and is endemic to most regions of Texas (Young 1958; Turner and Pollard 1959). Without naturally occurring forms of biological control, GWSS have established populations in new areas and have

negatively affected the yields of the grape industry (de Leon et al. 2004). GWSS is a voraciously-feeding, xylem-limited pest that has been reported to feed on host plants from at least 35 families, including both woody and herbaceous types (Hoddle et al. 2003), and can impact the plant's health directly by depriving the plant of nutrients and damaging the xylem sufficiently to preclude vascular flow. However, indirect plant damage occurs during feeding and subsequent transmission of the xylem-limited bacterium *Xylella fastidiosa (Xf)* Wells (Xanthomonadales: Xanthomonadaceae). The invasion of GWSS into grape growing regions of California, namely the Temecula valley, produced an enormous risk to the California wine and table grape industry by spreading the phytopathogen *Xf*, the causative agent of Pierce's disease (PD) (Purcell 1997, de Leon et al. 2004). Additionally, many other economically important plants including citrus, almond and oleander are affected by separate strains of *Xf* resulting in a multitude of plant diseases including citrus variegated chlorosis (Chang et al. 1993; Pooler and Hartung 1995), almond leaf scorch (Mircetich et al. 1976) and oleander leaf scorch (Purcell 1999).

A search of the National Center for Biotechnology Information (NCBI) for GWSS genes or protein sequences revealed less than 25 complete, non-mitochondrial genes or complete proteins. Although the complete mitochondrial sequence of GWSS has been described (Genbank AY875213), the genomic DNA sequence is incomplete. Over 20,000 Expressed Sequence Tags (ESTs) from GWSS have been submitted to NCBI; however, many of these EST's are duplicates and this study is an initial step in examining the potential use of this information by examining the utility of these heat shock proteins to describe the phylogeny of leafhoppers in relation to other insects. Further management approaches have been proposed to disrupt HSP in insects as a means to suppress leafhopper populations.

OBJECTIVES

1. Identify the phylogenic relationship of important leafhopper species using heat shock protein sequences.
2. Develop a methodology to distinguish between populations of GWSS.

RESULTS AND DISCUSSION

Mining of the 5,906 cDNA clones produced from cDNA library constructed from 140 adult and fifth-instar GWSS using Stratagene's ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA) resulted in 4,445 high-quality (i.e., ≥200 bases with a TraceTuner™ score of 20 or better) GWSS ESTs sequenced by ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence alignment of these ESTs resulted in a Unigene set of 2,123 total assembled sequences, at Phred 20 score, 40bp overlap, 100 bp minimum length, using Sequencher™ 8.0, (Gene Codes Corp, Ann Arbor, MI, 48108). Translated proteins were analyzed with National Center for Biotechnology Information's (NCBI) BLAST*p*, Pfam (www.pfam.org), InterProScan (www.ebi.ac.uk) and Expert Protein Analysis System (www.expasy.org). Four partial protein sequences were analyzed for phylogenetic relationships with homologous HSP sequences. A BLAST*p* and BLAST*n* analysis showed that WHWTC-contig[1627] and WHWTC-contig[1325] showed significant homology with the sHSP, while WHHC-contig[1333] displayed homology with HSP70, and WHWTC-contig[1285] was homologous with HSP90 (**Table 1**). A homology search conducted in the Pfam database identified protein sequences with their respective HSP families (**Table 2**). A functional analysis and homology search using PantherDB annotated and classified the sequences as belonging to the HSP superfamily (data not shown).

Table 1. Protein sequence similarities from GWSS contigs. Nucleotide matches (accesion|protein description organism) and e-values for query contigs using National Center for Biotechnical Information (NCBI)'s BLAST*x*.

GWSSclones	**Descriptor**	**E-Value**
WHWTC-Contig[1627] 694 bases	gb|ABC84494.1| heat shock protein 20.7 *Locusta migratoria*	3.00E-43
WHWTC-Contig[1325] 954 bases	gb|ACH85196.1|heat shock protein 20 *Bemisia tabaci*	2.00E-44
WHHC-Contig[1333] 1047 bases	gb|AAZ17399.2| 70 kDa heat shock protein *Bemisia tabaci*	2.00E-149
WHWTC-Contig[1285] 1144 bases	gb|AAZ17403.1| 90 kDa heat shock protein *Bemisia tabaci*	6.00E-179

Table 2. Hidden Markov models (HMM) homology search of *in silico*-translated protein sequences using Pfam protein database (www.pfam.org) with protein family description, Pfam identification, sequence coverage and corresponding e-value.

Contig Number	Description	Pfam Family ID	Sequence		HMM		E-value
			Start	End	From	To	
WHWTC-Contig[1627]	Hsp20/alpha crystallin family	PF00011	86	182	1	109	1.50E-40
WHWTC-Contig[1325]	Hsp20/alpha crystallin family	PF00011	63	159	1	109	2.30E-38
WHHC-Contig[1333]	Hsp70 protein	PF00012	1	325	291	619	2.20E-201
WHWTC-Contig[1285]	Hsp90 protein	PF00183	3	380	101	489	0

The heat shock proteins (HSPs) from GWSS had homology to the HSP from other insects, and grouped most closely with other Hemiptera when subjected to phylogenetic analysis. Phylogenetic trees illustrated accurate grouping of taxa into clades relative to known HSP from closely related Hemipteran species (**Figures 1-3**). The clades were separated according to taxonomic Order. Two small heat shock protein sequences (sHSP) from GWSS grouped with another sharpshooter *Graphocephala atropunctata* sHSP (**Figure 1**). The HSP70 sequence from GWSS grouped with two HSP70 sequences from the pea aphid (*Acyrthosiphon pisum*) (**Figure 2**). Finally, the HSP90 sequence from GWSS grouped with three sequences from the pea aphid (*A. pisum*) (**Figure 3**).

These phylogenetic analyses corroborate evidence from Pfam and PantherDB protein databases that describe the GWSS partial protein sequences as HSP. Additionally, the phylogenetic trees created using these protein sequence comparisons show that HSP can be used to determine phylogenetic and cladistical associations.

Heat shock proteins, HSPs have a variety of functions within the cell including the prevention of protein aggregation and denaturation due to heat and are well conserved across all taxa, and are present in every species analyzed (Feder and Hofmann 1999, Sorensen 2003). HSP families are organized via their level of expression in the cell (i.e. inducible or constitutive expression) as well as the complexes formed by the HSP; however, the greatest organization criteria is the molecular weight of the HSP which include families of 20kDa, 40kDa, 60kDa, and 90kDa proteins (Gething 1997). Finally, conserved domains exist in these families, including alpha-crystalline structure in sHSP, an N-terminal pentapeptide sequence in HSP70, and a highly conserved N-terminal domain in HSP90. The HSP sequences collected from GWSS contain these conserved domains permitting significant *in silico* comparisons (data not shown).

Phylogenetic analysis and alignment searches of the four HSP sequences were confounded by the overwhelming number of HSP sequences isoforms submitted to NCBI. However, with careful consideration paid to HSP isoforms, phylogenetic comparisons showed accurate clades of GWSS HSP with those of other closely related insect taxa (**Figures 1-3**). Phylogenetic trees formed on HSP comparison verified other phylogenetic analyses based on mitochondrial DNA. Although many ESTs in this study were found to be HSP homologues, there remain many as yet unanalyzed HSP in the GWSS genome, including members of the small Heat Shock Proteins (sHSP), HSP60, and HSP70 families. Additionally, many members of the HSP90 family and its co-chaperones have yet to be sequenced. The need for more in depth sequencing of GWSS is evident by the paucity of HSPs currently identified in the GWSS genomic database. In *Drosophila melanogaster*, whose genome is completely sequenced, over 200 HSPs have been identified and submitted to the National Center for Biotechnology Information (NCBI). GWSS has a predicted genome size of ~1.24 pg (Hunter, unpublished), similar to the haploid male Whitefly, *Bemisia argentifolii* at ~1.1pg (Leshkowitz et al. 2006), three times the size of the Asian citrus psyllid ~0.35pg (Hunter et al., 2009) and roughly five times the size of the fruitfly *D. melanogaster*, which is ~0.18pg (Brown et al. 2005). Thus, we suspect that sharpshooters will have a number of HSPs that would approximate the number in other insect genomes.

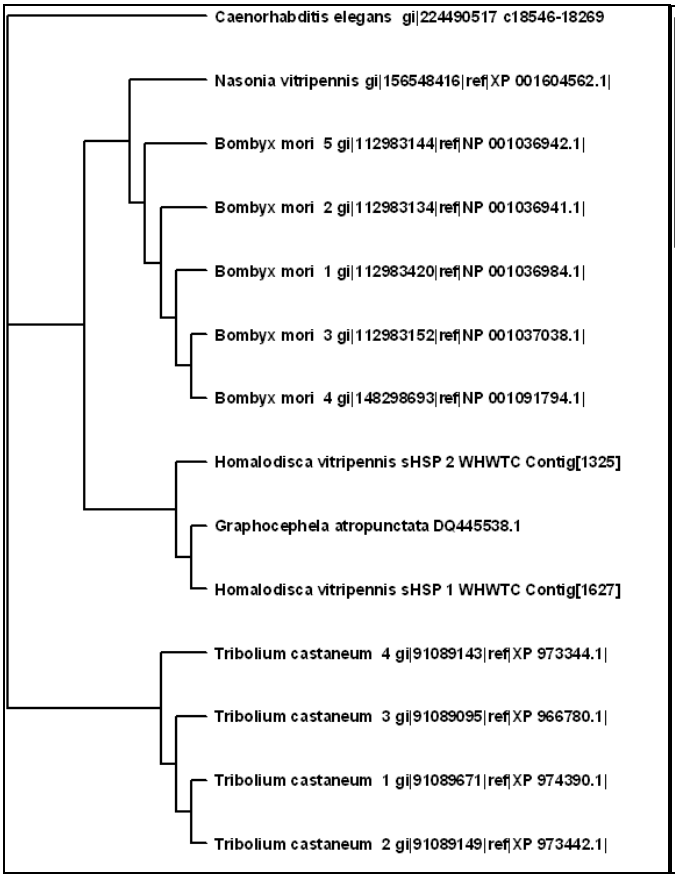

Figure 1. Cladogram of small heat shock proteins constructed using Glassy-winged sharpshooter sequences WHWTC-Contig[1627] and WHWTC-Contig[1325]. Subject sequences were analyzed using NCBI BLAST*p* search and aligned using T-Coffee multiple alignment tool (www.tcoffee.org) and visualized using Treeview v1.6.6. (Organism name|accession number| reference number).

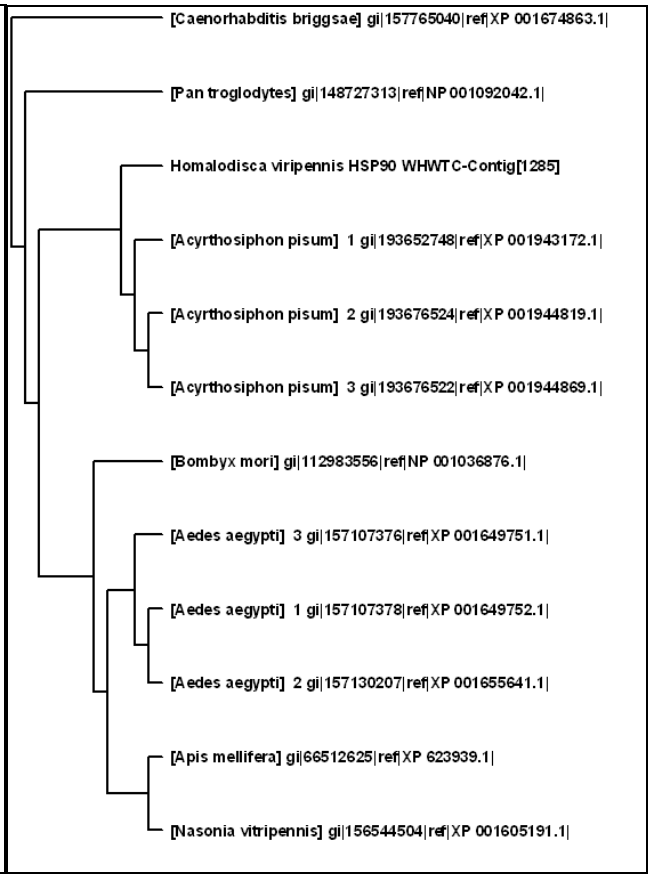

Figure 2. Cladogram of heat shock 70 proteins constructed using sequence WHHC-Contig[1333]. Subject sequences were analyzed NCBI BLAST*p* search and aligned using ClustalW2 multiple alignment tool (www.ebi.ac.uk/Tools/clustalw2/index.html) and visualized using Treeview v1.6.6. (Organism name|accession number| reference number).

Systematic biases can distort evidence via improper gene sampling. Therefore, it is necessary to limit the effects of these entanglements by analyzing multiple genes that undergo relatively uniform evolution. Ribosomal DNA is a useful molecule for examining phylogenetic relationships among many eukaryotes, primarily because no other molecule has been sequenced as extensively (Stechmann 2003). However, phylogenetic analysis using ribosomal DNA can cause artefactual groupings of unrelated genera that have undergone rapid rRNA evolution (Philippe and Adoutte 1998; Philippe et al. 2000). Previous studies have used HSPs to elucidate phylogenetic relationships in eukaryotes (Plesofsky-Vig 1992, Stechmann 2003). The ubiquitous and metropolitan prevalence of HSP allow for comparison of organisms as distantly related as that of bacteria, *Escherichia coli* and flies, *Drosophila melanogaster* (Lindquist 1986). Additionally, the importance of HSP in evolution and speciation has been well documented (Sorensen 2003). Finally, the difference between families of HSPs allows researchers many options in determining precision and resolution in describing phylogenetic relationships by utilizing the more conserved HSP90 domain or the more varied sHSP family to define relationships at any level of categorization, from kingdom to species (Feder and Hofmann 1999). One of the greatest determining factors in host range for an invasive species is stress tolerance; an attribute directly related to HSP expression (Feder and Hoffman 1999). As such, the sequence variation of heat shock proteins offer an excellent resource to apply in defining phylogenetic relationships, and also to aid in revealing pest species range.

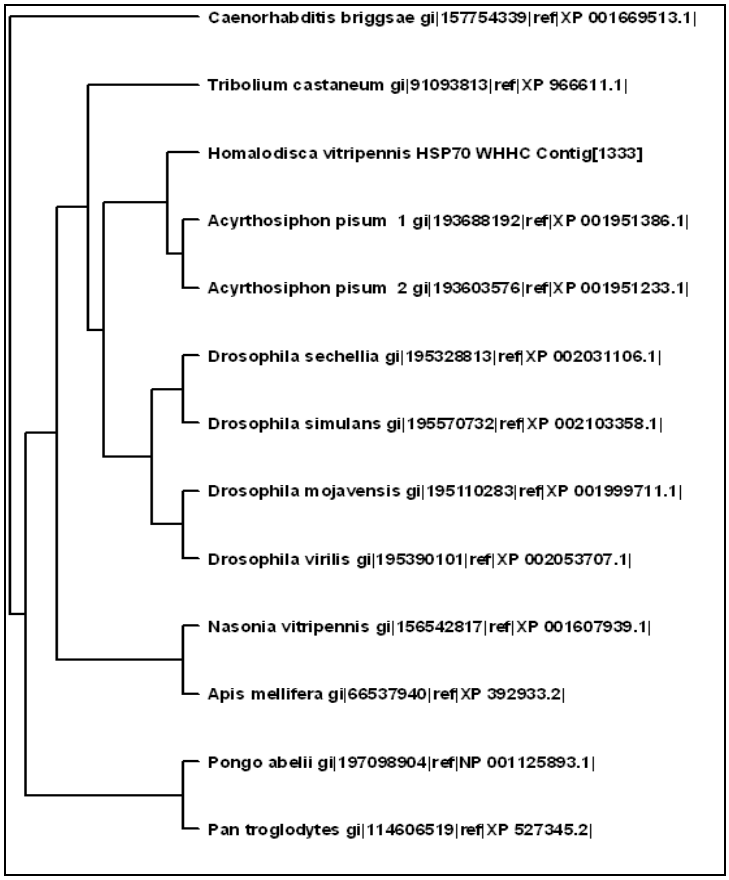

Figure 3. Cladogram heat shock 90 proteins constructed using sequence WHWTC-Contig[1285]. Subject sequences were analyzed using NCBI BLAST*p* search and aligned using ClustalW2 multiple alignment tool (www.ebi.ac.uk/Tools/ clustalw2/ index.html) and visualized using Treeview v1.6.6. (Organism name|accession number| reference number).

CONCLUSION

These results show that HSP can be used to accurately describe the phylogenetic history of GWSS, thus offering a novel target for molecular systematics. Additionally, this study is the first to describe any of the HSP sequences found inGWSS. We believe that understanding and sequencing heat shock protein encoding genes is an important step elucidating the underlying genetic determinants of pest species range and stress tolerance of GWSS.

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

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Reporting Period: The results reported here are from work conducted March 2002 to October 2009.

ABSTRACT
Glassy-winged sharpshooter (GWSS) population densities have been steadily declining over a 7.5 year period in organic lemons grown in an experimental study plot at UC Riverside Agricultural Operations. Peak adult GWSS populations in September 2009 were just 16% of those observed around August 2002. It is uncertain if egg parasitism, which has consistently averaged ~25% 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.

LAYPERSON SUMMARY
Glassy-winged sharpshooter (GWSS) populations in an organic lemon orchard in Riverside, southern California have been declining steadily since 2002. In September 2009, GWSS densities at their peak were only 16% of those observed at a similar peak in August 2002. This downward trend seems to have occurred in most of southern California but there are occasionally flare ups of GWSS as populations undergo localized outbreaks. The exact reasons for the significant decline in GWSS population densities is unknown, but could be due to the activity of natural enemies like egg parasitoids, or the weather, especially winter conditions, could be responsible. Consequently, the goal of this study is to figure out why GWSS populations have largely collapsed in southern California.

INTRODUCTION
Data collected from bi-weekly monitoring over the last 7.5 years from organic commercially-managed lemons at Agricultural Operations (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 *Gonatacerus 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 ~25% parasitism of GWSS eggs over the entire 7.5 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 over winter). (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. Understory management may be an important cultural strategy to benefit GWSS parasitoids if it can be demonstrated not to enhance GWSS and *Xylella* populations. (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* and populations of other parasitoids 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.

OBJECTIVE
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. These data will be analyzed to determine if density-dependent (e.g., natural enemies) or density-independent (e.g., winter temperatures and rainfall) influence observed GWSS population trends at the study site at 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 feedback 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 UC Riverside. Percentage parasitism of GWSS eggs by mymarid parasitoids, in particular, *G. ashmeadi*, has remained relatively constant from year to year at ~25%. It is unknown if this level of parasitism is sufficient to have caused the steady decline in GWSS numbers observed over the past 7.5 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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FUNDING AGENCIES

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

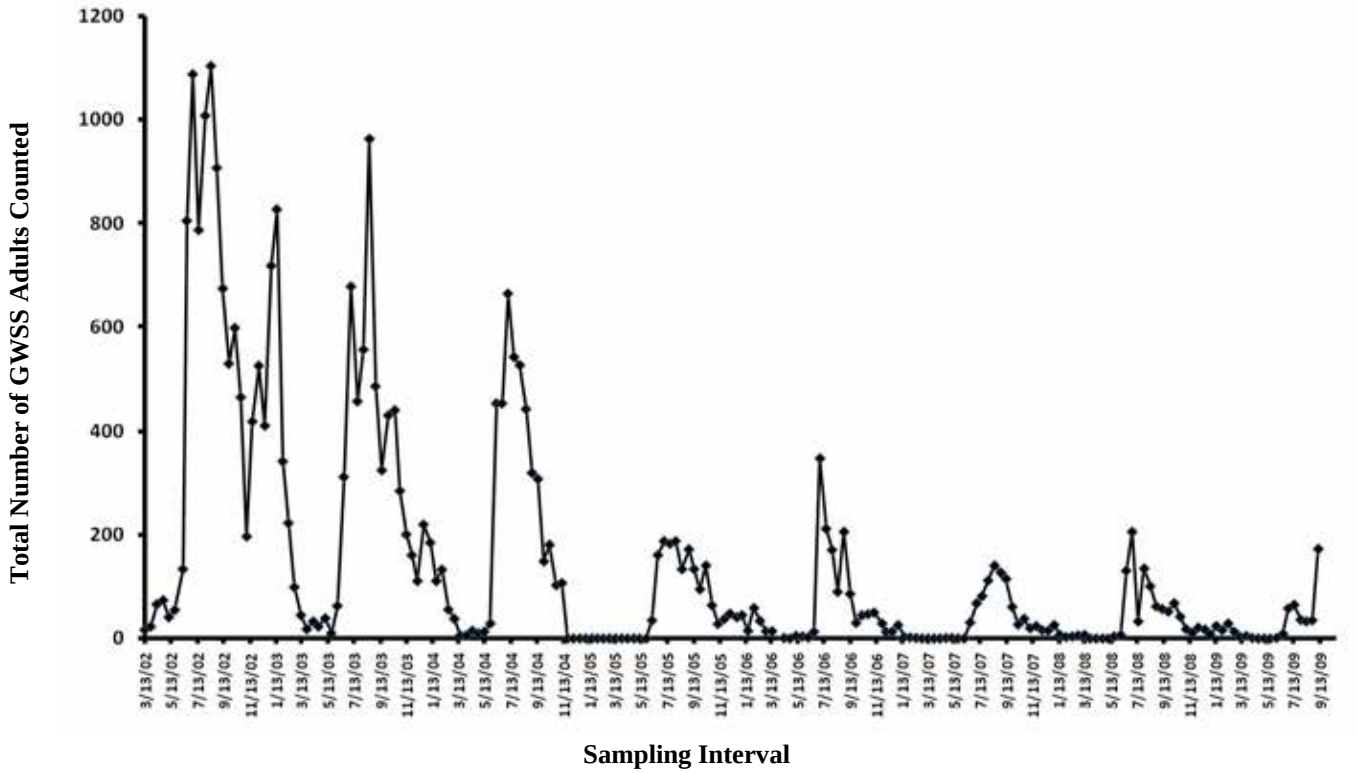

Figure 1. Phenology of adult GWSS in organic Eureka lemons. Data are total counts from timed five minute surveys made every two weeks of 10 mature lemon trees at UC Riverside Ag. Ops.

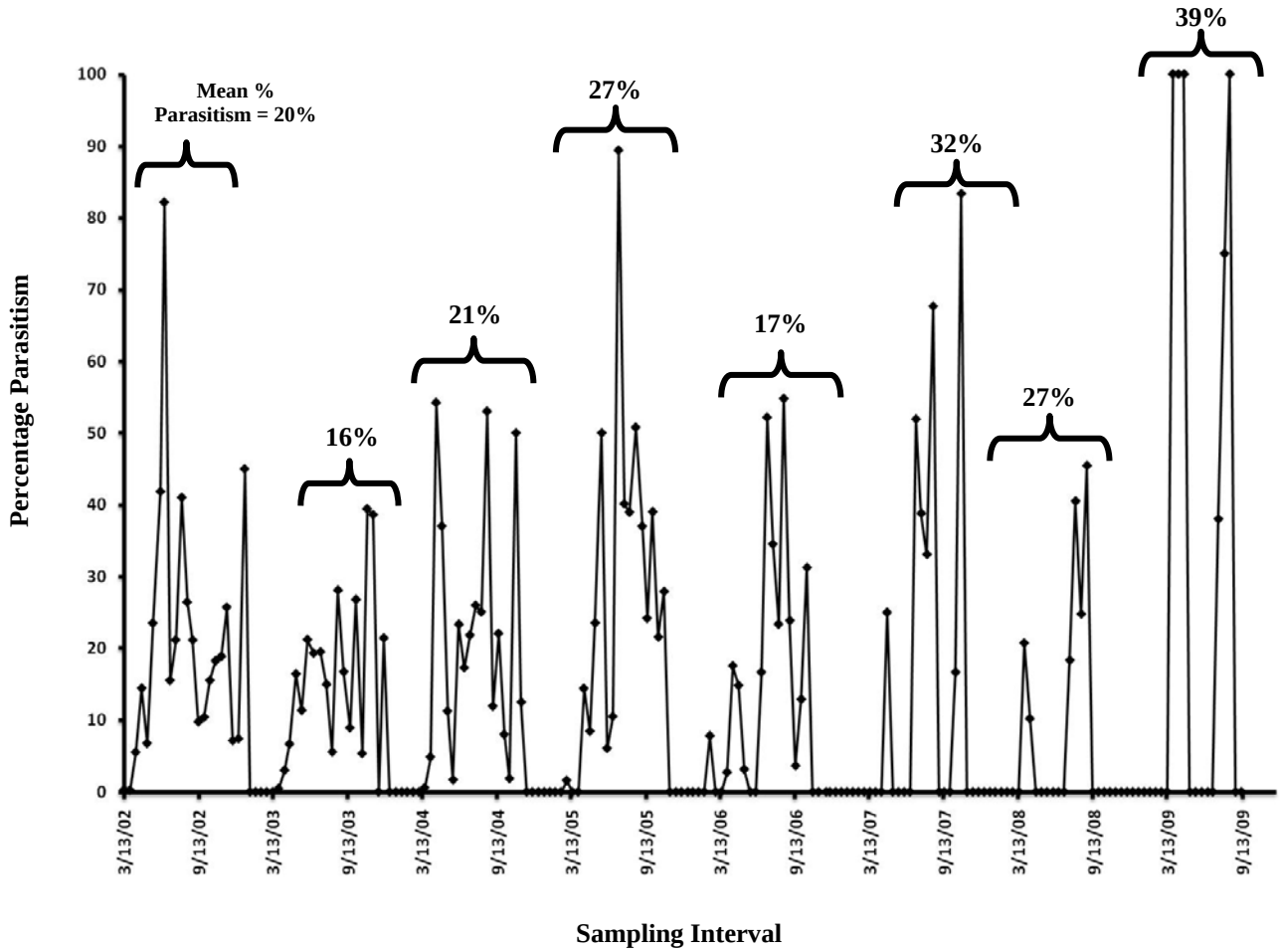

Figure 2. Percentage parasitism estimates of GWSS eggs in Eureka lemons. GWSS egg masses are collected from timed five minute surveys made every two weeks of 10 mature lemon trees at UC Riverside Ag. Ops. Harvested leaves are returned to the laboratory, the number of eggs per egg mass are counted and parasitoid emergence and species identity is determined. Percentage parasitism of GWSS eggs across all years has averaged ~ 25%.

EFFECT OF CONSTANT TEMPERATURE ON GLASSY-WINGED SHARPSHOOTER LIFE CYCLE

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ABSTRACT
The glassy-winged sharpshooter (GWSS) is a vector of *Xylella fastidiosa (Xf)*, the causal agent of Pierce's disease of grapevine. It is the most common leafhopper associated with vineyards in Texas, with the exception of the High Plains. In the Hill Country grape growing region of Central Texas, the insect overwinters in the adult stage. The earliest egg masses are laid in February and March when relatively cool temperatures prevail. Our interest was to determine the effect of ten constant temperatures on GWSS egg development and the effect of exposure periods of 6 to 120 hours in duration to subfreezing temperatures. The effect of temperature on nymphal growth and development was also measured. However, adverse artificial rearing conditions in the growth incubators negatively affected nymphal development which was twice as long as expected in the optimal temperature range under natural conditions. We were unable to draw conclusions without modifying the rearing conditions. The study on the effect of temperature on adult survival is underway and results are not available for this report.

LAYPERSON SUMMARY
The glassy-winged sharpshooter (GWSS) is an insect pest which can transmit the bacterium responsible for Pierce's disease of grapevine. The insect and the disease are commonly associated with vineyards in Texas and are the main limiting factor to grape production in the State. We studied the effect of ten temperatures on the life cycle of GWSS and the effect of short exposure periods to subzero temperatures on the eggs. Tests using the adults are underway. The data generated by these studies are critical to optimizing rearing techniques and for developing control strategies for grape growers.

INTRODUCTION
Grapevine hybrids of *Vitis vinifera* which are traditionally associated with the highest quality wines are varieties susceptible to various degrees of damage by Pierce's disease (PD). This disease is an incurable, debilitating and often fatal bacterial infection caused by *Xylella fastidiosa (Xf)* and disseminated by xylem fluid-feeding insects such as the glassy-winged sharpshooter (GWSS). In Texas we also recorded an additional 28 xylem fluid-feeding species associated with vineyards (Lauzière et al 2008). Many of these insect pests can vector the bacterium (Mitchell et al in press). PD is the most important limiting factor to grape production in Texas (Texas Pierce's Disease Task Force 2004). Funded by the U.S. Department of Agriculture, a statewide research program was initiated in 2002 to study vectors in their natural habitat, their interaction with cultivated vines and other vegetation, and investigate their biology in order to develop pest and disease management strategies.

GWSS is native to the Gulf Coast states of the USA (Young 1968). Indigenous populations of GWSS depend on the insects' ability to survive under the environmental conditions prevailing in the different grape growing regions of Texas. Among abiotic factors, temperature plays a major role influencing an insect's life cycle. Temperature interacts jointly with other factors such as humidity, food availability and light and since temperature is easily measured and controlled, it is common practice to examine its influence upon species of economic importance (Howe 1967). Thorough knowledge of the effects of temperature on development and survival, among other aspects of the biology and behavior of GWSS, is also critical for developing and optimizing rearing techniques under carefully regulated environmental conditions and for conducting field research aimed at developing control strategies.

OBJECTIVES
1. Determine the effect of constant temperatures on the life cycle of GWSS (embryo, nymph and adult)
2. Determine the effect of subzero temperatures on embryos and adults

RESULTS AND DISCUSSION
These studies were conducted at the Texas AgriLife Pierce Disease Research and Education Program facility in Fredericksburg, TX. Containerized *Euonymus japonica* were grown in a greenhouse setting and placed into cages with reproductively mature GWSS. The plants were monitored daily for leaves bearing egg masses. These were left *in situ* and enclosed in an organza pouch fastened with twist-ties. The egg masses were incubated in a growth chamber at the following temperatures: 12, 15, 18, 21, 24, 27, 30, 32.5, 35, and 37.5°C. Development of the eggs was monitored twice daily and emergence of nymphs was recorded. Undeveloped eggs were dissected under a stereomicroscope after 21 days and the total number of eggs was recorded. Because of the non linearity of development rates (the reciprocal of the developmental

period), only temperatures between 12 and 30˚C were used to compute the linear regression for embryonic development of GWSS. Nonlinearities of insect development at high temperatures justified the development of a nonlinear regression model. Therefore, embryonic development rate was fitted to the model of Logan et al. (1976). Embryonic survival was subjected to one-way analysis of variance (ANOVA) to test for temperature effect. When significant *F*-values were obtained, treatment means were discriminated using the Student Newman Keuls (SNK) test ($P < 0.01$).

We also studied the effect of freezing temperatures (-0.9± 0.21°C) on GWSS eggs exposed for 0, 6, 24, 48, 72 and 120 consecutive hours. After each specified time period, the plants bearing egg masses were transferred into a second growth chamber held at a constant 25°C and the eggs were monitored daily for nymphal emergence. Mortality was assessed as described above, 21 days after the mean emergence period when the eggs were assumed to be non-viable. We used a one-way ANOVA to estimate the effect of the exposure period on development time and survival. When significant *F*-values were obtained, treatment means were discriminated using the SNK test ($P < 0.01$).

Embryonic development of GWSS was successful to nymphal emergence between 15 and 35°C. Ultimate embryonic survival varied with temperature ($F = 3.09$; $df = 7, 156$; $P = 0.004$). The proportion of egg hatching was not significantly different for temperatures between 18 and 35°C and averaged 74.2% ± 2.9. At 15°C, percent survival was significantly lower with 43.8% ± 8.8 of the embryos developing into nymphs. Embryos continuously exposed to 12 or 37.5°C did not develop. The lower embryonic development threshold was calculated using a linear regression over the 15-30°C range and was estimated at 12.1°C. Using the Logan model and all temperatures tested, we determined that the optimal development temperature for GWSS eggs is 30.6°C. Embryonic development time decreased linearly between 15 and 30°C, ranging from 22.7 to 4.7 days.

Exposure to freezing temperatures delayed embryonic development for all exposure periods as compared to unexposed egg masses ($F = 201.17$; $df = 4, 384$; $P < 0.0001$). Eggs exposed to freezing temperatures for 6 and 24 hours required about nine days to complete their development, whereas embryogenesis of eggs treated for 48 hours and 72 hours took 11.8 and 13.2 days, respectively. Lethal effects occurred when eggs were kept below freezing for a consecutive 120 hours. This was also the only treatment which affected the plant. Percent survival was significantly different among the 24-120-hour exposure periods ($F = 16.99$; $df = 5, 113$; $P = 0.001$) with 26.6% survival measured after a 24-hour exposure down to 0% survival at 120 hours.

Nymphal development of GWSS fed black-eyed pea plants was studied by M. Sétamou at Weslaco in a similar fashion under controlled constant temperatures of 18, 21, 24, 27, 30, 34°C. In the optimal temperature range, development to adulthood required 45 days. In a previous study, nymphs reared by Lauzière and Sétamou (2009) at 25°C developed in 29.8 ± 0.7 days. We are critical of the data obtained from the temperature study and are concerned that food and light may have deeply affected the development of the nymphs reared under artificial light without sun light. Steps are being taken to correct our methodology so that we may draw conclusions that are more applicable to insect populations under natural conditions.

Temperature data summarized for the Hill Country of Central Texas indicated that the coldest months are usually December, January and February, with average temperatures of 8.8 to 15.1°C for the years 2003 to 2008. The warmest months are usually July and August which ranged 26.8 to 30.9°C in 2003-2008. During the past six years, the coldest year was 2004. Data from a previous study indicated that during the winter of 2004, temperatures in the vineyards remained below 0°C for a total of 100-140 hours (Lauzière et al. 2008). The winter of 2005 was not as cold as it was the previous winter, however, temperatures remained below zero for a total of 200-240 hours. On average, in this region, temperatures remain below zero for 8.6 ± 7.7 consecutive hours at a time and minimal temperatures of -2.8 ± 2.23°C (range -13.5-0.16°C) are recorded. It would be interesting to study this insect's development under cyclical temperature fluctuations in the low to freezing temperature range.

CONCLUSIONS

These studies showed that temperature had a strong influence on growth and development of GWSS. Embryonic development times decreased with increasing temperatures whereas mortality increased with increasing temperatures. For rearing purposes, temperatures of 28-30°C are optimum for the eggs and will yield the highest percentage of emerging nymphs.

Field data from 2005-2008 indicated that GWSS females actively produced eggs from February to September, with highest egg loads observed in March (13.8 ± 7.2 eggs/female; n = 155; (Lauzière 2008). During these months in Central Texas, relatively cool temperatures are usually observed which will affect glassy-winged sharpshooter development. However, the data suggest that egg development is possible and is correlated by field observations of the first generation of adults in early April.

Bioclimatic studies on insect hosts and their natural enemies can help explain their geographic distribution and also provide insight into the potential physiological limitations for their spread into other regions, either naturally or through unintentional translocations.

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EXPANSION OF THE GLASSY-WINGED SHARPSHOOTER IN NORTH CAROLINA VINEYARDS AND ITS ASSOCIATION WITH THE MIMOSA TREE

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Reporting Period: The results reported here are from work conducted May 2006 to September 2009.

ABSTRACT
The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis)* was found in several North Carolina counties that were not previously reported between 2006 and 2009. Data from this four-year study showed that GWSS has been expanding to new sites; this movement appeared to start in the south coastal region and move to the northern and western parts of the state. Several GWSS specimens were found in Currituck Co. (in the most northeastern part of the state) in 2006 and none was found in Wake Co. (Piedmont) the same year in a vineyard that was heavily monitored since 2004, but GWSS appeared in this vineyard in 2007 and 2008. In addition, we found that GWSS preferred the mimosa tree, *Albizia julibrissin.* In areas close to creeks, large numbers of adults and nymphs were recorded in these plants. In the laboratory, this insect laid eggs and completed its life cycle feeding only on this plant. Currently, GWSS appears to have established populations in most of the Coastal Plain and in several areas of the Piedmont. However, GWSS was not detected in the Yadkin Valley (major vinifera growing region of NC) in the northwestern Piedmont, or the Mountain region of NC in 2009.

LAYPERSON SUMMARY
Data collected in this study showed that the glassy-winged sharpshooter (GWSS) has expanded its range to new sites in North Carolina. This movement appeared to start in the southern coastal region and move to the northern and western parts of the state. The GWSS was found in Currituck Co. (northeastern part of the state) but none was found in Wake Co. (Piedmont) in 2006 in a vineyard that was heavily monitored since 2004, but GWSS appeared in this vineyard in 2007 and 2008. In addition, we found that GWSS preferred the mimosa tree, as large numbers of adults and nymphs were recorded in these plants close to creeks. In the laboratory, this insect laid eggs and completed its life cycle feeding only in this plant. Currently, GWSS appears to be established in most of the Coastal Plain, and several areas of the Piedmont. However, GWSS was not been detected in the Yadkin Valley in the northwestern Piedmont (major vinifera growing region of NC) or the Mountain region of NC in 2009.

INTRODUCTION
Leafhoppers are vectors of *Xylella fastidiosa* (*Xf*), the causal agent of Pierce's disease (PD) in European grapes (*Vitis vinifera*). The glassy-winged sharpshooter (GWSS) has become a well known subject of study since its introduction to California. There, the grape growers faced a dire situation after the arrival of GWSS - vines infected with *Xf* increased and the disease became widespread. Reports comparing the transmission of *Xf* by GWSS with native Californian species such as *Graphocephala atropunctata* showed a lower transmission capacity of the former (Hill and Purcell 1995); however, its dispersion capacity (ability to fly long distances) facilitated the expansion of the disease.

In *vinifera* growing areas of NC, four species of sharpshooters were reported prior to the beginning of this study (Villanueva et al. 2007). The presence of the GWSS may increase the incidence of PD, and further limit development of the vinifera industry in North Carolina. In a preliminary study, we observed that the mimosa tree (*Albizia julibrissin*) was apparently a good host of GWSS and leafhoppers were monitored with yellow sticky traps placed in several vineyards of NC and in areas where mimosa trees grows.

OBJECTIVES
1. To evaluate the distribution of GWSS in North Carolina.
2. To study the importance of *Albizia julibrissin* as preferred host of GWSS.

RESULTS AND DISCUSSION

GWSS was first reported in NC in Pender Co. in 2002 (David Stephan, personal communication) prior to this study (**Figure 1**). Pender Co. is located in the southeastern Coastal Plain. In 2006, GWSS was found on yellow sticky traps collected from Currituck Co. (the most northeastern county of NC), which indicates that GWSS has moved to the north. Myers et al (2007) using yellow sticky traps, did not collect any GWSS in two Piedmont vineyards, one located in Wake Co. and the second in Alamance Co. in 2004 and 2005 (**Figure 1**). However, in this study we sampled intensively (>12 traps/vineyard) the same vineyards from 2006 to 2009 and GWSS was found in large numbers in 2007 and 2008 in the Wake Co. vineyard and in addition, live specimens were collected in the NC State University campus in 2009 (in the same county). Also, one GWSS was found in Alamance Co. in August 2009 (**Figure 1**). These results indicate that GWSS has moved from the eastern NC to the west over the past three years. GWSS could have been present in the Coastal Plain counties before this study started in 2006. This area is where muscadine grapes are grown and large numbers of GWSS were detected in traps near muscadine vineyards from mid-June to October in 2007 and 2008. Additionally, they were more abundant in muscadine vineyards than *Oncometopia orbona* the most well distributed sharpshooter in NC. The cause of this migration may be the warmer temperatures which have been observed in recent years (Anas et al. 2008).

We also sampled areas where *Albizia julibrissin* grows; these sites were near vineyards or beside roads and creeks. In most cases we captured GWSS, especially in areas close to creeks. More GWSS were captured in traps hung close to young *A. julibrissin* than plants of *Lonicera albiflora* (honeysuckle), a *Rhus* sp. (sumac) and *Rubus* sp. (blackberry) growing nearby, cherry (*Prunus* sp.), wild grape (*Vitis* sp.) or an old *A. julibrissin* tree (20 ft tall) (**Figure 2a**). The young *A. julibrissin* plants were cut every year from the base and trunks by highway maintenance crews and can grow 2.5 to 3.5 m from April to September. Also, by mid-June large numbers of *O. orbona* were caught in traps in old *A. julibrissin* (~20 ft height) plants. The reason for this is unknown but this plant may be important not only as preferred host of GWSS but it may be a temporary host of *O. orbona* during this time of the year. However, in live counts, GWSS was generally found in greater numbers compared with other species in *A. julibrissin* plants (**Figure 3**). In addition, GWSS females -placed in cages containing six-eight month old *A. julibrissin* plants- were able to laid eggs, and complete their development on these plants alone.

CONCLUSION

In this study we found that GWSS has expanded to new areas of North Carolina. This insect might have been undetected along the coast and southern part of the state for many years. However, in spite of intensive monitoring, it was not detected in the central part of the state until 2007 when we captured it in a vineyard in Wake Co. and 2009 in Alamance Co. Many species of leafhoppers that are vectors of *Xf* are endemic to NC, but GWSS may cause *Xf* to spread more rapidly. Additionally, we found that the mimosa tree is a preferred host of GWSS. GWSS was able to lay eggs, and completed its life cycle on this plant. Also, direct visual counts and trap catches showed a preference for it compared to the surrounding vegetation. Additionally, large numbers of *O. orbona* were found in old *A. julibrissin* trees.

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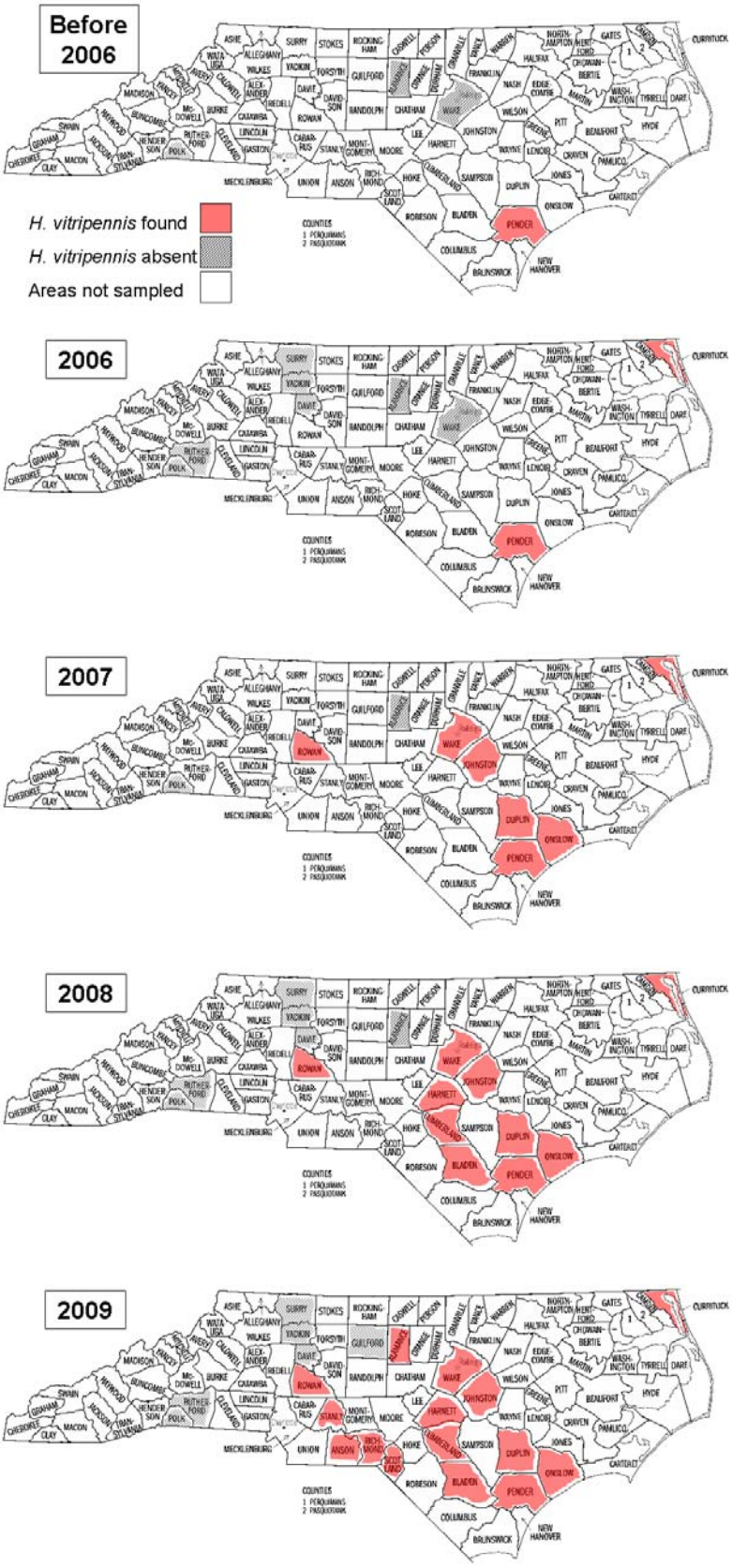

Figure 1. North Carolina maps showing counties where GWSS were present, absent, and not sampled.

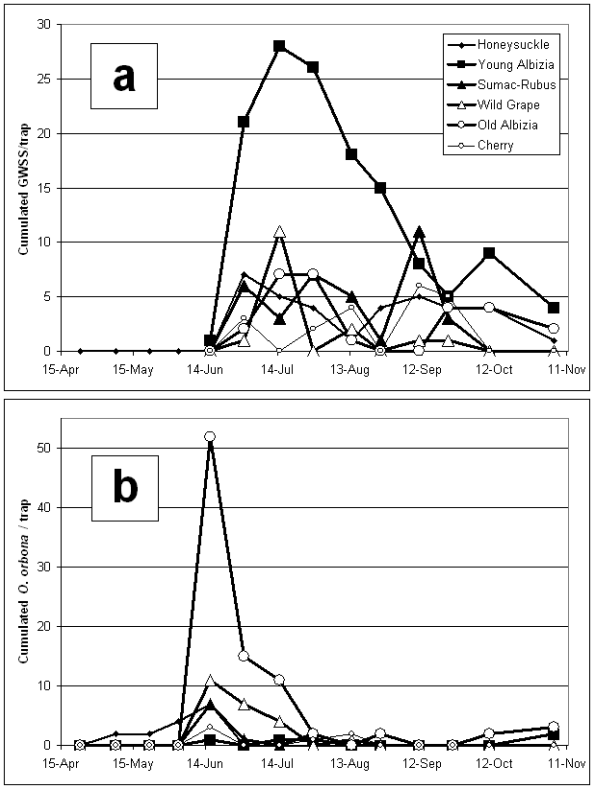

Figure 2. Cumulated numbers of (a) *GWSS* (GWSS) and (b) *Oncometopia orbona* caught in yellow sticky traps placed in honeysuckle, sumac- Rubus, wild grape, cherry, and old and young *A. julibrissin* trees in 2008. Traps were replaced every 2 weeks.

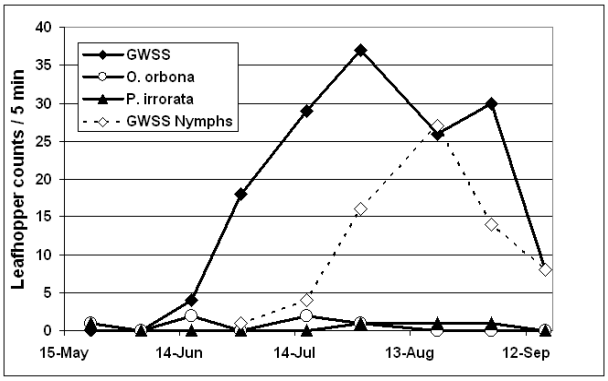

Figure 3. Numbers of GWSS (GWSS adult or nymphs), *Oncometopia orbona*, and *Paraulacizes irrorata* found during 5-min interval counts in *Albizia julibrissin* plants in 2007

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Section 2:

Vector Management

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

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Reporting Period: The results reported here are from work conducted July 2009 to September 2009.

ABSTRACT
The impact of systemic treatments of dinotefuran on the adult and egg stages of the glassy-winged sharpshooter (GWSS) 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. In bioassays with adults exposed to grapevines treated systemically with dinotefuran, we quantified concentrations within the xylem by ELISA and related the concentrations to insect 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.

LAYPERSON SUMMARY
The systemic neonicotinoids imidacloprid and dinotefuran are effective insecticides that growers can use for long-term management of glassy-winged sharpshooter (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. One of the interesting observations from this study has been that the concentrations of insecticide present within the xylem can be managed by choosing the appropriate application rate. This is a very powerful tool that can be used to optimize insecticide applications and manage insecticide use more effectively. In this study, we are determining the concentration of dinotefuran that is needed within the xylem of plants to kill a feeding GWSS. We have already demonstrated that dinotefuran is toxic to the GWSS adults, and we have also shown that nymphs emerging from an egg mass are susceptible to the contact activity of the insecticide before they commence feeding.

INTRODUCTION
Our research program focuses on the use of chemical insecticides for the management of glassy-winged sharpshooter (GWSS). 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. 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). Dinotefuran offers a potential solution to overcoming the problems encountered with imidacloprid use – its rate of uptake is faster and it can reach higher concentrations at peak uptake than imidacloprid under the more challenging situations. It also exhibits favorable persistence. Having established that the uptake and persistence of dinotefuran is superior to imidacloprid in terms of insecticidal titers reached in the xylem, it is important to ensure that the levels attained in the xylem are active against sharpshooters. Comparative data on the efficacies of systemic dinotefuran against GWSS are not available.

OBJECTIVE

1. Determine target thresholds for systemic neonicotinoids against GWSS in grapevines.

RESULTS AND DISCUSSION

The concentrations of dinotefuran in cotton plants used for bioassays of adult GWSS can be effectively controlled (**Figure 1**). In three independent experiments, the concentrations of dinotefuran in extracts of xylem fluid were consistent with the application rates used. After dilution of samples to eliminate matrix effects, the lower limit of detection of the ELISA was 30 ppb dinotefuran. This result shows that there is potential to control the levels of insecticide present in plants, provided there is a good understanding of the environment (soil type, irrigation, etc) under which the insecticide is being used.

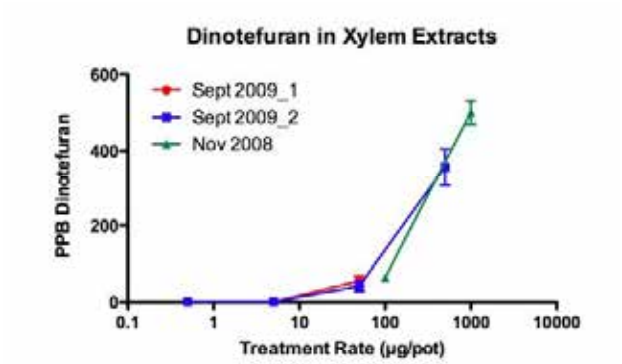

Figure 1. Concentrations of dinotefuran in xylem fluid sampled from cotton plants. Plants were treated with Venom 70 SG insecticide at different rates, and the xylem fluid extracted by pressure bomb at 5 days post-treatment when GWSS bioassays were completed. Not all concentrations were repeated for each bioassay.

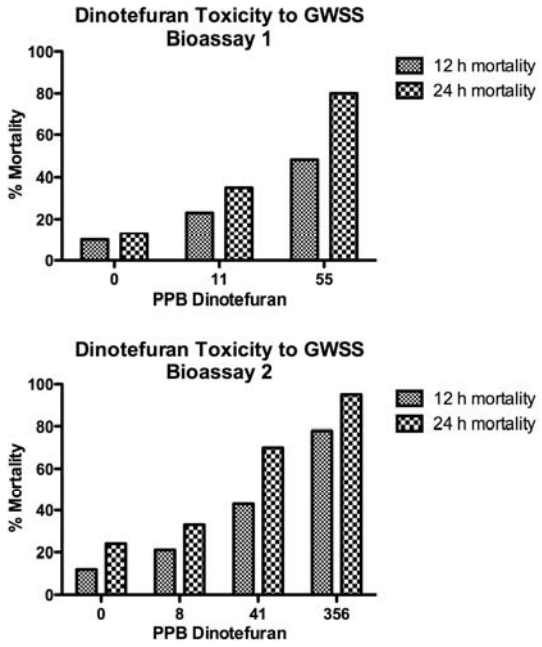

Figure 2. Toxicity of dinotefuran to GWSS adults. Mortality was assessed at 12 and 24 hours after the insects were confined on the treated plants. Data are from 2 independent bioassays.

The results of two independent bioassays are shown in **Figure 2**. Adult GWSS were caged on treated plants and mortality was assessed at 12 and 24 hours. In both bioassays, the control mortality was high. GWSS adults are difficult to work with because of the high control mortality, so it will be necessary to increase the number of replicates in subsequent bioassays in order to minimize this effect. Nevertheless, the results indicate the toxic nature of dinotefuran, with a clear dose response. The results from both bioassays were very consistent, indicating a robust bioassay system.

In the second set of experiments, we evaluated the effect of dinotefuran against the eggs of the GWSS. Adult GWSS were confined in cages with cotton, which is an excellent host for GWSS oviposition. Leaves with egg masses (not older than 24 hours) were cut from the plants and the petioles inserted into vials containing a range of insecticide solutions. The uptake of insecticide into each leaf was allowed to proceed for 24 hours and the leaves are then transferred to leaf boxes. The leaf boxes were maintained under lights until the normal period of embryonic development was completed. Mortality was assessed at the time of emergence of the first instar.

As with imidacloprid, the nymphs developed fully within the egg mass and only succumbed to the effects of contact with dinotefuran during emergence. In contrast to our previous data for imidacloprid, where we observed an LC_{50} of 39 ng/cm^2 leaf, the indications from our current data set show that dinotefuran is slightly more toxic to the first instars than imidacloprid (**Figure 3**). Also, the slope of the dose-response curve is extremely steep, as was observed for imidacloprid.

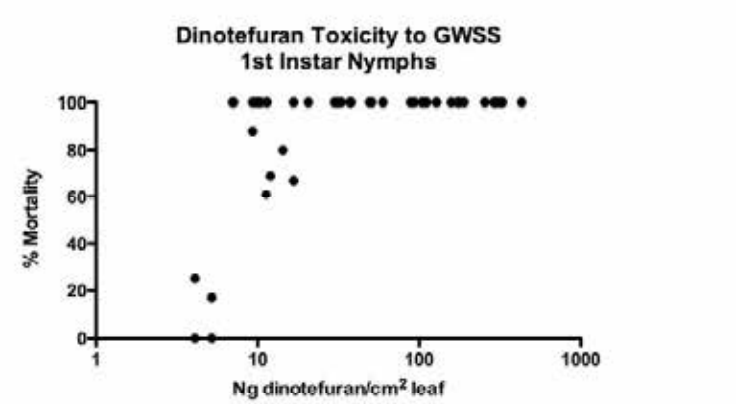

Figure 3. Toxicity of dinotefuran to emerging 1st instar GWSS. The petioles of leaves containing egg masses were placed in vials containing different concentrations of insecticide for 24 h systemic uptake. Leaves were then transferred to leaf boxes where the egg masses were allowed to continue their development. The survivorship of nymphs was determined for 2 days after emergence.

CONCLUSION

At current label recommendations, the rate of uptake of dinotefuran into grapevines is faster than imidacloprid and concentrations of dinotefuran at peak uptake are higher (Toscano et al., 2007). These two properties make dinotefuran a strong candidate for inclusion in a sharpshooter management strategy, provided that effective concentrations are reached within the xylem. Our bioassay data are inconclusive at this point due to high control mortality, which is making the true insecticidal effects difficult to ascertain. It will be important to minimize the impact of control mortality in order to derive an effective target concentration for dinotefuran against adult GWSS, so further bioassays are needed. Dinotefuran is highly toxic to emerging first instars, and our data suggest that the insecticide is slightly more toxic than imidacloprid. As with imidacloprid, the toxic effect is not manifested until the nymphs emerge from the egg mass, suggesting that dinotefuran and imidacloprid act as contact insecticides.

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. One of the interesting observations from this study has been that the concentrations of insecticide present within the xylem can be managed by choosing the appropriate application rate. This is a very powerful tool that could be used to optimize insecticide applications and manage insecticide use more effectively.

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FUNDING AGENCIES

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DEVELOPMENT OF EFFECTIVE MONITORING TECHNIQUES FOR SHARPSHOOTERS AND THEIR PARASITOIDS

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Reporting Period: This project is awaiting receipt of funding.

ABSTRACT
This project relates to ongoing efforts of the Pierce's Disease Control Program to assess the efficacy of the sharpshooter egg parasitoid biocontrol program. *Gonatocerus morgani*, *G. morrilli*, and *G. triguttatus* have been reared and released by the program at sites throughout southern California and the southern Central Valley since 2000. California Department of Food and Agricultiure (CDFA) reports, the most recent in 2007, have demonstrated the effectiveness of these efforts. However, current methods to assess released species populations, the extent of parasitism by native competitors, and host preferences of the parasitoids involved are limited. D. Cooksey conducts research in comparative and functional genomics of *Xylella fastidiosa (Xf)* to identify key virulence factors through construction of specific mutations in the bacterial genome. To facilitate this work, his laboratory has developed a multiplex PCR system for the simultaneous identification of *Xf* strains (Hernandez-Martinez *et al.*, 2006). D. Morgan, Supervisor of the release program, is thoroughly familiar with the biology, ecology, systematics, and identification of the host and parasitoid species targeted in the proposed study. The development of the proposed multiplex PCR system will greatly enhance the data acquisition of the CDFA parasitoid release biocontrol program.

LAYPERSON SUMMARY
The suppression of glassy-winged sharpshooter (GWSS) populations is accomplished in part by biological control agents. An accurate and rapid method for identification of the eggs of sharpshooter species, determining whether eggs are parasitized, and by which parasitoid species, is essential for estimating success. Current methods are flawed and expensive. Development of a single-step multiplex real-time PCR assay for sharpshooters and their parasitoids would allow for accurate reporting of GWSS occurrences and facilitate development of effective control agents.

OBJECTIVES
1. Develop primer pairs that can be used in a multiplex PCR system for each species of sharpshooter and parasitoid. Several genes have been partially sequenced for GWSS and smoketree sharpshooter and for a number of their parasitoids. These sequences will be analyzed for primer design.
2. Clone the target genes from those species of parasitoid for which there is no sequence data available. This will be accomplished through the use of published primers or the development of degenerate primers.
3. Determine the limits of detection of each species of sharpshooter and parasitoid. Based on other studies, we are confident we will be able to detect developing parasitoid embryos in sharpshooter eggs. We hope to be able to determine both the host and parasitoid species from sharpshooter egg cases from which the parasitoids have eclosed by amplifying the layer of cells which remain in the parasitoid egg (Oda and Akiyama-Oda, 2008).

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FUNDING AGENCIES
Funding for this project will be provided by the University of California Pierce's Disease Research Grants Program.

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

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Reporting Period: The results reported here are from work conducted March 2009 to October 2009.

ABSTRACT
This report presents the progress obtained in the development and application of an RNA interference (RNAi) based system aimed to target genes of the vector of *Xylella fastidiosa(Xf)*, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*). After demonstrating that RNAi induction in GWSS cells and insects is achievable, in the year 2008-2009 we started screening a large pool of candidate genes to find the best target to control the survival of the insect vector. These data will be used to develop transgenic plants expressing dsRNAs of the target genes in their xylem tissues via EgCAD2, a xylem-specific promoter. Transgenic plants will be evaluated for their ability to induce RNAi effects on GWSS and other sharpshooter vectors of *Xf*.

LAYPERSON SUMMARY
This work presents fundamental efforts towards long term application of using RNA interference, RNAi, to help combat a plant disease of great economic importance. The disease, Pierce's disease (PD) of grapevines, is a significant threat to grape production in California and other parts of the U.S., and the causal agent of the disease, *Xylella fastidiosa (Xf)*, a xylem-limited bacterium, also causes several other extremely important plant diseases worldwide. Our effort here does not directly target *Xf*, but instead targets one of its most significant insect vectors, the glassy-winged sharpshooter (GWSS), and we combine the use of an *in vivo* system (GWSS whole insects) with an *in vitro* GWSS cell based system and demonstrate genetic and phenotypic RNAi effects. RNAi is an extremely important and broadly studied area in contemporary biology, and terms such as "magic bullet" for human medicine, and "genetic insecticide" for targeting insects have been used in the literature. Our work represents the first demonstrated RNAi effort in GWSS and our data will help to expand the possibilities to study plant-associated insects and at the same time to target the sharpshooter vectors of *Xf*, the causal agent of PD.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) is among the most robust and thus most threatening vectors of *Xylella fastidiosa (Xf)*, the bacterium that causes Pierce's disease (PD) (Davis, Purcell et al. 1982), a devastating disease occurring in wine grapes from California to Texas to Florida (Myers, Sutton et al. 2007). New strategies that will lead to environmentally sound approaches to control GWSS and other insect vectors are needed. RNA interference (RNAi) has been suggested as a strategy to develop "insect-proof plants" (Gordon and Waterhouse 2007) and even referred to as a "genetic insecticide" (Scharf 2008). RNAi is a eukaryotic gene regulation/defense mechanism in which small RNA segments, small interfering RNAs (siRNAs) (21-25 nt), generated by processing of dsRNA molecules often of viral origin, specifically down-regulate complementary RNA sequences (Meister and Tuschl 2004). Recent efforts demonstrate that RNAi is inducible in many insects. Intrathoracic injection of dsRNAs has been shown to be the most effective way to induce RNAi in whole insects of many species including *Anopheles gambiae* (Blandin, Moita et al. 2002; Blair, Sanchez-Vargas et al. 2006), *Blattella germanica* (Ciudad, Piulachs et al. 2006), *Drosophila melanogaster* (Dzitoyeva, Dimitrijevic et al. 2001), *Spodoptera litura* (Rajagopal, Sivakumar et al. 2002), *Culex pipiens* (Sim and Denlinger 2009), *Lutzomyia longipalpis* (Mauricio R.V. Sant'Anna), *Cecropia pupae* (Bettencourt, Terenius et al. 2002), *Acyrthosiphon pisum* (Mutti, Louis et al.), *Rhodnius prolixus* (Araujo, Soares et al. 2009), *Aedes aegypti* (Cooper, Chamberlain et al. 2009), *Bemisia tabaci* (Murad Ghanim), *Dermacentor variabilis* (Mitchell Iii, Ross et al. 2007) and *Tribolium castaneum* (Arakane, Dixit et al.). Oral induction has also been demonstrated in several of these same species. Our effort demonstrates for the first time that RNAi activity can be induced in a leafhopper species, but also is inducible in GWSS cell lines. In the long term, RNAi can be used as an effective fundamental tool to better understand the dynamics of plant: pathogen: vector interactions as well as GWSS physiology and of course we hope as a strategy to complement overall efforts for PD control.

OBJECTIVES

The specific objectives of our effort are:

1. To identify RNAi-inducers capable of killing or reducing the survival and/or fecundity of GWSS and other sharpshooter vectors of *Xf*.
2. To generate transgenic plants capable of expressing GWSS deleterious interfering RNA molecules within their xylem.
3. To evaluate transgenic plants for their ability to induce RNAi effects vs. GWSS and other sharpshooter vectors of *Xf*.

RESULTS AND DISCUSSION

RNAi in GWSS cells and insects. Initially, we used 14 GWSS GenBank cDNA sequences corresponding to known proteins in order to synthesize RNAi inducer molecules, dsRNAs. We then tested whether RNAi was inducible in GWSS cells and insects, and we were able to show that RNAi activity is inducible in GWSS. Sets of dsRNA molecules were delivered to GWSS cells via lipid-based transfection, and to GWSS nymphs via intrathoracic injection or by feeding on cuttings immersed in a solution containing dsRNAs. Real time RT-PCR, semi quantitative RT-PCR, Northern blot of small and large RNA fractions showed that RNAi was achieved in cells and insects injected with dsRNA, where target mRNAs were partially degraded and specific siRNA, hallmarks of RNAi, were detected.

Because there are several potential sharpshooter vectors of *Xf*, the sequences isolated from GWSS were also amplified from the blue-green sharpshooter (BGSS; *Graphocephala atropunctata*) and from the green sharpshooter (*Draeculacephala minerva*) and cloned. This demonstrated a high degree of sequence conservation among these distinct sharpshooters, and the resulting sequences could be used to develop a general RNAi strategy to control multiple *Xf* vectors.

The above results showed anticipated reductions in target mRNAs. Therefore we evaluated if corresponding encoded proteins were reduced and if visible phenotypic results were induced. Western Blot analysis also showed a reduction of actin protein in GWSS nymphs injected with actin dsRNA (**Figure 1**). In addition, some of the injected nymphs did not complete ecdysis, demonstrating a striking phenotypic effect in whole insects vs. those injected with control gfp dsRNAs (**Figure 2**). A visible phenotypic effect was also obtained in GWSS cells transfected with actin dsRNA, where aberrations of the actin filaments occurred starting 72 hours post transfection. (**Figure 3**).

Because our results so far were dependent on a limited number of GWSS sequences so far available in Genbank, we analyzed three EST libraries deposited in GenBank. Twenty thousand thirty (20,030) EST sequences were analyzed using the Arthropod EST analysis pipeline at Kansas State University. One thousand nine hundred seventeen (1917) contigs were assembled and 6561 input reads were retained. The average length of the assembled contigs was 570 bp. NCB BLASTX was used to find sequence similarities in GenBank for the assembled contigs and singletons. One thousand seventy three (1073) contigs and 2057 singlets returned significant hits from GenBank, for a total of more than 3100 sequences. As expected, the great majority of these sequences correspond to structural and housekeeping genes, but a great number correspond to genes of potential interest as RNAi targets, including genes for cuticle formation, larval development, juvenile hormones, central nervous system development, eye morphogenesis and development, lipid and carbohydrate metabolism expressed in gut tissues and genes expressed specifically in salivary glands. Experiments are underway to begin assessing these potential RNAi targets.

Xylem specific promoter cloning.

The specific xylem promoter EgCAD2 was cloned from *Eucalyptus gunii*. The sequence was fused to the GUS reporter gene in the binary PCB 301 vector. Then, GUS expression driven by the xylem specific promoter was accessed in a transient *Agrobacterium tumefaciens* assay in *N. benthamiana* plants. Upon staining for GUS activity, results showed that blue product was restricted to the main vascular tissues. This gives confidence in this promoter, which will now be used to attempt to express specific interfering RNAs in the xylem of transgenic plants. Choosing which plant to use initially is difficult. However, citrus has been implicated as an important GWSS host plant in southern California, and Carrizo citrange is one of the plants easily transformed and manipulated at UC Davis. It also is a host of GWSS in our studies (**Figure 4**), thus it will allow us to rapidly test our hypothesis for xylem delivery of RNAi molecules.

CONCLUSIONS

Xf is an important bacterial pathogen of economically important crops such as grape, but also citrus and almond. The ability to minimize the economic impact of this bacterium depends on the presence and abundance of its biological vectors and GWSS is the most effective vector of *Xf* transmission in some agricultural areas. RNAi-based efforts directed toward the control of insect plant pests are now becoming more feasible, and RNAi for insects as GWSS has great potential application.

The results presented here show that RNAi can be induced both *in vitro* (GWSS -Z15 derived cell line) and *in vivo* in GWSS nymphs. We showed that GWSS -Z15 cells can be used to screen candidate gene silencing targets, and that since RNAi is active in cells, it could also be used to study GWSS gene function via mRNA knockdown. The mRNAs targeted for RNAi in this study were chosen from a limited number of sequences currently available for GWSS, but the same approach can be applied to the other genes identified in the analysis of the GenBank GWSS EST libraries. More notably, the employment of RNA silencing in whole GWSS insects could offer help towards a potential solution for control of the vector. Future work

includes the screening of more RNAi targets, the production of transgenic plants expressing dsRNAs in their xylem and the study of GWSS insects grown on the transgenic plants.

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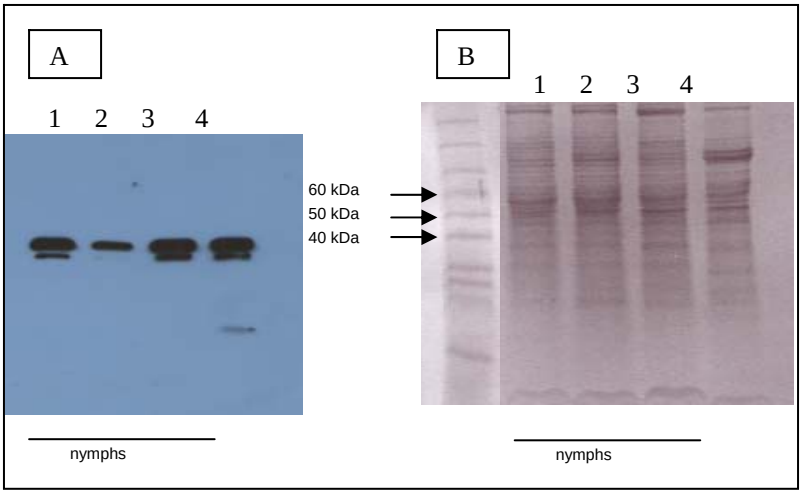

Figure 1. GWSS nymph injected with actin dsRNA shows decreased actin protein level. Fifteen third and fourth instar GWSS nymphs were injected with 1µg actin dsRNA in 1µl volume, with 1 µg GFP dsRNA or with 1 µl injection buffer and left on basil plants for five days. Then, proteins were extracted from three living and one dead insect and subjeted to Western blot analysis, using actin antibodies specific for *Drosophila melanogaster* . Results show a decrease in actin protein in the nymph injected with actin dsRNA and alive five days post injection (gel lane 2 panel A), compared to the other insects (gel lanes 1, 3 and 4 in panel A). Coomassie staining shows equal amounts of proteins loaded for each sample (panel B), 15 µg total proteins were loaded in each lane. Treatments: Lane 1: Actin dsRNA injected nymph, collected dead one day after injection. Lane 2: Actin dsRNA injected nymph collected dead five days after injection. Lane 3: GFP dsRNA injected nymph collected dead five day after injection. Lane 4: Uninjected adult.

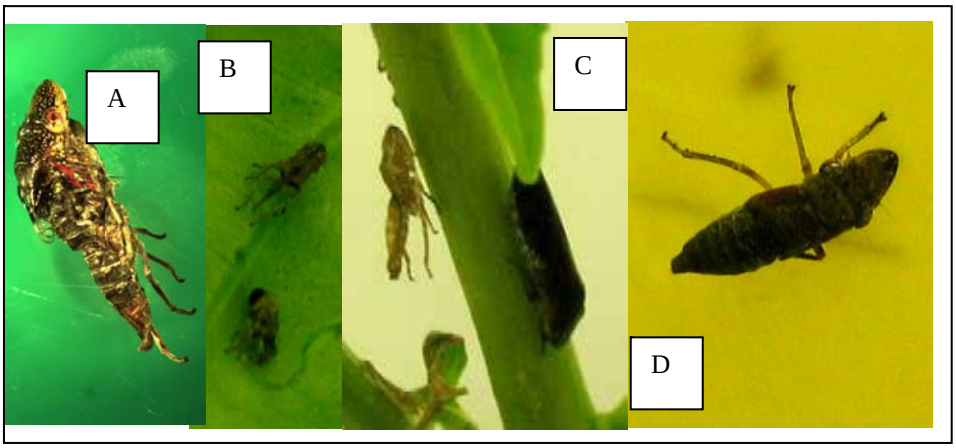

Figure 2. GWSS nymphs injected with actin dsRNA died during molting. Fifteen third and fourth instar GWSS nymphs were injected with 1ug actin dsRNA in 1ul, or with 1 ul injection buffer and left on basil plants for five days. During this period, two of the actin dsRNA injected insects couldn't complete molting and died. In panel A, one of the nymphs with incomplete molting is shown. In other panels the presence of exoskeletons on a basil leaf indicate the completion of molting in the observed group of nymphs (picture B). Shot of an exoskelton close to an adult that succesfully completed molting (picture C). Injected nymph showing a normal phenotype (picture D). Experiment was repeated three times with similar outcome.

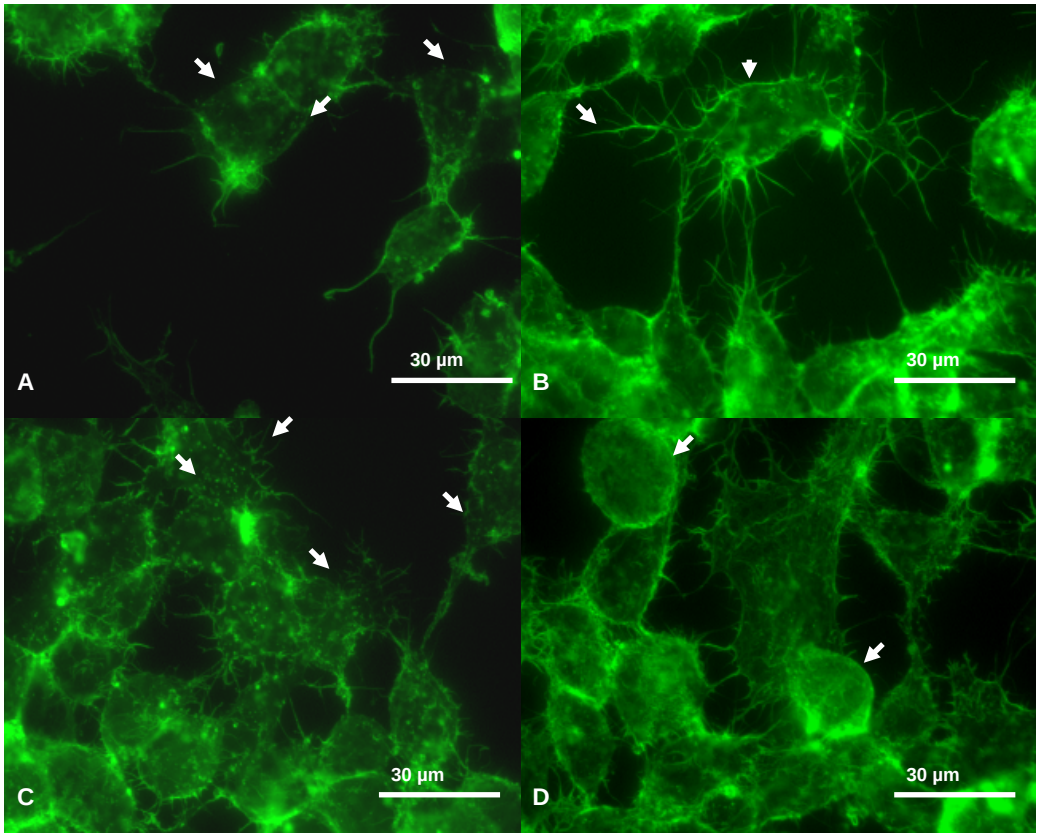

Figure 3. Actin representative morphology in GWSS -Z15 cells after transfection with actin dsRNA. Cells were transfected with 2 µg of actin dsRNA (A and C), or GFP dsRNA (B and D) and harvested 72 hpt. Actin filaments in the cell membrane and cytoplasmic area were largely disturbed (arrows in A and C). (A) GWSS cells showing partial disruption of the actin organization at the cell plasma membrane. Some filaments began to break and the cells failed to branch out. (B) GWSS cells showing no changes in actin filament distribution and polymerization. Healthy isolated cells were connected through a densely branched actin filament network. (C) GWSS cells showing severe disruption of actin filaments. The short fragments of actin filaments were scattered throughout the cytoplasm. Some actin fragments tended to aggregate into clusters below the plasma membrane and obvious twisted actin cables could be observed. (D) Actin filaments were found primarily in the cell cytoplasm as a continuous and organized net in the control cells. All observations were at 72 hours post treatment.

Figure 4. GWSS adults and nymphs feeding on Carrizo seedlings. Two GWSS adults and two nymphs were introduced in cylindrical plastic cages containing one month old Carrizo seedlings and left feeding for one week. After this period of time, all insects were alive and were feeding on the seedlings. Plants did not show any damage caused by the GWSS feeding.

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

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Reporting Period: The results reported here are from work conducted July 2007 to October 2009.

ABSTRACT
Efficient and precise methods for detection of new colony infestations and for monitoring glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis* (Germar)) populations are lacking. This proposal addressed detection and monitoring methods and accompanying leafhopper behavior toward improved management of GWSS.

LAYPERSON SUMMARY
Management of the vector glassy-winged sharpshooter (GWSS) and Pierce's disease (PD) is contingent on the availability of efficient field-sampling methods. This proposal aimed to improve upon the current monitoring methods. GWSS behavior in response to various types of traps in combination with host plants and other factors with potential to increase trap efficiency were investigated. Current trapping relies on use of the yellow Seabright trap which is flat with two sides covered with stickem to capture the insects. The trap attracts in two directions and has a total yellow attraction area of 653.8 cm^2 and a trapping surface area (stickem covered) of 409.4 cm^2. We have found that a yellow cylinder (tube) trap 7.6 × 30.5 cm (3 × 12 in, 730 cm^2 area) that samples in all directions (360^0) usually improves trap capture rate by two-four times for males and somewhat less for females. We have used Glidden Alkyd Industrial Enamel 4540, Safety Yellow, as the standard color and Tangletrap™ (Gemplers.com) as the standard sticky substance. Trap capture efficiency is inversely proportional to the distance from a host plant. GWSS respond to other leafhoppers when searching for and landing on a host plant and apparently in response to traps. Adding a leafhopper or model of a leafhopper to a trap can increase trap catch by 20-50% under low vector populations. Trap capture efficiency does not correlate well to leafhopper numbers found on host plants with the exception of within large blocks of citrus (Castle and Naranjo 2008), and may be inversely related on host plants with high nutritional quality such as crape myrtle when the plant is at peak quality with respect to xylem nutrients.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) 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 critical uninfested locations. Due to the unique biology and behavior of 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 addressed the detection and monitoring needs.

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
A series of data are provided to indicate some of the approaches we have undertaken. In brief, we have looked at trap size, color, height, shape, orientation, background contrast, placement relative to vegetation, distance from vegetation and a number of other factors relative to GWSS behaviors with the objective of understanding and improving trap efficiency. We have also made some novel discoveries about GWSS behavior in response to congeners. We have used the commercially-available Glidden Alkyd Industrial Enamel 4540 Safety Yellow, as the standard color in all tests versus commercial traps, Tangletrap™ (www.Gemplers.com) as the standard sticky substance, and a standard height of 1 m from the ground for trap placement.

We have shown that GWSS capture rate may be increased by changing the trap configuration from a flat two-sided trap into a cylinder (tube) shape which apparently samples the entire surrounding 360^0. A comparison of safety yellow mailing tubes 5.1 cm width × 15.2 cm or 30.5 cm length, 7.62 cm width × 15.2 cm or 20.5 cm width, and 10.2 cm width × 15.2 cm length or 20.5 cm length indicated that total GWSS trap capture increased approximately 40-50% in response to each incremental

increase in trap size either in width or length. All tube sizes captured significantly more GWSS than the Pherocon AM trap used as the standard in these experiments. We choose a 7.62 cm × 15.2 cm as a standard size of the tube trap because it improves capture rate, is easy to work with and less expensive. In other experiments we tested the tube trap versus the Pherocon AM and the Seabright flat, 2-sided yellow traps as well as versions of the commercial traps configured into a cylinder shape with mixed results. The tube trap always captures numerically greater numbers of GWSS but it was usually only the male leafhoppers that were captured in significantly higher numbers. For example in one test the tube captured 125 (total) leafhoppers (93 males, significantly greater than other treatments, t-test, $P<0.001$) while 31 (24 males), 25 (17 males) and 37 (23 males) were captured by the Pherocon AM, Seabright and the Seabright cylinder, respectively. In another test, the Pherocon AM captured 10.5 ± 3.5 (mean ± SEM)/trap/sample period, Pherocon AM cylinder captured 12 ± 3.5 and the tube captured 21 ± 3.4. In another test we deployed 10 traps of the tube, the standard Seabright and two Seabright traps together formed into a cylinder with staples to provide the same surface area and relative profile as the tube trap. We placed the traps on one m high stakes in a RCB design in a block of large crape myrtle. The Seabright captured a total of 142 GWSS (122 males), the Seabright cylinder 295 (260 males) and the tube captured a total of 389 (337 males) over the duration of the test. All the trap types were significantly different ($P<0.0001$) using SAS PROC GENMOD and contrast tests to compare means. These results appear to indicate that the cylinder shape provides an advantage but we did not control for the difference in yellow hue inherent in the trap colors. Nevertheless, color does not appear to be the main factor because it is likely that the Seabright cylinder capture rate was in part lower due to our inability to make it completely smooth when we constructed it from two separate traps. However, the Seabright cylinder trap presented a larger target overall because we formed the trap such that the area covered with stickem was equal to that of the tube trap. All things being equal, the tube trap provided a significant increase in capture rate.

Another method to potentially increase trap capture rate and efficiency may be to increase the number of traps used together or to enhance the attraction of the main sticky trap with some additional visual cues. Size matters as explained above. The addition of a host plant clearly increases the number and/or the residence time of responding leafhoppers thereby bringing them in closer contact with the trap. This might function by increasing the active distance of the trap to the leafhoppers, thus in effect sampling more area around the trap. We tested this concept in several ways in several tests with both Seabright and tube traps as follows but found no significant increase in trap capture rate. We placed traps in the field using an randomized complete block (RCB) design with treatments of one, two and three traps together two m apart. We placed sticky traps ≤ 0.5 m away from another yellow tube without stickem that was 7.6 cm in width but 91 cm in length (bottom 30 cm next to trap and top 60 cm above the sticky trap). We also placed the larger yellow tube without stickem directly above the target sticky trap. These results appear to indicate that GWSS respond directly to traps and do not spend time in any behavior around traps once they respond such as moving down, repeated flying around into the trap, etc. that may be exploited. We also tested a treatment that placed the larger tube without stickem below the target trap (ground -one m) without significant improvement in trap catch.

GWSS respond strongly to host plant quality and change host plants often. Optimum trap placement relative to host plants was considered as one potential method to improve trap capture rates. Several tests were conducted to investigate GWSS response to traps relative to the presence of a host plant, host plant quality and the trap distance from a host plant. **Figure 1** shows the effect of host plant presence on trap catch using tube traps and a poor host peach vs a good host crape myrtle. Twenty-five container plants each of peach and crape myrtle with adjacent traps and 25 traps without plants were placed in the field in adjacent blocks in a five × five m grid. GWSS were recorded each day in the morning and afternoon by position either on the plant or trap. The treatments were re-randomized one time per week to remove any positional effects in the field plot. Clearly, the presence and quality of the host plant affected the number of GWSS trapped with the better host crape myrtle attracting more and inducing a higher trap capture rate than peach, the poor host plant, and the traps alone. In a different study we used tube traps in combination with an array of host plants in containers and compared trap capture between traps with plants within one m distance and traps without plants. Host plants used were apple, red oak, 'Tonto' crape myrtle, 'Flordaking', and 'Elberta' peach, redbud, 'Santa Rosa' plum and 'Bradford' pear. We used seven replicates of each treatment in a RCB design and conducted the test for 38 days. The trap alone captured 135 GWSS and all trap + plant treatments, except for 'Flordaking' peach and 'Santa Rosa' plum which captured less, numerically captured a higher number, on average 47% more, GWSS than the control. Apple, oak, redbud and Bradford pear traps captured statistically significantly higher GWSS than the control ($P<0.05$, LSD).

Along with plant quality differences, the distance a trap is placed from a plant may also be a factor potentially affecting GWSS trap capture rates. In another test we placed a seven × seven m grid of tube traps 10 m apart centered on a large planting of 'Natchez' crape myrtle of ca. two m in height (see other results from this test reported in Northfield et al. 2009). Response of GWSS to traps located at different distances from the crape myrtle plants are shown in **Figure 2**. A linear relationship inversely related to distance was significant at $P< 0.001$ with an $r^2 =0.58$. In another test we placed five replicates of tube traps and Seabright traps at one m and five m from large 'Natchez' crape myrtles. In this test GWSS response was: tube 8.7/trap period ± 0.86 (mean ± SEM) at 1 m, and 4.4 ± 0.6 at 5 m, and Seabright 5.2 ± 1.24 at one m and 4.6 ± 0.75 at 5 m. The tube at one m had significantly higher GWSS ($P < 0.0006$) using SAS Proc GENMOD and contrast statements. Again, the majority of leafhoppers captured were males. Finally, trap efficiency is directly related to how much surface area the trap actually samples termed here as the trap's "active distance". In the case of a cylinder this would be

circle of some size around the trap. This parameter is directly related to the size of the trap given that color and height are optimized. We attempted to determine the active distance of the tube trap by placing a set of five traps in a configuration where a center trap was surrounded by four other traps in a square around it. We varied the distance of the surrounding square of four traps by six, eight, 10 and 12 m. We also placed a single control trap 35 m away from the treatment trap sets. We used five replicates in a RCB design. Theoretically, the active distance is determined by comparing the treatment capture rates in the center trap to the control trap rates and by comparing the total capture rate between treatments. When the center and control rates are equal then the active distance of the trap is somewhere near this spacing because at lower distances the four companion traps interfere with the center trap capture rate which is lower than the control as a result. When the total capture rates per treatment decrease, the trap groupings change at that distance into independent traps rather than acting together (overlapping as in a single visual presentation) as they would at lower distances. Our results were unclear, however, we observed no differences between the center and control traps but total GWSS trap capture rate by treatment did increase linearly from six, eight and 10 m and then declined in the 12 m treatment. This suggests that the tube trap may have an active distance of approximately 10 m.

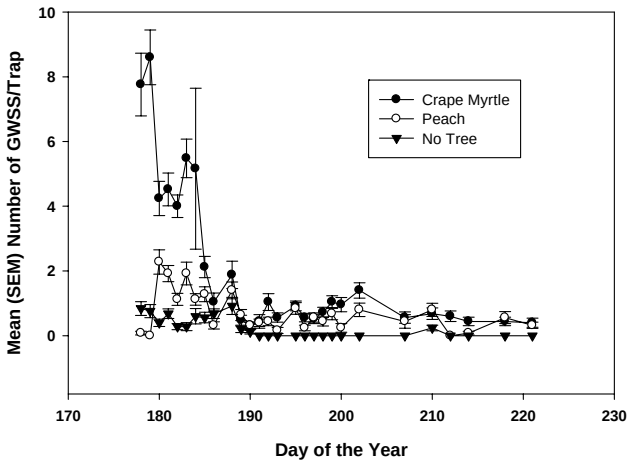

Figure 1. Relationship of GWSS trap capture rate to the presence and absence of host plants: peach and crape myrtle represent poor and good host plants, respectively. Traps used were standard tube traps.

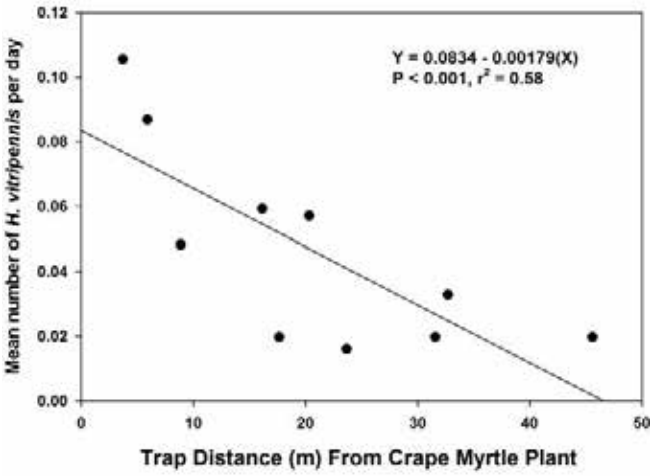

Figure 2. Response of GWSS to traps located at different distances from large 'Natchez' crape myrtle plants. Data points have been normalized by placing them in increments of 10 m centered on the values along the X axis.

The above results, observations of GWSS aggregation behavior in the field on host plants, in response to host plants and on traps led us to examine in depth the response of leafhoppers to traps and to other leafhoppers. The distribution of GWSS landing on traps was investigated with and without other GWSS present. **Figure 3** indicates the natural distribution of both sexes of GWSS on the Seabright trap and shows that GWSS tend to aggregate naturally in the lower right hand quadrant. That is in blocks three-five (left to right) of the Seabright trap. **Figure 4** shows how the GWSS responded to the Seabright trap when there was a dead GWSS carcass added to the center block on the trap. In this test we allowed only one leafhopper to respond each time to eliminate the confounding effect of previously trapped leafhoppers on the behavior of newly arriving leafhoppers. The presence of a leafhopper carcass shifted the distribution of the arriving leafhoppers to the center area of the trap with males landing at significantly higher numbers in blocks one and two from the center while females landed in higher numbers in blocks two and four. In another test, GWSS response to an unbaited control Seabright trap was compared to treatments of one, two or three GWSS carcasses or one, two or three black plastic models similar in size to GWSS added to the center boxes of one side of Seabright traps. Five replicates of each treatment were used in a RCB design. Responding GWSS were recorded by the block number away from the carcass where they landed as was the number of GWSS that landed on the unbaited side of the trap. Overall the baits increased trap catch by 55% on the baited side versus the unbaited side (data not shown). Other tests conducted included the addition of a small 15 cm long tree branch with GWSS carcasses on it to tube traps in parallel with the trap orientation and the addition of a similar small branch with carcasses attached to the tube trap in the middle and sticking out at a 45^0 angle away from the trap versus an unbaited control. Neither of these treatments provided significant increase in trap captures. Thus, the response to congeners appears to be a short range landing orientations. Nevertheless, we are pursuing this response to congener behavior by GWSS to further describe what is actually occurring and our results will be reported orally at the symposium.

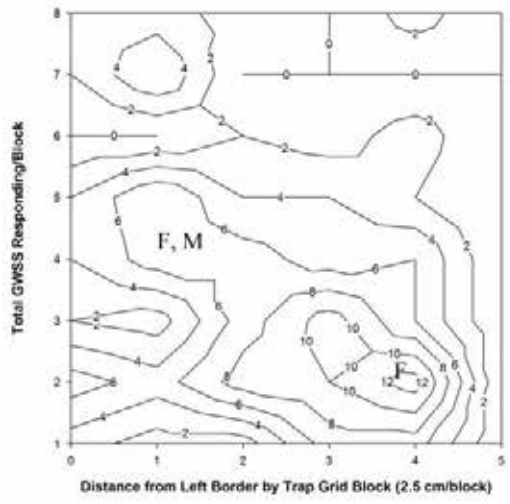

Figure 3. **GWSS landing distribution on a Seabright** trap without any other GWSS present. M is peak point of males and F indicates female peaks.

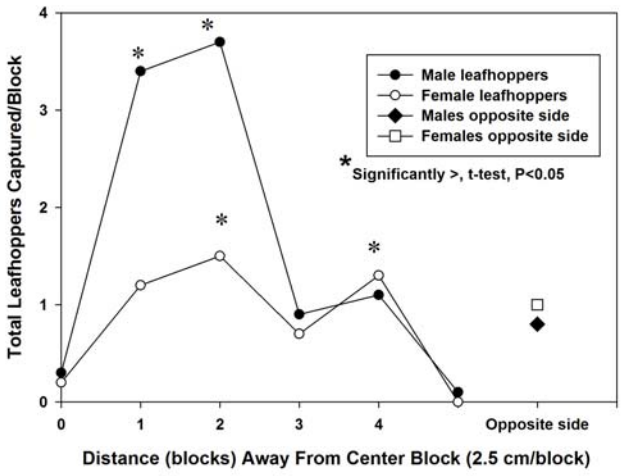

Figure 4. Response of GWSS to Seabright traps baited in the center block with a GWSS carcass.

CONCLUSIONS:

The experiments conducted in Florida under this grant were all completed in locations with relatively low GWSS populations. These low population conditions typify what would occur under a regulatory/quarantine function when the objective is to detect newly establishing populations when they first likely occur in very low numbers. The collective results of these studies suggest that the detection and monitoring efficiency of trapping of GWSS may be improved in a number of ways. Some are highly practical, whereas others would require a much different approach than the conventional deployment of traps haphazardly in some fashion in the field using a transect or a grid of traps. Use of traps for estimating populations within plants appears to be highly ineffective in most cases with the exception of crops in large acreage blocks where there is little immigration and emigration occurring in the sample location (Castle and Naranjo 2008, Northfield et al. 2009). Changing from the Seabright trap to a cylinder tube trap would improve detection levels but may be impractical relative to costs and logistics required shipping, moving and storage. Response by GWSS to congeners is novel and may have significant value. We are pursuing this aspect of GWSS behavior. The addition of a printed black silhouette GWSS model to the middle of the Seabright trap may improve its efficiency but this requires more testing under high populations. An increase in trap capture rate may accrue simply from the model's result of shifting the position of GWSS landings on the trap to nearer the center where they are surrounded by larger areas of sticky surface which may decrease their ability to escape the trap. Once leafhoppers arrive on the trap the model effect may lose value. However, from a regulatory perspective this change may be of value, but requires more tests. The attention to trap placement relative to host plant quality, distance from plants and trap size all can be considered for improving trap efficiency and the probability of early detection of low GWSS populations. It does not appear that GWSS exhibit any unusual and exploitable behaviors in either long or short range response to single or multiple trap configurations that may be exploited to improve trap capture rate.

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Northfield, T., R. Mizell, T. Riddle, P. Andersen and B. Brodbeck. 2009. Dispersal, patch leaving and aggregation of the glassy-winged sharpshooter, *Homalodisca vitripennis* (Hemiptera: Cicadellidae). Environ. Entomol. 38: 183-191.

FUNDING AGENCIES

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

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

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Reporting Period: The results reported here are from work conducted October 2008 to September 2009.

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 1,000 acres of citrus are vulnerable to Pierce's disease (PD). The grapes in the Coachella and Temecula areas of Riverside County are in jeopardy because of the glassy-winged sharpshooter (GWSS), 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 890,000 acres of vineyards and $30 billion dollar 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 40% 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 *Xf*, 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. The GWSS area wide management program is needed 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. Approximately 1,600 acres of citrus in Riverside County were treated for the GWSS in April through September, 2009 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 2010.
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 Agricultural Research Service, USDA Animal and Plant Health Inspection Service, California Department of Food and Agriculture, Riverside County Agricultural Commissioner's Office, University of California-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, 2008, 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 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 in the Riverside county area wide program. Based on trap counts and visual inspection, approximately 1,000 acres of citrus were treated in Temecula valley for GWSS in 2009. In 2009, 600 acres of citrus were treated in Coachella Valley for GWSS area wide management. Because of high Temecula GWSS trap catches in the late summer and early autumn of 2008 and GWSS trap catches in January, 2009, imidacloprid (Admire Pro) applications in citrus were initiated in April, 2009 (**Figure 3**). Admire Pro was applied at the rate of 14 oz/acre. Of the 1,000 acres of treated citrus, 72 acres of organically farmed citrus were treated with Omni Oil 6E at the rate of 1%/acre and PyGanic (1.4% Pyrethrins) at 18 oz/acre. Because of the low residual of the organic insecticides the organic citrus was treated three times during the season. Omni Oil was applied in June on the citrus, followed by PyGanic treatments in July and September.

For a successful area-wide GWSS management program with large acreages of citrus, a management program has to be maintained. Organic insecticides are not as effective as the neonicotinoid insecticides such as imidacloprid for controlling GWSS. Therefore, organic insecticides will have to be applied more frequently than its synthetic counterpart. In our Riverside County GWSS area wide program organic citrus groves pose challenges to area-wide GWSS management programs.

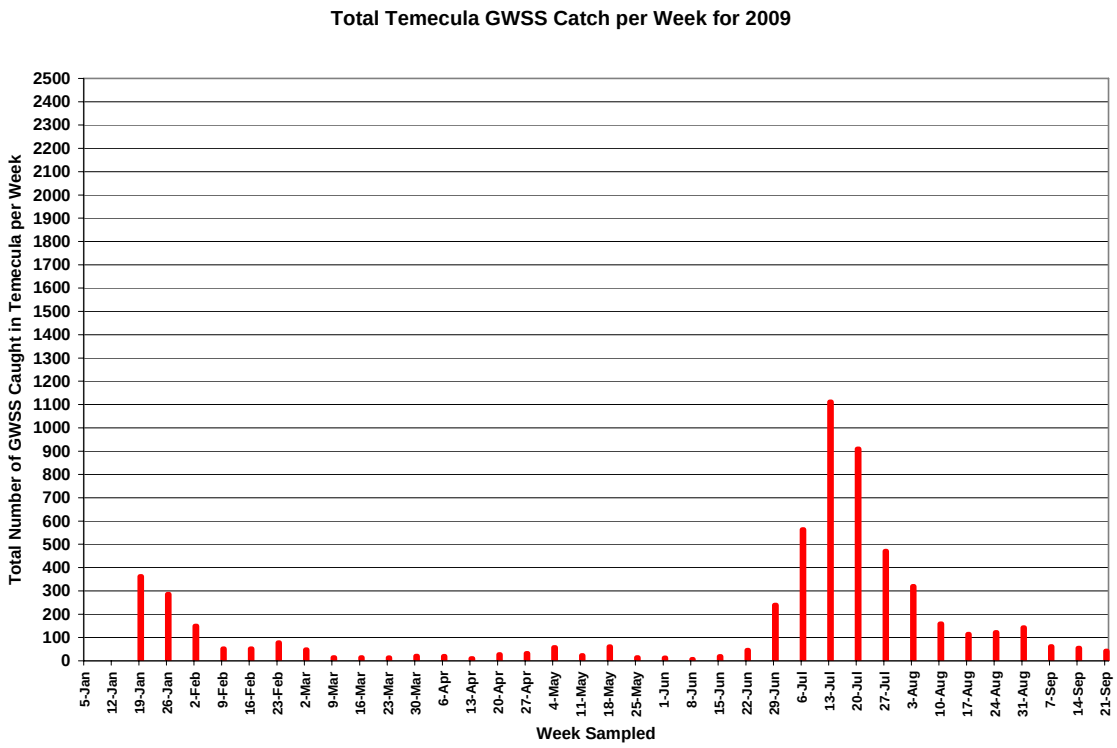

Figure 1. In 2009, high numbers of adult glassy-winged sharpshooters were caught on the yellow sticky traps in Temecula, with populations peaking in July reaching a total of approximately 1,100 trapped.

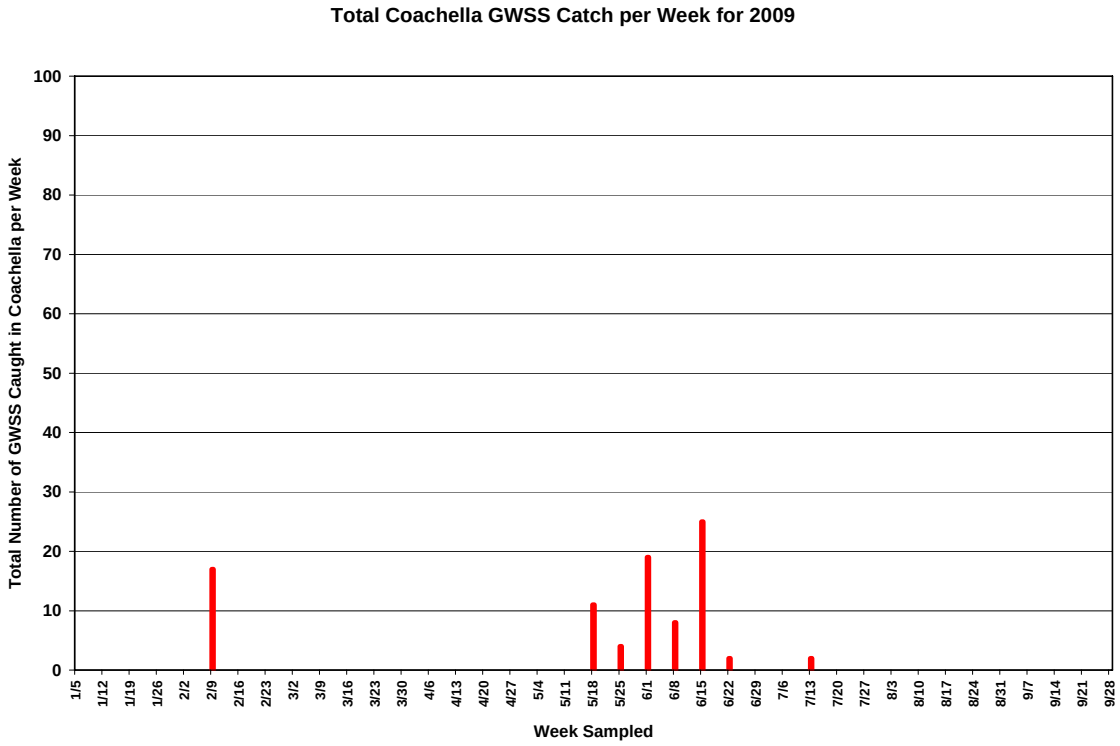

Figure 2. Glassy-winged sharpshooter populations in Coachella Valley peaked in June with a high of 25 trapped.

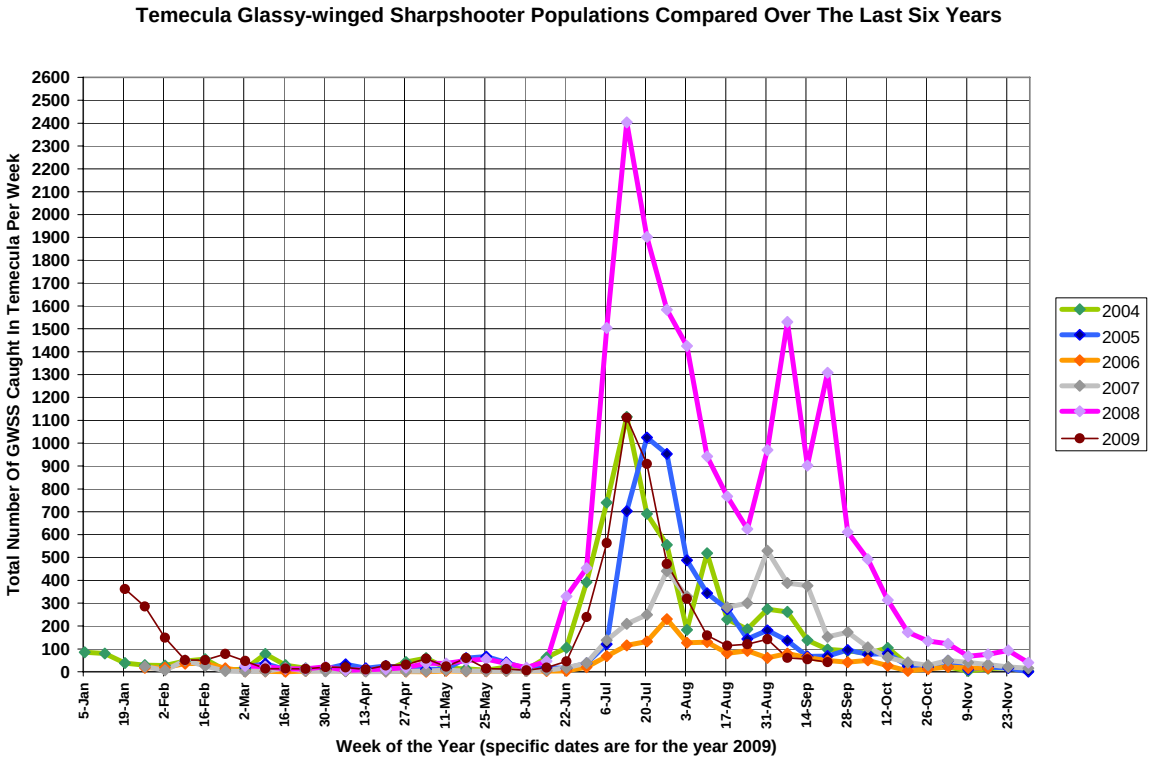

Figure 3. Glassy-winged Sharpshooter populations compared over the last six years

FUNDING AGENCIES
Funding for this project was provided by the USDA Animal and Plant Health Inspection Service, and the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

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Section 3:
Pathogen Biology
and
Ecology

BIOLOGY OF THE *XYLELLA FASTIDIOSA*-VECTOR INTERFACE

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ABSTRACT
Xylella fastidiosa (*Xf*) has complex life histories because it must colonize both plant host and its vectors for successful dissemination. The switch from host to vector environments may require changes in gene expression prior to the *Xylella*'s departure from the plant. We found that structural polysaccharides of plant host origin are important in regulating *Xf* gene expression and mediating vector transmission of this pathogen. Through the addition of pectin and glucan to a simple defined medium we showed dramatic changes in *Xf*'s phenotype and gene expression profile. Cells grown in the presence of pectin become more adhesive than in other media tested. In addition, the presence of pectin and glucan in media result in significant changes in the expression of several genes previously identified as important for *Xf*'s pathogenicity in plants. Furthermore, vector transmission of *Xf* is induced in the presence of both polysaccharides. Our data show that host structural polysaccharides mediate gene regulation in *Xf* which results in phenotypic changes required for vector transmission. A better understanding of how vector-borne pathogens shift from host to vector, and vice-versa, may lead to novel disease control strategies.

INTRODUCTION
The interactions between *Xylella fastidiosa (Xf)* cells and the foregut of vectors probably are complex and specific, as other xylem-limited bacteria such as *Leifsonia xyli* are acquired but not transmitted by insects (Barbehenn and Purcell 1993). However, little is known about the specific interactions between *Xf* and the foregut of vectors. In previous work, we used different approaches to determine how *Xf* cells interact with the cuticular surface of the foreguts of vectors. We demonstrate that *Xf* binds to different polysaccharides with various affinities and that these interactions are mediated by cell surface carbohydrate-binding proteins. In addition, competition assays showed that *N*-acetylglucosamine inhibits bacterial adhesion to vector foregut extracts and intact wings, demonstrating that attachment to leafhopper surfaces is affected in the presence of specific polysaccharides. *In vitro* experiments with several *Xf* knockout mutants indicated that hemagglutinin-like proteins are associated with cell adhesion to polysaccharides (Killiny & Almeida 2009). These results were confirmed with biological experiments in which hemagglutinin-like protein mutants were transmitted to plants by vectors at lower rates than that of 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 that of the wild type, suggesting that these proteins are important for initial adhesion of *Xf* to leafhoppers. It seems that *Xf* colonization of leafhopper vectors is a complex, stepwise process similar to the formation of biofilms on surfaces. In order to more characterize these complex interactions, setting up an artificial diet system with which *Xylella* cells can be acquired by the insect vector, is essential. The artificial diet system would allow the delivery of cells directly to sharpshooters without the requirement of source plants. Because cells interact with plants, are unevenly distributed in the xylem network, and have different gene expression profiles depending on individual vessel colonization stage, it has always been challenging to study how *Xf* colonizes its vectors. In addition, an artificial diet system would permit us to control the delivery of cells and compounds that may block transmission, to sharpshooters under various conditions. We and other groups have tried to develop this protocol, on and off, for 30 years. Here we report on our successful efforts to deliver transmissible *Xf* cells to vectors. This is an essential component of our project, as now we can easily compare cells that are transmissible if grown on certain conditions with others that are not transmissible. Here we describe for the first time a successful *Xf* transmission through sachet.

OBJECTIVES
1. Establishment an artificial diet system to deliver *Xf* to vectors.
2. Characterization of chitin-binding proteins in the *Xf* surface.
3. Identification of molecules that disrupt *Xf* adhesion to vector foregut surface.

We focus on Objective 1 in this report due to space limitations. We have preliminary data for objective 2 and have already set up experiments to test a large number of molecules to block *Xf* transmission (Objective 3, results pending).

RESULTS AND DISCUSSION

Host polysaccharides induce phenotypic changes necessary for the vector transmission of *Xf*.

Cells were grown in vitro, suspended in buffer, confined in parafilm sachets and given to sharpshooters in small cages. Insects are then transferred to grape seedlings for an inoculation access period. Our group and others have successfully shown acquisition of cells from such a system before. However, those cells were never transmitted to plants. Our work started with two assumptions. First, the most commonly used medium to grow *Xf*, PWG, is too rich, does not mimic conditions cells encounter in nature, and likely results in pathogen gene expression profiles that are not correlated with those in plants or insects. Therefore, we modified a previous published medium (Almeida et al. 2004) to obtain XFM, a simple defined medium to grow *Xf*. Our second assumption was that a plant component induced pathogen transmission, as cells colonizing plants are obviously transmitted by insects. We highlight that, although our work focuses on transmission, these results should be useful for the *Xf* community in general, especially those interested in how this bacterium colonizes plants. Our approach to understand the biology of our system was to compare 4 different media for different phenotypic, biochemical and molecular characteristics (**Figures 1** and **2**). These media are: PWG (rich medium, most commonly used for *Xf* research), XFM, XFM-glucan (XFM with glucan), and XFM-pectin (XFM with pectin). We choose glucan and pectin as plant polysaccharides because they occur on pit membranes and xylem cell walls, surfaces with which *Xf* interacts and is capable to degrade. The *pglA* mutant was also used to confirm results (**Figure 3**)

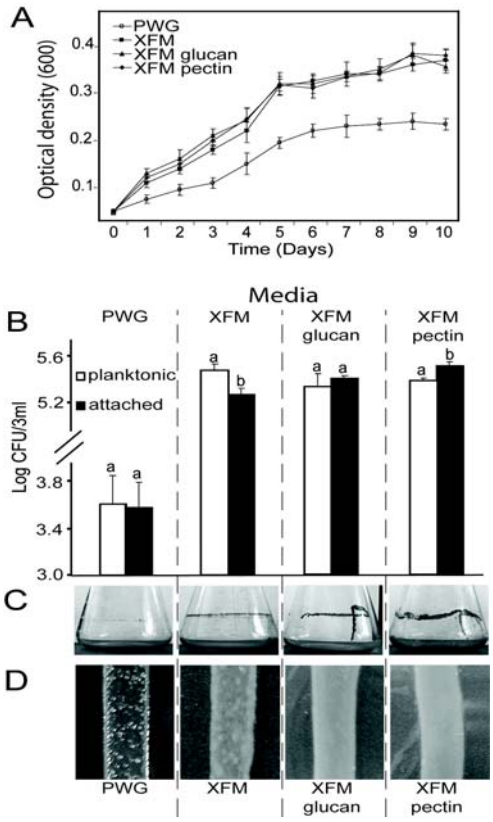

Figure 1. Growth characterization of *Xf* on XFM media. *Xf* (Temecula isolate) cells reached higher populations on all XFM-based media compared to PWG (A). These results are interesting, suggesting that although PWG is the most robust medium for growth of different *Xf* strains, it either suppresses growth or does not provide adequate growth conditions as the much nutrient poorer XFM. Similar results can be observed in (B), where populations were higher on XFM-media than PWG.

Of interest to our work, and of many other groups, are *Xf*'s adhesion phenotypes. In (B) we compared the number of planktonic versus attached cells grown in tissue culture tubes in these media. Both glucan and pectin induced cell adhesion, compared to the basal XFM medium. *Xf* adhesion is mediated by a large number of surface proteins, such as pili and hemagglutinins.

Figure (C) shows adhesion 'rings' on culture flasks when cells were grown on these different media, illustrating the data presented in (B) In addition, colony phenotype on these media were different on solid surfaces. On solid media (D), cells formed a uniform, glossy lawn on XFM-glucan and –pectin, when compared to individual colonies on PWG, which were more typical of *Xf*. Colonies on XFM medium were intermediate among these treatments. These results suggest, among other things, that gum is up-regulated in XFM media.

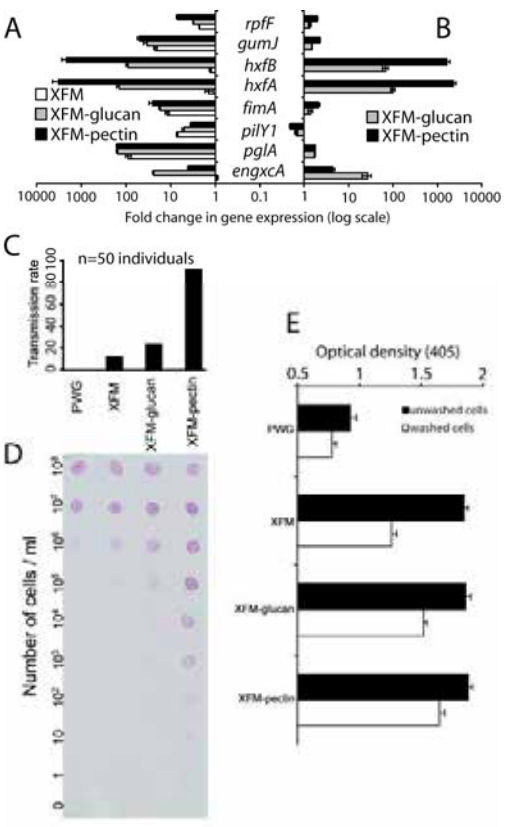

Figure 2. In (A) we compared gene expression of multiple *Xf* genes when cells were grown in different XFM-based media compared to PWG. Equal gene expression was represented by the value of '1.' In (B) we compared the role of glucan and pectin on transcription, in this case '1' represents expression equal to XFM. We focused on adhesion and pathogenicity genes. It is clear that all these genes are repressed in PWG. In relation to XFM, however, hemagglutinins were up-regulated many fold, while other genes were up-regulated but not as much. The type IV pilus gene tested (*pilY1*) was down-regulated. These data suggesting that these carbohydrates induce a cell 'adhesion' phenotype and limit movement within plants; similarly to *Xf* occurring in high cell density. In (D) we quantified the amount of hemagglutinin-like proteins on cells grown in the four media. We have previously shown that these proteins were associated with vector transmission of *Xf* to plants. The results show that the proteins were expressed at higher rates in cells grown on pectin and glucan compared to PWG and XFM. Our in vitro transmission tests (C), using the artificial diet system described above, show that transmission was above 90% efficient if cells were grown on XFM-pectin, while no transmission was observed on PWG. It is interesting to note a good correlation between the quantity of hemagglutinin on cells and vector transmission of *Xf* using the in vitro system. Lastly, in (E), we compared the amount of gum produced by cells on these different conditions. Gum production was similar in all XFM media, but much lower on PWG, which may explain why colonies have different phenotypes on solid media (previous Figure)

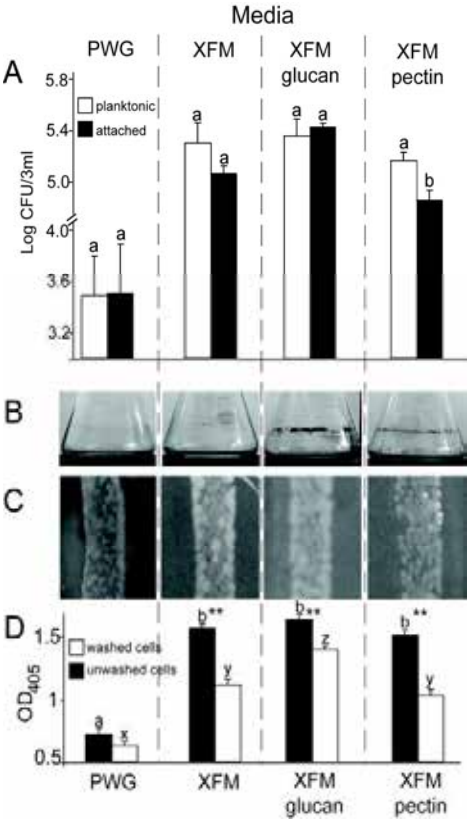

Figure 3. Growth characterization of *Xf pglA* mutant grown in four different media. (A) Populations of planktonic versus glass-attached cells grown *in vitro*, bars with the same letter are not different from each other within media treatments (t-test, $P < 0.05$); and B) biofilm formation at air/broth interface in different media. (C) visual aspect of bacterial lawns on solid media, 'glossy' phenotype likely associated with exopolysaccharide production. D) EPS production quantified immunologically in four media (unwashed cells – filled bars, washed cells – empty bars), asterisks ($P < 0.05$ for one, $P < 0.001$ for two) indicate within media differences, bars with the same letter are not different from each other within wash treatments.

The main subunit of pectin is responsible for gene induction in *Xf*.

We showed that pectin mediates the transmission of *Xf*, but pectin is a very large and complex molecule. Thus, we also studied the effect of different subunits of pectin in the changes in phenotype and gene expression we observed. Pectin is primarily composed of galacturonic acid with rhamnose side chains; the ratio of these sugars is host plant species dependent (Cho et al 2001, Coutinho & Henrissant 1999). We tested the effect of rhamnose and galacturonic acid separately. Results are described in **Figures 4 and 5**.

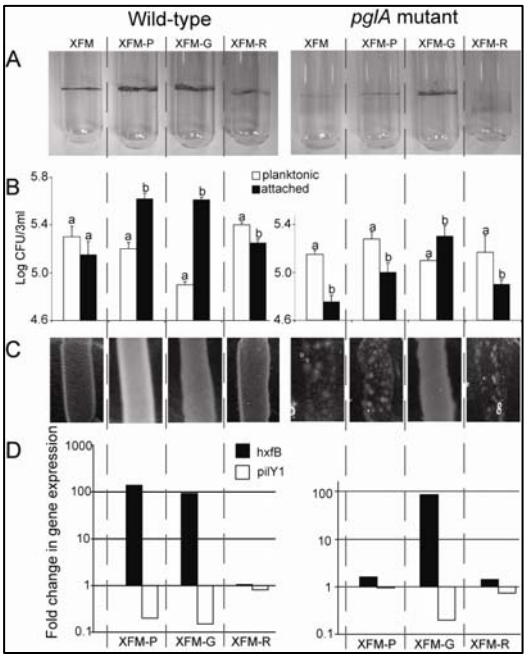

Figure 4 . The main subunit of pectin is responsible for gene induction in *Xf*. A) Biofilm formation at air/broth interface for the *Xf* wild-type and polygalacturonase (*pglA*) mutant in four media; XFM, and XFM supplemented with -P(ectin), -G(alacturonate-Na) and -R(hamnose). B) Populations of planktonic versus glass-attached cells grown in four media, bars with the same letter are not different from each other within media treatments (t-test, $P < 0.05$); and C) visual aspect of bacterial lawns on solid media. D) Quantification of *hxfB* and *pilY1* transcription under the same conditions for the wild-type and mutant; value of 1 indicates transcription level equal to cells grown in the basal medium XFM.

Taking together, it seems that galacturonic acid is the selective sugar for *Xf* and it is the responsible of the change in phenotype and gene expression profiles. In order to confirm these results we used the *pglA* mutant, the mutant grow in the presence of galacturonic acid just like the wild type in the presence of pectin, which corroborates other observations that this sugar is responsible for the phenotypic changes we found.

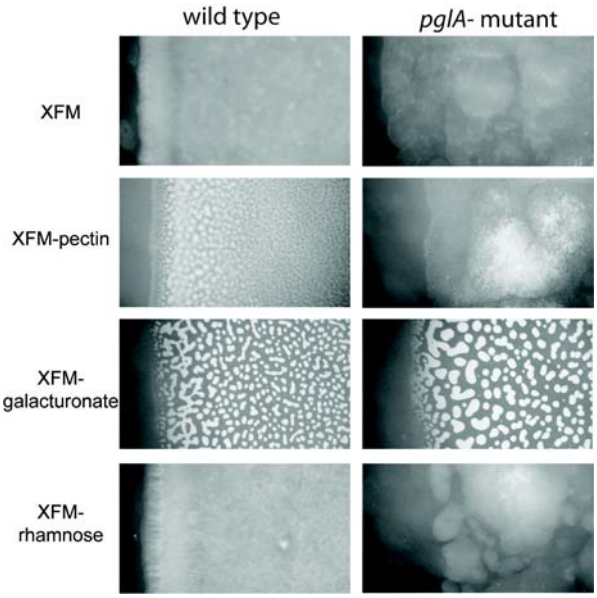

Figure 5. Phenotype of wild type and *pglA* mutant colonies on XFM media supplied with pectin or one of its major component. Pictures focus on the edge of plated cells for comparative purposes Images were taken using LEILKA M125 steromicroscope with 100X total magnification. Inoculums were adjusted to OD600 of 0.2 before plating on the media.

Host polysaccharides induce changes in gene regulation profiles.
In order to better understand the changes to cells when grown on XFM-pectin, we carried out a gene expression microarray analysis for *Xf*'s whole genome. Not surprisingly given the phenotypic changes observed, we found the presence of pectin induced many changes on the transcriptome of *Xf* (**Figure 6**).

(*A*) Hierarchical clustering analysis of microarray expression data for 187 genes found to be differentially regulated during growth in PWG, XFM, XFM-pectin, and XFM-glucan, respectively. (*B*) Up-regulated genes in the presence of pectin. (*C*) Down-regulated genes in the presence of pectin when compared to XFM. Note the gradient increasing or decreasing gradient of expression data are the average of 4 independent replicates of each treatment. The most intense red and blue colors correspond to increased or decreased expression values respectively. Genes having ratios of ≥ 1.6 or ≤ 0.6 fold for expression were selected as up-regulated or down-regulated respectively. PWG was used as baseline for other media. (*D*) Scatter plots of XFM media against PWG showing difference on gene expression.

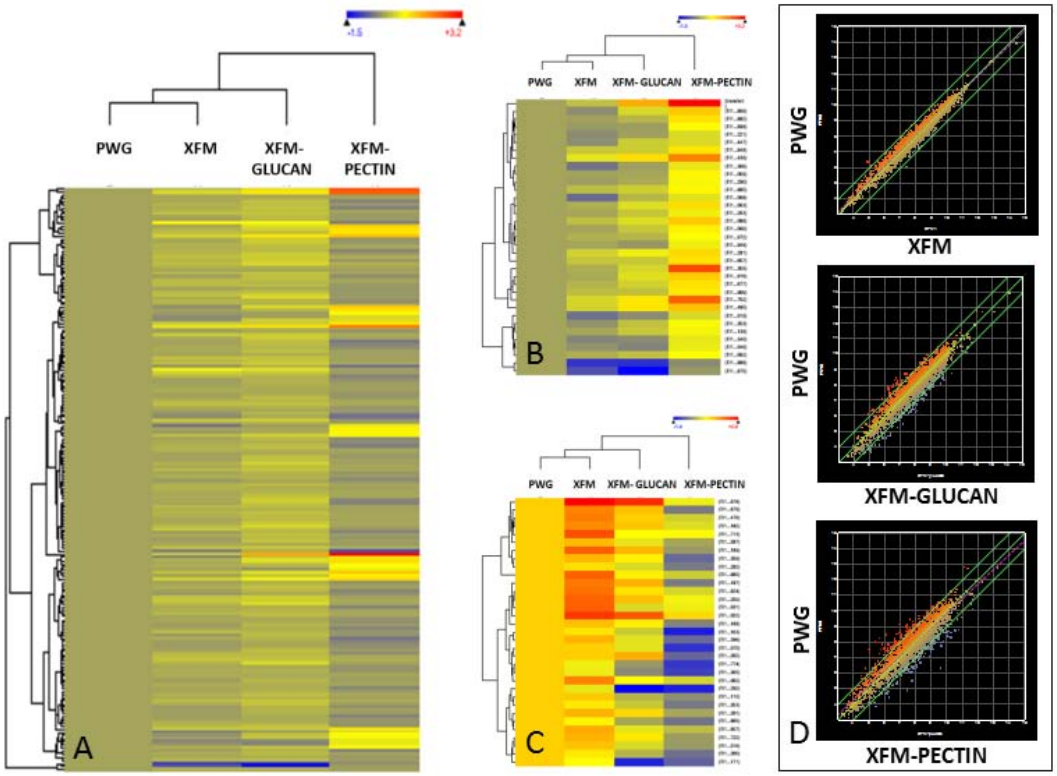

Figure 6. Microarray analysis for gene expression of cells grown in different media.

CONCLUSIONS

In this report we described the first successful transmission through artificial diet systems for *Xf*. We found that *Xf* cells grown in a minimal media XFM supplied with polysaccharides from host, especially pectin were capable to be acquired and transmitted by the vector to grape plants. Pectin induced gene regulation and subsequently, a different growth phenotype than cells grown in the complex medium PWG. Hemagglutinin-like proteins and exopolysaccharides were found to be significantly over expressed and thus associated with the transmission. These results support our previous work (Killiny & Almeida 2009). This artificial diet system (transmission through sachet) will be a powerful tool to study and identify molecules may disrupt the transmission using competition assays. Furthermore, we can study the transmissibility of certain mutants that may grow in lower populations in plants than required for the transmission from plant to plant by insects. Lastly, our results should be useful to other *Xf* research groups interested in plant-pathogen interactions.

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WHICH GRAPE VARIETALS ARE SOURCES OF PIERCE'S DISEASE SPREAD? DECOUPLING RESISTANCE, TOLERANCE, AND GLASSY-WINGED SHARPSHOOTER DISCRIMINATION

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ABSTRACT
Plant resistance to pathogens and the feeding behavior of the pathogen vectors can influence patterns of disease spread. This study is aimed at evaluating the two above ecological factors that could limit the negative impact of Pierce's disease (PD) on the economically valuable grapevine, *Vitis vinifera*. Eighteen commonly used varieties are being scored for symptom severity and quantified for *Xylella fastidiosa (Xf)* infection level. We will investigate the vector, glassy-winged sharpshooter (GWSS), feeding-choice behavior for infected versus healthy plants. In addition, we will compare the vector transmission efficiency among different grape varietals. Our preliminary results for symptom severity, in weeks eight and 12 post-inoculation, indicates that Rubired has the least symptom development score among 17 other tested varieties. Chardonnay and Chenin Blanc also show a relatively slow symptom development. Our study on vector feeding behavior showed that, while GWSS does not discriminate against water stressed plants (which mimic PD symptoms); they had a greater tendency to position themselves on lower and woody parts of the stem due to its response to brown colors. Our experimental setup and preliminary finding will be described and discussed.

LAYPERSON SUMMARY
The degree of resistance (and tolerance) to Pierce's disease (PD) is under evaluation for 18 commonly used grapevine varieties. We also quantify feeding and host-choice behaviors of glassy-winged sharpshooter, a key vector for the pathogenic bacteria. This project has been just started. Our preliminary results showed that the variety Rubired has the least symptom development score among 18 varieties. Chardonnay and Chenin Blanc are also showing slower symptom development relative to the other tested varietals. The insect vector did not discriminate against water stressed plants (with similar symptoms as PD) though showed a distinct preference for the brown coloration of the lower main shoot. Our experimental setup and preliminary finding will be described and discussed.

INTRODUCTION
Plant resistance to pathogens is important for limiting disease in many agro-ecosystems (e.g. Kolmer 1996; Leung et al. 2003). The levels of resistance and tolerance (infected with limited or no symptoms) exhibited among host genotypes typically vary continuously (Kover & Schaal 2002). The used definition of resistance and tolerance is important because each may differ in its effect on disease spread, especially plant pathogens that are transmitted by insect vectors (see below).

Pierce's disease (PD) is caused by the bacterial pathogen *Xylella fastidiosa (Xf)*. This bacterium is xylem-limited and pathogenic to a wide range of agricultural crops, such as alfalfa, almond, citrus, coffee, peach, plum, and also infects dozens of native, ornamental, and weedy species in the U.S. (Purcell 1997, Hopkins and Purcell 2002, Costa et al. 2005, Wistrom and Purcell 2005). PD symptoms include leaf scorch, irregular maturation of the cane, and dieback of the apex of the plant (Krivank et al. 2005). Typically, infected susceptible vines die within two to three years. Xylem sap-feeders are known to be the vectors for the bacterial pathogen. In the 1990s severe outbreaks of PD occurred in southern California that was attributed to the invasion by the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis)* (Sorensen and Gill 1996, Blua et al.1999).

Vector preference for infected versus uninfected plants can dramatically impact disease prevalence (McElhany et al. 1995). For example, vector preference for healthy plants (i.e. discrimination against infected plants) should limit pathogen spread. Under this scenario, relatively tolerant varieties that are infected but yet have no (or limited) symptoms may potentially act as a source for the bacteria. Needless to say that this can potentially promote disease spread in nearby healthy vines. Likewise, a lack of discrimination against infected plants would also increase the pathogen spread. Whether susceptible varieties can act as a source for *Xf* spread also depends on GWSS behavior and acquisition efficiency. Two aspects of GWSS feeding behavior may be important for *Xf* acquisition and spread: 1) preference for feeding on certain grape varietals (e.g., Purcell 1981), and 2) discrimination against infected vines of a given varietal (e.g., McElhany et al. 1995, Marucci et al. 2005). Thus, understanding vector feeding-choice behavior and varietal resistance versus tolerance are directly relevant to understanding the epidemiology of PD in vineyards.

In general, *Vitis vinifera* cultivars are susceptible to *Xf* infection (Krivanek and Walker 2005). While anecdotal field observations (Raju and Goheen 1981, A.H. Purcell and J. Hashim-Buckey, personal communication) are indicative of

differences in symptoms severity among varietals, studies quantifying *Xf* infection levels have also documented substantial variability among cultivars (Raju and Goheen 1981, Krivanek and Walker 2005). Thus, in order to predict which grape varietals are most likely to act as *Xf* sources for vectors we need to independently measure both the varietal infection level (i.e. resistance) as well as symptom severity for that particular variety in relation to vector transmission efficiency.

In this project our goal is to evaluate: *i* the differential response of *V. vinifera* to *Xf* infection, and *ii* feeding-choice behavior of the pathogen vector GWSS.

OBJECTIVES

1. Measure the relative levels of both resistance and tolerance for important California grape varietals.
2. Evaluate GWSS feeding behavior in grapevine and measure discrimination against infected vines and *Xf* spread for different grape varietals.

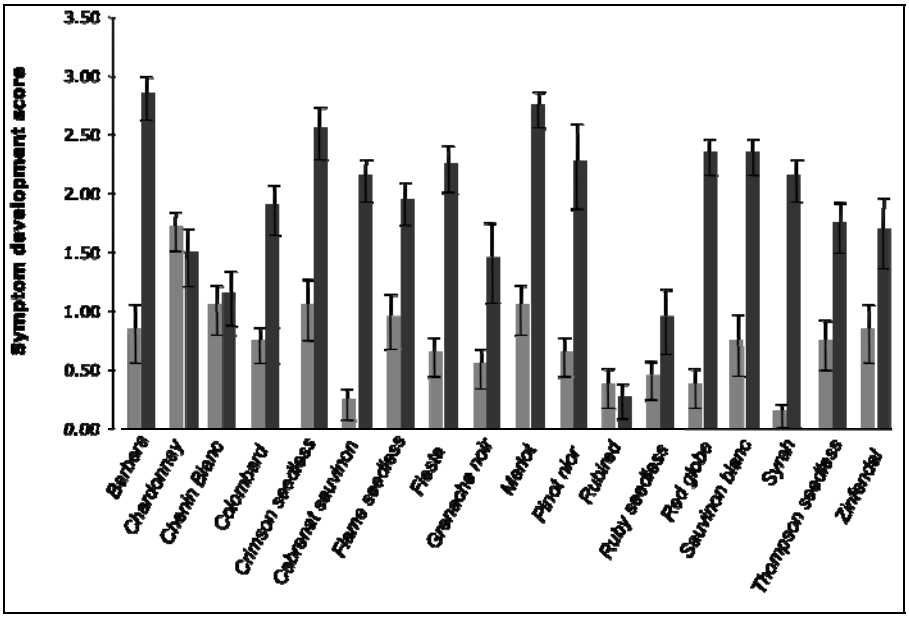
RESULTS AND DISCUSSION

The project currently is at the beginning and therefore this report presents some preliminary results and experimental setup and preparation for upcoming time blocks scheduled for mid-October and mid-December 2009.

In order to quantify the levels of resistance and tolerance we have obtained and planted dormant cuttings of 18 different commonly used varieties of *Vitis vinifera* in March 2009. We now have an estimated 100 plants per genotype for each of those varieties. We have mechanically inoculated *Xf* into 22 plants of each variety in two different time blocks within 24 hrs. All plants were inoculated at the base of the main shoot.

Inoculated plants were quantified for symptoms on the weeks eight and 12 post inoculation. The same plants will again be quantified on weeks 16 and 20. The disease symptoms were scored following Guilhabert and Kirkpatrick (2005). The PD symptom development appears to be most aggressive for the varieties Flame Seedless, Red Globe, Crimson Seedless, Cabernet Sauvignon and Syrah consistently across the two time blocks. The varieties Chardonnay and Chenin Blanc should a weaker symptom development compare to the other tested varieties Red Globe seem to show a slight but non-significant recovery in its symptom severity (see **Figure 1**). However, PD symptoms are sometime hard to distinguish from water stress or nutrition stress symptoms. Therefore, a final conclusion needs to be made following culturing and qPCR, which verify the bacterial presence and quantify the infection level, respectively.

On weeks eight and 12 post-inoculation we sampled leaf petioles of all inoculated varieties at +10 cm above the inoculation point. In addition, we took one sample at +30 cm above the inoculation point (per plant) to also compare the bacterial movement within different grape varietals. All samples are stored in -80^{o}C freezer for later processing. Plants will also be sampled on weeks 16 and 24. For the sake of consistency, we will start qPCR after the labor-intensive greenhouse experiments are finalized. This approach would provide us with a sufficient measure of bacterial populations at the three different stages of disease progress, which allow us to track within host disease spread over time for each variety and enables us to compare population growth of bacteria among our varietals.



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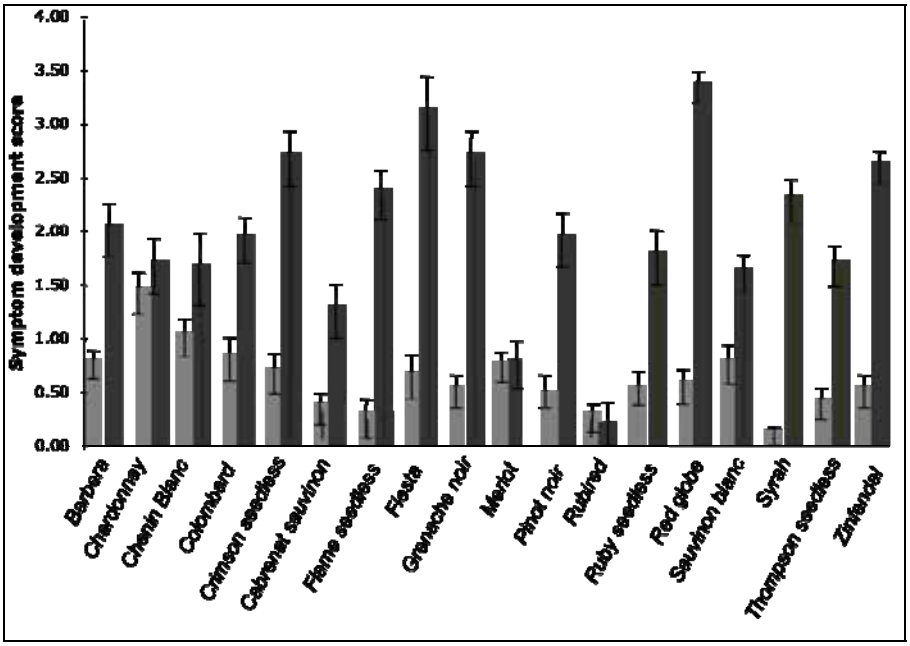

Figure 1: Symptom development scores for all tested grape varieties following a 0 to 5 scale (Guilhabert and Kirkpatrick 2005): *a)* time block 1; n= 10, *b)* time block 2; n= 12. Light-Gray fill marks the symptom development score on week eight and dark-gray marks the symptom development score on week 12.

By contrasting bacterial growth rate within the plant and visible plant symptoms we will provide an accurate classification for susceptible, tolerant and resistant varieties. Tolerance and resistance are used as relative terms within *V. vinifera* among its varietals.

No-choice transmission experiments (in progress)

Adult GWSSs were caged individually on the 22 mechanically inoculated plants (see above-block 1: n=10; block 2: n=12) per variety (18 varieties in total) for 48 hours (acquisition period). After 48 hours insects were gently removed from the infected plants and caged (again individually) on healthy plants of corresponding variety.

Insects were kept on healthy plants for six days (transmission period). Insects were removed and plants are currently being monitored for symptoms. Transmission rates will be calculated and compared among varieties. We stored the collected insects in -80^{o}C freezer for future qPCR. Through this process we will be able to quantify the bacteria acquisition by insects across the 18 varieties.

The same procedure will be repeated on weeks 16 and 24 post-inoculation (using the original mechanically inoculated plants). Thus, the transmission/acquisition rates will also be compared across three different stages of bacterial growth within plants for each variety.

Feeding behavior of H. vitripennis- choice experiments

Morphological, physiological and nutritional changes in plants due to pathogen infection has bee suggested to influence insect vector choice of the host plant (Hammond and Hardy 1988). For example aphids that are attracted to yellow color show preference to feed on yellow leaves caused by virus infection (e.g. Baker 1960). Although such behavior maybe an adaptation to detect higher amounts of nitrogen in plant resources (see Kennedy et al 1961), it also could lead aphids to plants that are more susceptible due to weakness.

In a previous study, however, Marucci et al. (2005) showed that sharpshooter vectors, *Dilobopterus costalimia* and *Oncometopia facialis* (Hemiptera: Cicadellidae) both prefer to feed on healthy asymptomatic plants rather than symptomatic plants infected by the pathogenic *Xf*. Likewise, anecdotal observations are suggestive of a similar host choice pattern in GWSS. Based on these observations GWSS is expected to choose healthy hosts more frequently compared to a symptomatic plant. However, this proposed behavior has never been formally tested.

PD symptoms are similar to water stress possibly due to xylems being blocked by the *Xf* colonies. Thus, GWSS response to water-stressed plants is expected to follow that of *Xf* infected plants, if indeed GWSS chooses its host plant based on visual signals. We conducted this experiment as a preliminary test preparing for assays to be conducted as part of Objective 2, which we are preparing plants and insects for at this point.

One water-stressed and one healthy *V. vinifera* (var. Zinfandel) plant was placed an observational bugdorm cage. A plant was considered water stressed when at least two thirds of its leaves showed wilting and scorching symptoms. One adult GWSS was gently aspirated into the cage and its movement with in the cage was monitored every 30 minutes until a choice was made. The choice was recorded and the individual's position was double-checked after two hours to make sure that no host plant switches occurred. None of the GWSS in our 20 trials moved to a different plant once they made their host-choice. Unexpectedly, GWSS did not discriminate against water stressed plants (Sign test (two-tailed): $P = 0.66$). Similar pairwise experiments will be conducted with inoculated plants on week 24 post-inoculation, when PD symptoms are visually detectable, to a considerable extend, to human eye.

In addition to between-plant choice, within-plant feeding site may also influence the transmission efficiency of an insect vector. Insect preference for a particular feeding site may coincide with the pathogen colonization and thus increase the acquisition and transmission rates, as a result. We have shown this to be the case for sharpshooter transmission of *Xf* from alfalfa plants (Daugherty, Lopes and Almeida, submitted). Anecdotal observations indicated GWSS preference for feeding on the lower parts of the grapevine stem. However, the exact reason for such a choice is yet to be confirmed. Here we tested the possibility of color matching in GWSS since the lower parts of grapevines turn into brown following maturation. Detecting a link between GWSS feeding site choice is significantly important because: *i)* cane color in grapevine changes in response to *Xf* infection (Krivanek et al. 2005) and *ii*) different grape varieties may vary in their time of cane maturation and the amount of brown color of the stem. We hypothesize that this may explain the lower transmission rates of *Xf* from grape to grape by the GWSS (Almeida and Purcell 2003).

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To test for this possibility we presented individual sharpshooters with grape cuttings *Vitis vinifera* (var Cab Sauv), 20-30 cm in length and with equal proportions of green and brown colors (to the experimenter eye). Our experimental data verified the fact that GWSS prefers to feed on the lower and brownish-colored parts of the stem (sign-test (two-tailed): $P= 0.011$; n=20).

To confirm the existence of background color preference, we also caged GWSS individually in a 30x30x30 cm cage covered with paper sheets of brown and green checkers and observed them for 15 minutes. We showed not only insects chose to jump to brown first (sign-test (two-tail): $P=0.04$; n=30), but also showed that they spend significantly more time on brown squares compared to the green squares (**Figure 2**). This additional test of the feeding site preference addresses questions directly associated with those Objectives related to GWSS feeding site preference and within-plant behavior and color attraction, which may explain the lower transmission rates observed in previous experiments by this species compared to other native CA sharpshooters (Almeida and Purcell 2003; Almeida et al. 2005).

We have ongoing experiments addressing the Objectives proposed. Monitoring and sampling of field experiments is also ongoing.

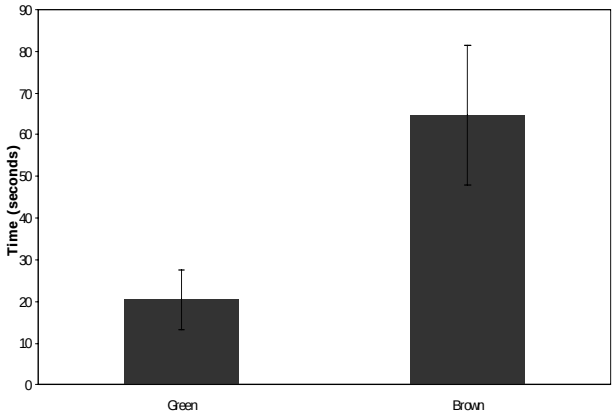

Figure 2. *H. vitripennis* individuals prefer to spend more time on brown colored- compared to green colored-squares (Wilcoxon brown-green: Z= -2.407; n=30; P=0.015).

CONCLUSIONS

This work directly addresses a recommendation by the PD advisory panel for more research to distinguish between *Xf*-resistant and *Xf*-tolerant varietals and the role of GWSS in spreading *Xf*. Our early visual inspection of the PD symptom development indicates that the varieties Flame Seedless, Red Globe, Crimson Seedless, Cabernat Sauvignon and Syrah show more susceptibility to *Xf* infection. The varieties Chardonnay and Chenin Blanc showed slower symptom development compared to the other tested varieties. Rubired seems to show a slight but non-significant recovery in its symptom severity. However, final conclusions cannot be made without further analysis upon a completed data collection. While, our finding, so far, indicates that GWSS does not discriminate against water stressed plants, which more-or-less mimic PD symptoms, they show a strong preference to feed on the lower parts of the stem where the colonization of the bacteria may be more difficult. Experiments are ongoing. Our final results will directly benefit growers by evaluating which varietals are most likely and least likely to promote spread of PD by GWSS. Targeted planting of more resistant varietals could be used as a vineyard management strategy to reduce PD incidence in high-risk areas.

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DEVELOPMENT OF QRT-PCR PROTOCOLS FOR RAPID *XYLELLA FASTIDIOSA* SUBSPECIES DIAGNOSTICS

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ABSTRACT
Xylella fastidiosa (Xf) is a plant pathogenic bacterium that is transmitted between hosts by the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis)*. Multiple subspecies of *Xf* occur and are host specific. *Xf* subsp. *fastidiosa* is the causal agent of Pierce's disease (PD). *Xf* subsp. *multiplex* and *Xf* subsp. *sandyi* are commonly found in North America but do not cause PD. Rapid diagnostics to determine presence of *Xf* and differentiation of these subspecies is necessary for effective management of PD. In this study, three methods by which the subspecies can be distinguished by QRT-PCR were compared. SYBR green, Eva Green®, and Takara SYBR Green® melt curve analysis of partial gyraseB amplicons, Zot gene amplicons, and five TonB amplicons were evaluated for consistency and quality. Multiple melts were performed to find the ideal conditions for distinguishing between the subspecies. Emphasis was placed on a Ragweed insertion in the *Zot* gene, for which a probe was designed to increase the reliability of rapid diagnostics and differentiation of *Xf* subspecies. These new methods provide a more reliable protocol by which the subspecies of *Xf* can be determined.

LAYPERSON SUMMARY
Detection of the pathogen in an agronomic disease system is an important component of a management strategy. This is especially true in cases where incredibly mobile insects (like the glassy-winged sharpshooter) are the primary vectors of a pathogen (like *Xylella fastidiosa; Xf).* In short, it is important to know which plants are infected with the pathogen and which insects in the vineyards are carrying the pathogen. To further confuse our understanding of the system, three subspecies of *Xf* exist in our grape growing areas which are currently detectible by standard assays; however, only one of these subspecies will lead to Pierce's disease if it is in a vine. So in this study, we have developed a detection assay that is inexpensive and more sensitive than any detection protocols available. Furthermore, we have designed the protocol to distinguish between strains of *Xf.*

INTRODUCTION
Xylella fastidiosa (Xf) is a plant pathogenic bacterium residing in the xylem vessels and is the causal agent of many plant diseases including Pierce's disease (PD) of the grapevine (Hopkins and Purcell, 2002; Bextine et al, 2007). In the southwestern region of the United States, there are three common strains of *Xf*: *Xf* subsp. *fastidiosa* (PD), *Xf* subsp. *multiplex* (RW) (Schaad et al, 2004), and *Xf* subsp. *sandyi* (OLS) (Hernandez-Martinez et al, 2007). Previous methods of distinguishing *Xf* subspecies are slow and more prone to error. Initially, these previous methods, using SYBR Green, were evaluated and compared to new methods which used Takara SYBR Green® and Eva Green®. Melt analysis was used to analyze the strains. The *gyraseB* gene was used in the evaluation of subspecies determination protocols. In the second part of the experiment, new primers were designed for the *Zot* and *TonB* genes in order to attempt differentiation of the species based on melting temperature analysis. In the *Zot* gene of the Ragweed strain an insertion from base pair 379-426 (**Figure 1**) was the main focus of the *Zot* primers.

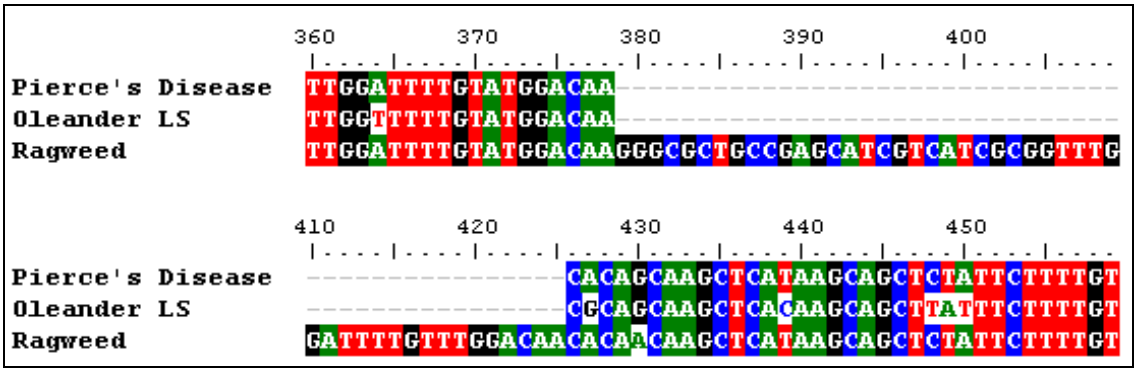

Figure 1: Section of Zot gene in PD, OLS, and RW *Xf* subspecies showing Ragweed insertion.

OBJECTIVES

1. Determine accuracy of currently used QRT-PCR methods for identification of *Xf*.
2. Compare to new methods using Eva Green and Takara SYBR Green.
3. Develop rapid and more reliable methods for identification of *Xf* subspecies.

MATERIALS AND METHODS

DNA Extraction. *Xf* colonies were collected from agar plates using a sterilized metal loop and placed in 100µl of PBS buffer in a MCT. The bacteria were vortexed until mixed into the solution and 100µl Lysis Buffer L6 was added to each MCT. The samples were centrifuged at 5000 rpm for five minutes. Next 53µl of silica slurry was added to each MCT and vortexed. The samples were incubated at room temperature for five minutes, followed by five minutes of centrifugation at 2000 rpm. The supernatant was drawn off and discarded and 200µl of wash buffer was added to each MCT. The MCTs were centrifuged at 2000 rpm for five minutes and the wash step was repeated three times for a total of four washes. The MCTs were then placed in an incubator at 60° C until the silica was dry, approximately 10 minutes. After the silica dried, 100µl of TE Buffer was added to each sample and mixed with the silica, followed by incubation for another five minutes at 60° C. The MCTs were centrifuged for five minutes at 5000 rpm. Afterward 70µl of the supernatant was drawn off, without any silica, and placed into a sterile MCT, labeled, and stored in the freezer.

QRT-PCR. Prior to use the, PCR hood was subjected to a minimum of 30 minutes of UV light and sterilization with 10% bleach. All sample MCTs were placed in a cold block during preparation and all master mix reagents were placed on ice. QRT-PCR was conducted in 10µl reactions consisting of 5µl iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA), 0.4µl forward primes, 0.4µl reverse primes, 1.0µl nanopure water, 1.0µl SYBR® Green nucleic acid gel stain (Molecular Probes™, Eugene, OR) and 2µl sample DNA. The sample DNA consisted of RW, OLS, and PD *Xf* strains. Two No Template Controls (NTCs) were included each time QRT-PCR was conducted and consisted of 10µl master mix. Eva Green (Phenix Research Products, NC) and Takara SYBR Green (Takara Bio USA, WI) were each switched with SYBR® Green nucleic acid gel stain for comparison. QRT-PCR reactions were carried out in 0.1mL PCR tubes and consisted of an initial denaturing step at 95 C for ten minutes, followed by 40 cycles of 30 seconds denature, 30 seconds anneal, and one minute elongation at varying temperatures. Melt analysis was performed after a final elongation step and consisted of ramping the temperature a varying number of degrees per second.

QRT-PCR Probe Prior to use the, PCR hood was subjected to a minimum of 30 minutes of UV light and sterilization with 10% bleach. A 96-well plate was placed on ice along with all reagents used during preparation. QRT-PCR was conducted in 10µl reactions consisting of 5µl iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA), 1µl forward primes, 1µl reverse primes, 0.6µl nanopure water, 0.4µl probe and 2µl sample DNA. Only the PD and RW strains of *Xf* were used with the probe. A minimum of two No Template Controls, consisting of 10µl master mix, and no positive controls were used. QRT-PCR was performed on a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA).

RESULTS AND DISCUSSION

Analysis of current QRT-PCR Protocols. Current QRT-PCR protocols consisted of using SYBR® Green to measure fluorescence of PCR products. After comparison of SYBR® Green to Eva Green and Takara SYBR Green, it was found that the Takara SYBR green provided the most consistently reliable results of the three during melt analysis (**Table 1**). The results from the best QRT-PCR run/melt from each method were used in the comparison, in order to compare the best possible outcome from each method.

Table 1: Average Tm & SD for *Xf* subspecies using each SYBR Green method under ideal melt conditions		
SYBR Green Type & *Xf* Subspecies	**Avg. T_m**	**Avg SD**
SYBR® Green		
PD	85.42° C	0.3869
RW	85.66° C	0.0520
OLS	85.89° C	0.0346
Eva Green®		
PD	86.58° C	0.0458
RW	86.43° C	0.0346
OLS	86.87° C	0.0458
Takara SYBR Green®		
PD	84.90° C	0.0346
RW	85.30° C	0.0346
OLS	85.57° C	0.0346

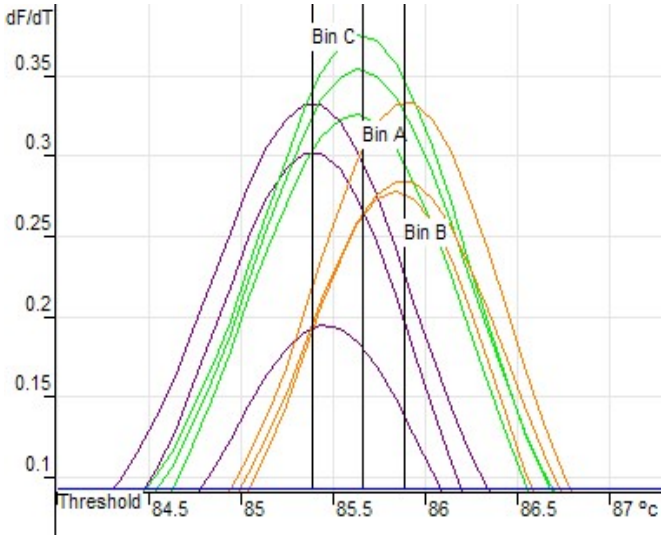

Figure 2. Melt analysis of OLS (Bin A), RW (Bin B), & PD (Bin C) using set6 primer set and Takara SYBR Green® melted at a rate of 0.3° C every five seconds.

Zot and TonB gene regions. The primers designed in the *TonB* gene region were better at differentiating between the different *Xf* subspecies using melt analysis then Sybr Green. The primers that did yield some seemingly useful differentiation of the strains were found to have standard deviations of equal or larger magnitude than the difference in melting temperature. Primers in the *Zot* gene region were designed in the Ragweed insertion sequence as well as bridging over the insertion site. Melt analysis after QRT-PCR found considerable differentiation of the *Xf* subspecies.

Zot Probe. Although primers in the *Zot* gene region provided a relatively reliable method to differentiate *Xf* strains, the melting temperatures did not separate far enough to conclude the identity of an experimental unknown. Dual-labeled probes were designed in the Ragweed insertion sequence and in the grape sequence around where the insertion would be. Using QRT-PCR and the Ragweed insertion probe, the Ragweed strain was successfully isolated from the grape strain.

Using the bridge probe, the PD strain was differentiated from the Ragweed strain, but not necessarily isolated as with the ragweed insertion probe (**Figures 2-4**).

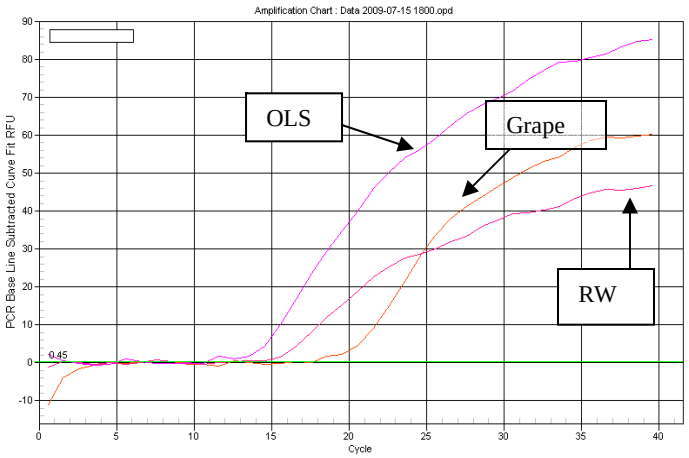

Figure 3. QRT-PCR using Grape bridge probe.

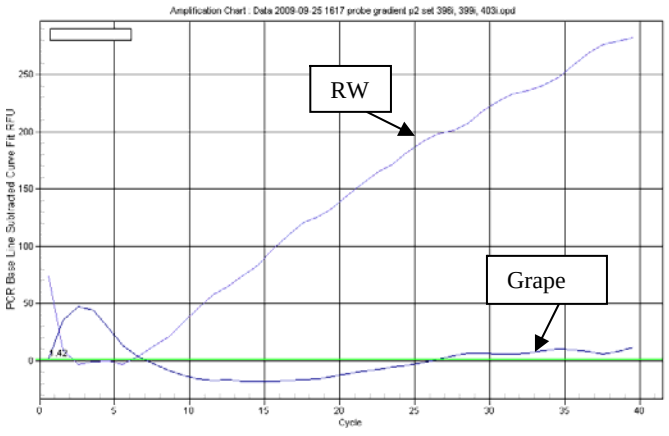

Figure 4. QRT-PCR using Ragweed insertion probe.

CONCLUSIONS

For QRT-PCR in general, Takara SYBR Green provides a statistically more accurate and consistent method of measuring fluorescence. Although more accurate, only using Takara SYBR Green instead of the previously used SYBR Green provides no significant advantage in identification of *Xf* subspecies in an unknown sample. Takara SYBR Green paired with primers

designed to take advantage of the insertion in the ragweed sequence provides a decent method of identifying strains, although the results were not consistent enough to come to conclude the *Xf* strain present based solely on QRT-PCR fluorescence or melt analysis.

The probe designed the using a bridge ragweed insertion provided an identification method while the Ragweed insertion provided a reliable and unmistakably accurate method of identifying between Grape and Ragweed strains. Utilizing the set6 primer set initially used in the comparison of the three SYBR green reagents with the Ragweed insertion probe provides a fast, reliable and accurate protocol by which strains of *Xf* can be identified using only QRT-PCR.

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FUNDING AGENCIES

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GENETIC ANALYSIS OF *ZONULA OCCLUDENS TOXIN* (*ZOT*) GENE IN TEXAS ISOLATES OF *XYLELLA FASTIDIOSA*

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ABSTRACT
Multiple subspecies of the phytopathogenic bacterium *Xylella fastidiosa (Xf)* exist which are pathogenic to distinct plant hosts, such as grapes, oleander, almonds, and citrus. Previously, DNA sequence analysis of the *mopB* and *gyr*B genes has been used to separate *Xf* strains into their subspecies groups. In this study, DNA sequence analysis of the *Zot* gene was used to corroborate the genetic variation found between three Texas strains of *Xf*, a grape strain, a weed strain, and an oleander strain (*BAN POL 055*, *GIL BEC 628A*, and *MED PRI 025* respectively). This approach provided variable gene sequences that allow for categorization of *Xf* at the population level. The *Xf* gene that encodes the z*onula occludens toxin* (*Zot*) is homologous to the *Zot* found in *Vibrio cholerae*, which is involved in tight junction modulation and disruption between host cells. The results of the analysis of this gene were consistent with the phylogeny found using the more conserved *mop*B and *gyr*B genes at the subspecies level and can be used to differentiate populations within subspecies. The analysis of these variable genes and gene regions provide additional opportunities for new diagnostic and disease management techniques.

LAYPERSON SUMMARY
In this study, we sequenced several regions of the *Xylella fastidiosa (Xf)* genome. *Xf* has been implicated as the cause of several plant diseases that cause plant death and crop loss, including PD, almond leaf scorch, and citrus variegated chlorosis. By identifying and comparing the sequences of *Zot*, we are able to determine the relationship of the different *Xf* subspecies, and may also be able to identify different populations of *Xf*, such as those from California versus those from Texas. This will allow researchers to track the spread of Pierce's disease (PD) among others, and may be useful in detailing the mechanisms by which *Xf* causes disease.

INTRODUCTION
Xylella fastidiosa (Xf) is a Gram-negative, xylem-limited, fastidious, insect-transmitted, gammaproteobacteria (26). Multiple subspecies have been described, including *Xf piercei* which causes Pierce's disease (PD), *Xf sandyi* which cases oleander leaf scorch (OLS), *Xf multiplex* which causes almond leaf scorch (ALS), *Xf 9a5c* which causes citrus variegated chlorosis (CVC) and others (19) (21) (23). *Xf* has distinctly different host ranges; though some strains of *Xf* are only pathogenic in a single host species, others cause disease in a variety of hosts (1) (10).

The *Zonula occludens toxin* (*Zot*) in *Xf* strains has been suggested as a new potential virulence factor in CVC caused by *Xf* strain *9a5c* (23). A homologous protein of the *Zot* family is found in many *Vibrio cholerae* strains and has been linked to disruption of tight junctions (11), and diarrheagenicity in *V. cholerae* that lack the cholerae toxin (5). Recently, a *Zot*-like protein was found in *Stenotrophomonas maltophilia* strains, which can cause severe health problems such as endocarditis and bacteremia (7). A *Zot* gene can also be found in strains of phytopathogens that are closely related to *Xf*, namely *Xanthomonas campestris*, which causes lesions and loss of water in plant tissue (2), and *Ralstonia solanacearum*, which causes bacterial wilt in a variety of plants (9).

Previous studies have shown that most of the sequence variation in *Xf* subspecies occurs in coding regions derived from bacteriophages (15). These regions are responsible for alterations to the chromosomes, including sequence rearrangements and deletions, of *Xf* subspecies, and therefore have an impact on the evolution of the genome of *Xf* (16) (24) (25). Several studies have shown that bacterial *Zot* genes have originated from bacteriophages (3) (7) (11) (13). The *Zot* gene found in *V. cholerae* has great sequence similarity to the protein product I (pI) of the filamentous phage Pf1, and is most likely derived from a Pf1-like phage (13). The pI protein, which shares similarity to many *Zot* proteins in *Xf*, has both an extracellular and intracellular region, and is necessary for phage packing and transport across the cell membrane in many filamentous phages (13) (22). The *Zot* gene discovered in *S. maltophilia* is reported to originate from the phage φSMA9, which is similar to the phage φLf which infects *Xanthomonas* species (3) (7). *Zot* genes in *Xf* share 55% identity with orthologs in the filamentous phage φLf of *Xanthomonas campestris pv. vesicatoria* and phage φSMA9 of *Stenotrophomonas maltophilia* , and with less than 30% identity to orthologues of *X. campestris pv. campestris* and RSM1 phage of *Ralstonia solanacearum* (15).

The *Zot* gene and respective protein are useful in comparing *Xf* because of its prophage origins, link to pathogenicity, cosmopolitan nature, and its variability in both nucleotide and amino acid sequences. In this work, the *Zot* genes of three Texas *Xf* strains, a grape strain (*BAN POL 055*), an oleander strain (*MED PRI 025*), and a multiplex strain (*GIL BEC 628A*)

were compared. These sequences were then translated and modeled *in silico* to compare the effects of the substitutions on protein structure. Finally, phylogenetic analysis was used to show the utility of the *Zot* gene in indentifying subspecies as well as populations within clades.

OBJECTIVES

1. Identify taxonomic differences between *Xf* strains that allow *Xf* population dynamics to be studied below the subspecies level.
2. Investigate the role of the ZOT protein in grape pathogenicity.

RESULTS AND DISCUSSION

Genbank accession numbers for *Xf BAN POL 055, MED PRI 025, GIL BEC 628A* were **GQ429146**, **GQ429147**, and **GQ891884**, respectively. These samples are labeled according to their subspecies (i.e. *Xf BAN POL 055* is labeled *Xf piercei* for ease of use). Sample *Xf BAN POL 055* had nucleotide sequences that align to the *Xf Temecula1* and *M23* with 99% identity while sample *Xf MED PRI 025* aligned with 99% identity the unfinished nucleotide sequence of *Xf Ann-1,* and *Xf GIL BEC 628A* aligned with to *Xf M23, Temecula1* and *Ann-1* with greater than 87% identity (**Table 1**).

Table 1. Nucleotides, Protein, and Homologous Structure Alignment Scores of *Zot1*.

Xf	Nucelotide								Protein			
	Temecula1		***M23***		***Ann-1***				e-value		samples	
	e-value	Identity	e-value	Identity	e-value	Identity	***BAN POL 055***		**d1ixza**	**c2r2aB**	***BAN POL 055***	
BAN POL 055	0	99%	0	99%	0	93%	Identity	***MED PRI 025***	9.7 E-16	NA	Identity	***MED PRI 025***
MED PRI 025	0	92%	0	92%	0	99%	92.50%	Identity	2.1 E-20	1.80 E-27	92.50%	Identity
GIL BEC 628A	0	91%	0	91%	0	90%	89.70%	89.10%	0.012	8.10 E-17	88.30%	89.90%

These results reveal homology between *Xf BAN POL 055* and *Xf M23 and Temecula1* and homology between *Xf MED PRI 025* and *Xf Ann-1*. Sequence variation between sample strains was also found. *Xf BAN POL 055* and *Xf MED PRI 025* shared 92.5% identity, *Xf BAN POL 055* and *Xf GIL BEC 628A* shared 87.9% identity, and *Xf MED PRI 025* and *Xf GIL BEC 628A* shared 87.1% identity. A hypervariable region was found between base pairs 40 to 160 in all three subspecies. Additionally, *Xf GIL BEC 628A* contained a large insertion at basepairs 344 to 387 not found in either *Xf BAN POL 055* or *Xf MED PRI 025* (**Figure 1**).

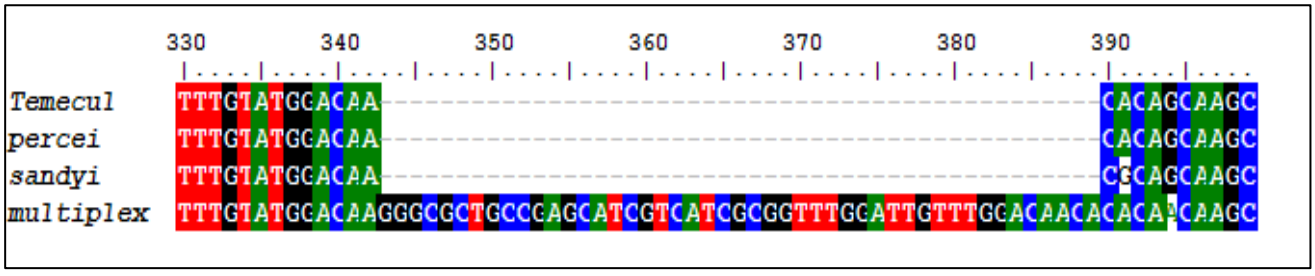

Figure 1. The insertion sequence appeared in multiple samples of *Xf GIL BEC 628A*, all collected in central Texas.

In silico translation of the *Zot* gene in all three subspecies yielded several amino acid changes and as well as changes in the predicted tertiary structure (data not shown). Sequence variation exists between members of *Xf* subspecies particularly in a hypervariable region found at the N-terminus of the *Zot1* protein (data not shown). Protein sequences submitted to Pfam returned significant results from the *Zot* family (PF05707) with e-values of at least $2.8e^{-11}$. In a homologous structure search,

the *Xf* subspecies were placed in two different groupings. *Xf GIL BEC 628A* and *MED PRI 025* matched the crystal structure of the N-terminus domain of the *Zot* protein of the *Neisseria meningitidis* (SCOP code c2r2aB) with an e-value of $8.1e^{-17}$ and $1.8e^{-27}$ respectively (**Table 1**). While *Xf BAN POL 055* matched a protein in the P-loop containing nucleoside triphosphate hydrolases superfamily (SCOP code d1ixza) with an e-value of $9.7e^{-16}$ (**Table 1**). As a reference, *Xf Temecula1* was also submitted to PHYRE and also matched the protein d1ixza in the triphosphate superfamily. *Xf GIL BEC 628A* and *MED PRI 025* also aligned to the protein d1ixza, but with a higher e-value than their respective alignments to c2r2aB (**Table 1**).

Phylogenetic comparison shows three distinct groupings of *Xf Zot* proteins (data not shown). These groupings were not consistent with current organization of *Xf* clades. Closer analysis of the individual *Zot* proteins revealed that three distinct homologues of *Zot* proteins exist in *Xf*. Each form was placed in the *Zot* superfamily according to HMM searches performed at Pfam. Additionally, these homologues of *Zot* are found across many taxa, including *Xanthomonas campestris* and *Stenotrophomonas maltophilia*. Finally, protein sequence comparisons between the four copies of the *Zot* gene found in *Xf Ann-1* show that only two copies share a high degree similarity (an error value of 0.0) while all other comparisons show positive error value scores, thus indicating high sequence variation. The *Zot* proteins thus form three distinct homologues hereby named *Zot1*, *Zot2*, and *Zot3*. *Zot1* includes the three sequences from *Xf BAN POL 055*, *MED PRI 025*, and *GIL BEC 628A* as well as two additional sequences from *Xf Temecula1* (**gi|28198830|** and **gi|28198817|**), two additional sequences from *Xf M23* (**gi|182681531|** and **gi|182681517**|), and one additional sequence from *Xf Ann-1* (**gi|71902114|**). *Zot2* includes one sequence from *Xf Temecula1* (**gi|28198835|**), one sequence from *Xf M23* (**gi|182681534|**), two sequences from *Xf Ann-1* (**gi|71901575|** and **gi|71728661**), one from *Xf Dixon* (**gi|71164684|**) and two sequences from *Xf M12* (**gi|170730250**| and **gi|170730245**|). *Zot3* includes one sequence from *Xf Ann-1* (**gi|71728117**) and two sequences from *Xf 9a5c* (**gi|15838473**| and **gi|15838468**|).

Phylogenetic analysis comparison of *Zot1* proteins corroborated analysis of the *mopB* and *gyrB* proteins. The *Xf BAN POL 055* sample grouped with *Xf Temecula1* and *M23* sequences, and the *Xf MED PRI 025* sequence grouped with the sequence from *Xf Ann-1*, while *the Xf GIL BEC 628A* sequence branched separately from the other sequences (**Figure 2**).

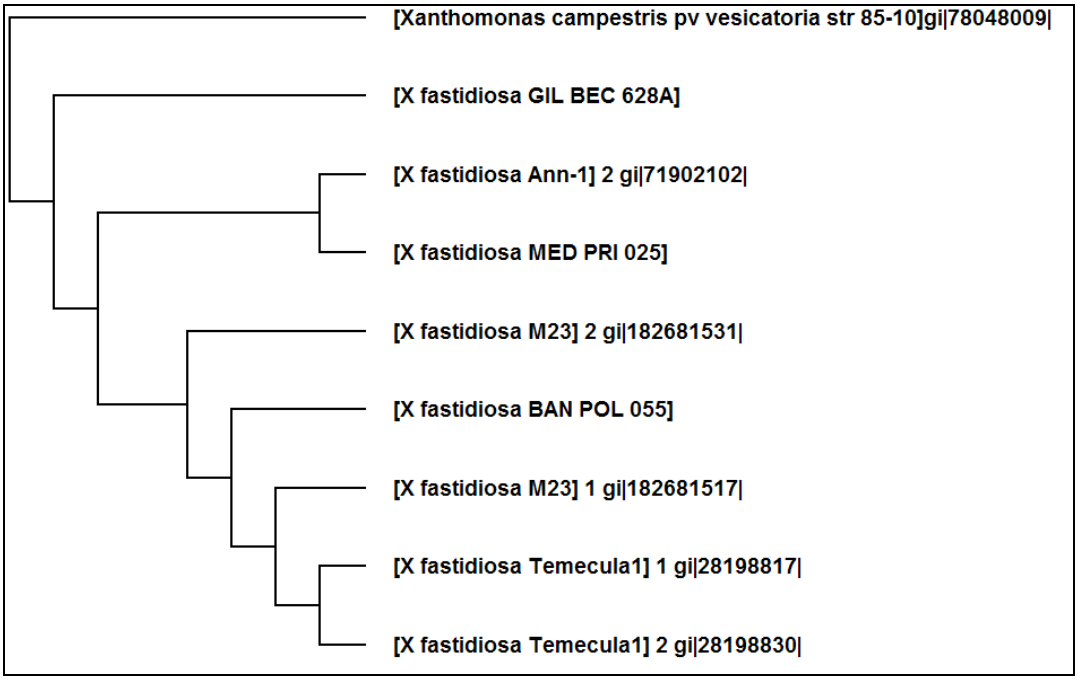

Figure 2. Cladogram of *Zot1* using sequences found in NCBI. Alignment performed using ClustalW and visualized using Treeview v1.6.6. Genbank accession numbers follow organism name. Sequences labeled according to source organism and genomic location. Sequence from *Xanthomonas campestris pv vesicatoria str 85-10* is a filamentous phage Cf1c related protein belonging to the *Zot* family.

The sequence variation in *Zot1* found in Texas subspecies of *Xf* is evident and can be exploited for classification and identification. All tested isolates contained *Zot1* representing all three clades of *Xf* present in Texas. *In silico* protein analysis revealed changes in amino acid sequence, as well as predicted changes in tertiary structure. Phylogenetic analysis using the *Zot1* protein accurately grouped the sample *Xf* strains to their respective clades. Finally, sequence variation within the *Zot1* gene was great enough to show differentiation between populations of *Xf piercei* populations in California and Texas, thus allowing a greater degree of classification of *Xf*.

Zot1 was chosen for this study as the *Zot1* gene is the most common form of *Zot* in *Xf Temecula1* and *M23.* Future study will elucidate the relationship between *Zot1, Zot2,* and *Zot3* and determine if they are paralogous, xenologous, or a combination of the two. Additionally, future study will describe which Texas isolates, if any, contain the *Zot2* or *Zot3* gene. Alignment analysis performed using BLAST*n* and BLAST*p* shows that several *Xf* genomes, including *Xf M12,* and *9a5c* do not contain *Zot1* based on searches conducted in the EST, Genome, Chromosome, and Nucleotide libraries of NCBI (data not shown). Additionally, *Zot1* does not appear in *Xf Dixon,* though this may be a result of an incomplete *Dixon* genome.

The exact origin of *Zot1* is not certain; however, by examining other, closely related *Zot* genes, we may make inferences. *Zot2* appears to have evolved from filamentous bacteriophages in *Vibrio cholerae* and *Stenotrophomonas maltophilia* (7) (13). The *Zot2* protein found in *Xf* has significant homology to another *Zot*-like protein found in strains of *S. maltophilia* (7). This *Zot* protein is the first virulence factor in clinical isolates of *S. maltophilia* strains, and shows a similarity to the *Zot2* protein in *Xf Dixon* with an error value of $1e^{-128}$, and appears to have arisen from infection from phage φSMA9 (7). Though, *Zot1* and *Zot2* share little identity (less than 5%), it can be deduced that *Zot1* arose in *Xf* through phage integration as in *Stenotrophomonas maltophilia* and *Vibrio cholerae*. In a BLAST*x* search at NCBI, *Xf BAN POL 055* matched *Xanthomonas* phage Cf1c protein labeled Cf1cp4, which contains a putative *Zot* protein. Additionally, several of the phage related proteins found in *S. maltophilia* strain c5 match phage related protein sequences surrounding a *Zot1* gene found in *Xf Temecula1*. Finally, the direction of the open reading frame of the *Zot1* gene in *Xf BAN POL 055* and *GIL BEC 628A* differs from *Xf MED PRI 025,* and the genomic context of the *Zot1* gene differs between each strain. Genomic rearrangements of *Xf* prophage regions have been described before (16). Initial analysis of the alignment of the *Xf Ann-1* partial genome to both *Xf Temecula1* and *M23* shows that the *Zot1* gene and its associated phage integrases are found in the opposite direction and in different locations in the genome as can be inferred from the necessary use of different primer sets at the beginning and end of the *Zot1* gene, as well as a study of the genomic context of *Xf M23, Temecula1,* and *Ann-1* found at NCBI. Reorganizations such as these have previously been attributed to large prophage regions that act as recombination sites (16). Thus, it is possible that some *Xf* ancestors received the *Zot1* gene after branching. Another possibility is that phage-related recombination removed the *Zot1* gene from some *Xf* subspecies after a branching event

Little is known about the *Zot* protein's structure due to the complexity of isolating *Zot* for analysis as a result of its transmembrane region (7) (13) (22). *In silico* translations and structure predictions offer great insights into protein function and classification and has been found to be accurate and sensitive. By using *in silico* analysis, the sequence variation found in *Zot* genes between subspecies has been shown to yield differences in protein structure. These differences appear near the N-terminus of the *Zot1* protein, which is extracellular in other species of bacteria (7) (13) (22). It is possible to infer that changes made in the *Zot* protein are driven by host-pathogen interactions. Further study regarding the location and expression of the *Zot1* protein is necessary to fully elucidate the relationship.

The first step in determining host range in differentially pathogenic bacteria is placing the bacteria into clades (17). Many techniques for identification and classification exist; however, the complexity of *Xf* pathogen makes categorization based on morphology or pathogenicity difficult (1). Additionally, *Xf* has been shown to have limited genomic variability within clades and region, and that the majority of strain specific genes occur in prophage regions, though they contain genomic islands which enable rapid evolution (25). Techniques that focus on classification based on well conserved regions shared by all *Xf* subspecies might then miss putative evolutionary growth and adaptation. The *Zot1* gene insertion is an excellent target for QRT-PCR, and the small sequence differences can be targeted by restriction enzyme digestion analysis for quick and accurate identification and classification of *Xf* subspecies and populations.

CONCLUSIONS

Comparative analysis *Zot1*genes and proteins provide accurate, population level differentiation therefore allows researchers greater ability to track the spread of economically important phytopathogens. Additionally, *in silico* translation and analysis of *Zot1* describes in greater detail differences between strains, and describes possible conformation changes that result from sequence changes between strains. Taken together, these results show that *Zot1* is a useful target for differential sequence analysis and can be used to elucidate the phylogenetic history of *Xf*, and its spread through the U.S.

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INFLUENCE OF HOST XYLEM CHEMISTRY ON REGULATION OF *XYLELLA FASTIDIOSA* VIRULENCE GENES AND HOST SPECIFICITY

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ABSTRACT
This project follows our previous work on virulence gene regulation in *Xylella fastidiosa (Xf)* and other plant pathogens using DNA microarray techniques (Shi et al., 2007; 2009; Shi and Cooksey, 2009). Virulence genes in bacterial pathogens of plants and animals are regulated by complex pathways that are often responsive to environmental and host signals. We have studied several of these regulatory pathways in *Xf* and have begun to understand the regulation of specific virulence genes that contribute to functions, such as growth, biofilm formation, gum formation, and motility, which are important for this pathogen to cause disease. At the end of that project, we began to look at the effect of different host xylem fluids on expression of virulence genes. Our work with a Pierce's disease (PD) strain of *Xf* showed that several virulence genes were more highly expressed in xylem fluid of grapevine vs. xylem fluid of citrus, a non-host plant for the PD strain (Cooksey, 2008). This finding suggests that host range of *Xf* may be influenced by differential expression of virulence genes in response to different host xylem chemistry. This proposal is to further explore that hypothesis with several strain/host combinations and to investigate components of xylem fluid that are responsible for either inducing or repressing virulence in *Xf*.

LAYPERSON SUMMARY
We have shown that genes involved in disease induction by a Pierce's disease (PD) strain of *Xylella fastidiosa (Xf)* were expressed differently in sap of a susceptible plant (grape) vs. a resistant plant (citrus). This raises the possibility that the host range of different strains of *Xf* is in part due to differential regulation of bacterial genes in response to differences in chemical components of plant sap. We propose to further test this idea by examining gene expression in *Xf* grown in sap with several different strain/host combinations for which we have already defined whether the particular combinations result in susceptibility, resistance, or tolerance. We will also examine specific chemical components of plant sap that influence bacterial gene expression, with the goal of discovering components that could be used for practical disease control by reducing expression of genes necessary for disease induction.

OBJECTIVES
1. Assess virulence gene expression of several different host-range strains of *Xf* in the xylem fluid of a comment set of plant hosts.
2. Assess the influence of specific components of plant xylem fluids on the expression of virulence genes of *Xf*.

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DIFFUSIBLE SIGNALING FACTOR AND C-DI-GMP LEVELS REGULATE TRANSITION BETWEEN MOTILE AND AGGREGATIVE BEHAVIOR IN *XYLELLA FASTIDIOSA*, THEREBY CONTROLLING VIRULENCE

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ABSTRACT
We have determined that an EAL domain containing protein (PD1617) of *Xylella fastidiosa* (*Xf*) plays an essential role in biofilm formation and the aggregative behavior. The *ΔealXF* and Δ*rpfF* mutants exhibited similar phenotypes such as enhanced twitching motility and virulence in grape. Surprisingly, the double mutant (*ΔrpfFΔealXF*) resulted in a significant loss of pathogenicity and exhibited an opposite phenotype to that of the individual mutants, having an absence of twitching motility and more highly aggregated cells. These findings suggest that C-di-GMP is involved in the regulatory pathway of the genes controlled by DSF. The higher virulence of this mutant reinforces the model that colonization and pathogenicity of Grapevines by *Xf* is facilitated by its capacity to move into the xylem; higher endogenous levels of c-di-GMP promotes higher motility while less c-di-GMP increases adhesion and biofilm formation. Curiously, these results are opposite to that observed in a majority of other bacterial species, where more c-di-GMP promotes biofilm formation and suppresses motility.

LAYPERSON SUMMARY
The mechanism of *Xylella fastidiosa (Xf)* pathogenicity is associated with its capacity to multiply and spread widely from the site of infection to colonize the xylem vessel. Cells of *Xf* attach to the vessel walls forming biofilms (large aggregate of cells), that can completely occlude xylem vessels and block water transport. In this work we have verified that *Xf* biofilm formation and movement are determined by signals that trigger the expression of specific genes. This regulatory pathway involves Diffusible Signaling Factor (DSF) and levels of a small intracellular molecule named cyclic-di-GMP (c-di-GMP). We determined that different levels of c-di-GMP in *Xf* cells may regulate genes associated with both biofilm formation and motility. Our data suggests that low levels of c-di-GMP suppresses virulence in *Xf*, therefore c-di-GMP is directly involved in the regulation of pathogenic behavior. This work thus has determined the internal mechanisms by which external signals which determine cell density-dependent changes in bacterial behavior (DSF) lead to such large changes in virulence.

INTRODUCTION
Cyclic di-GMP (c-di-GMP) is a novel second messenger in bacteria that is synthesized by a class of enzymes containing GGDEF domains and hydrolyzed by another protein family containing EAL domains. C-di-GMP regulates a range of cellular functions, mainly those related to developmental transitions, aggregative behavior, adhesion, biofilm formation, and virulence (Jenal and Malone, 2006). In *Xylella fastidiosa* (*Xf*) the formation of biofilms and movement through the xylem vessels have strong effects on pathogenicity, with adhesion and biofilm formation reducing virulence while motility generally enhancing virulence. Cell-cell signaling mediated by Diffusible Signaling Factor (DSF), synthesized by RpfF, is involved in the regulation of genes associated with these functions (Newman et al., 2004; Chatterjee et al., 2008). It was recently demonstrated in *Xanthomonas campestris* that DSF signaling is mediated by c-di-GMP, where the HD-GYP domain of RpfG also functions as a c-di-GMP phosphodiesterase (Ryan et al., 2006, He et al., 2007). Besides these results with *X. campestris*, analysis of gene expression in a *Xf rpf*F mutant, reveled that DSF negatively regulates a protein with a GGDEF domain (Chatterjee et al., 2008). These results suggest that the levels of c-di-GMP may have a role in the DSF cell-cell signalization network, which modulates the expression of several genes associated with pathogenicity and adaptation. Besides RpfG that can hydrolyze c-di-GMP, *Xf* has also three other genes (PD1617, PD1671 and PD1994), with EAL domains that may also hydrolyze c-di-GMP. However, only PD1617 has an EAL domain without an associated GGDEF domain, suggesting that it is dedicated only to a specific function in c-di-GMP degradation.

OBJECTIVES
The goal of this work was to investigate the role of PD1617 (EAL domain) in the DSF signaling pathway and its effect on the virulence of *Xf*. The work had the following specific objectives:
1. Generation of mutants for the gene encoding EAL domain protein PD1617 (ΔealXF) and the double mutant ΔrpfFΔealXF.
2. Evaluate the in vitro phenotypes of these mutants such as colony morphology, aggregative behavior and biofilm formation.
3. Study the virulence of *Xf* mutants (ΔrpfF, ΔealXF, ΔrpfFΔealXF) compared with the WT strain.

RESULTS AND DISCUSSION

Generation of mutants of the EAL domain protein PD1617 (Δ*ealXF)* and double mutants Δ*rpfF*Δ*ealXF*

A fragment from pBBR1MCS-2 encoding *nptII* which confers kanamycin resistance, was ligated into pUC19, resulting in pFXF. Genomic regions each of about 1kb both downstream and upstream from the PD1617 gene (which we named *ealXF)*, were amplified by PCR from the PD Temecula strain total DNA without its native promoter. These PCR fragments were ligated to pFXF, flanking the *nptII* marker gene, resulting in pFXF1. The PCR-amplified insert carrying *ealXF* was verified by sequencing to ascertain the absence of PCR amplification errors. The transformation of the WT strain Temecula with this suicide vector was done by exploiting the natural competence *Xf* by methodologies developed by Rodrigo Almeida (U.C Berkeley, personal communication), using XFM media (Almeida et al., 2004). Colonies were picked and then screened for interruption of *ealXF* by PCR using appropriate primers. The double mutant was obtained by deletion of *ealXF* in the genetic background of Δ*rpfF* (Chatterjee et al., 2008) to generate Δ*rpfF*Δ*ealXF*. Two colonies from each mutant Δ*ealXF* and Δ*rpfF*Δ*ealXF* were used in this study.

Evaluation of mutant phenotypes

The *ΔrpfF*, *ΔealXF* and *ΔrpfFΔealXF* mutants exhibited distinct growth patterns and colony morphologies from each other.

To evaluate the possible alteration in growth and colony morphology caused by the altered C-di-GMP and DSF levels in cells, the same concentration of cells (OD_{600} = 0.1) were placed as a drop of suspension on two different media, PWG and XFM (plus 1% pectin). Serial dilutions were also performed to obtain individual colonies. The different phenotypes of growth morphology can be observed in **Figure 1**. In both media (**Figures 1 A and B**) no strong difference was observed between the Δ*ealXF* and WT. In both a thick ring around the colony was observed. *ΔrpfF* also has this ring, but it was much thinner than that in the WT and Δ*ealXF* mutant. No ring was observed in the *ΔrpfFΔealXF* double mutant. The center of the WT and Δ*ealXF* colonies seems to be the same, with less growth and EPS accumulation apparent compared to that on the edges of the colony. The *ΔrpfF* mutant showed more growth at the center of the colony compared with WT and *ΔealXF*. The double mutant showed strong aggregative cells behavior at the center of the colony. Similar growth was also observed when the cell suspensions were spread on the plates (**Figure 1 C**).

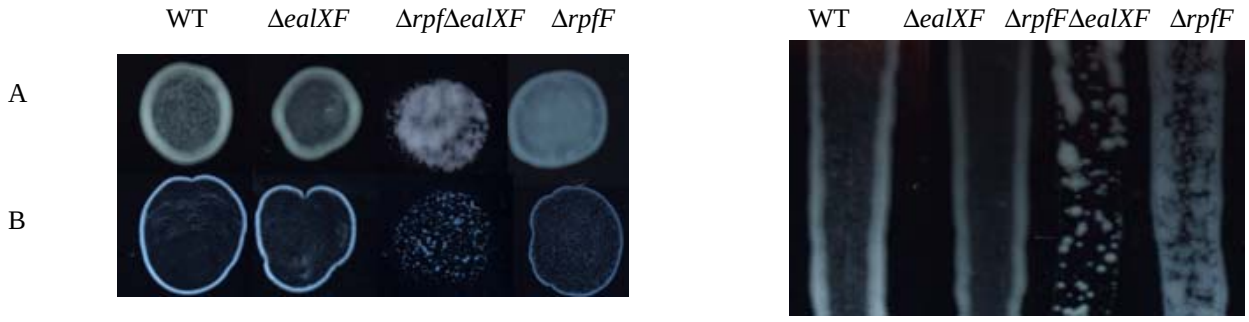

Figure 1. Growth morphology of WT and mutants from droplets of inoculum on PWG (A) XFM (B) media and spread on PWG (C).

When individual colonies were evaluated, a clearer difference in colony appearance was observed between the mutant and WT strains (**Figure 2**). The WT strain exhibited a circular colony with smooth surface with no apparent twitching motility around the colony evident as a lack of a "fringe of cells (minor twitching motility was observed only in the smallest colonies). The Δ*ealXF* mutant had small dome-shaped colonies with a wrinkled surface and obvious twitching motility around the cells. Twitching motility was also observed in the Δ*rpfF* mutant but the colony surface was smoother than Δ*ealXF*. The most interesting phenotype was observed in colonies of the double mutant that had a smooth surface and absence of twitching motility. The presence of twitching motility in Δ*ealXF* is an indication that under normal condition the degradation of c-di-GMP can negatively regulate genes associated with twitching motility; this is opposite to that in most bacteria, where less c-di-GMP is associated with motility.

We also investigated the mutant phenotype in comparison with wild type by scanning electron microscopy. Cells of the WT, Δ*ealXF* and Δ*rpfF* strain had more polar pili than the double mutant Δ*rpfF*Δ*ealXF* (**Figure 3**). These polar pili were more abundant in the Δ*ealXF* mutant. These results are consistent with the twitching motility colony observation, since the long pili are associated with this characteristic (Meng et al., 2005). Although polar pili were also occationally observed in the double mutant (data not shown) they are much less frequent than in Δ*rpfF* and Δ*ealXF*. On the other hand cells of the *ΔrpfFΔealXF* double mutant had more connected cells than the other strains (**Figure 3C**).

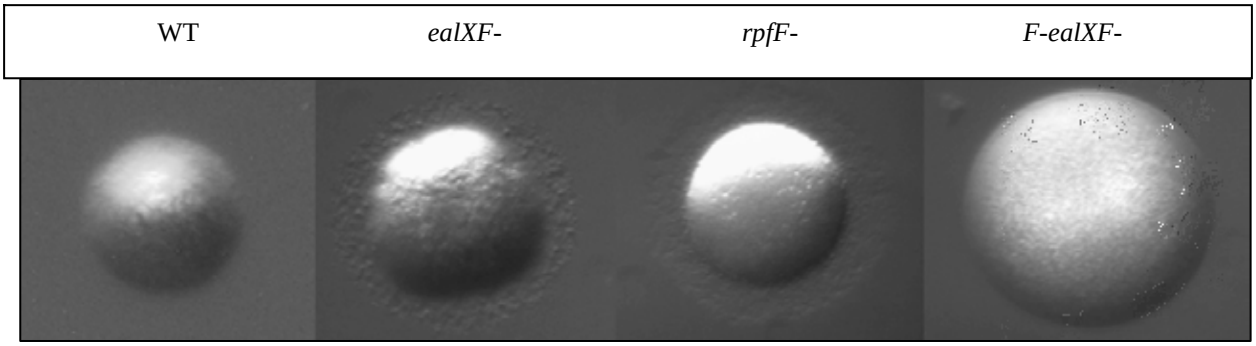

Figure 2. Colony morphologies of wild-type *Xf* Temecula strain and mutants grown on PWG for 4 days.

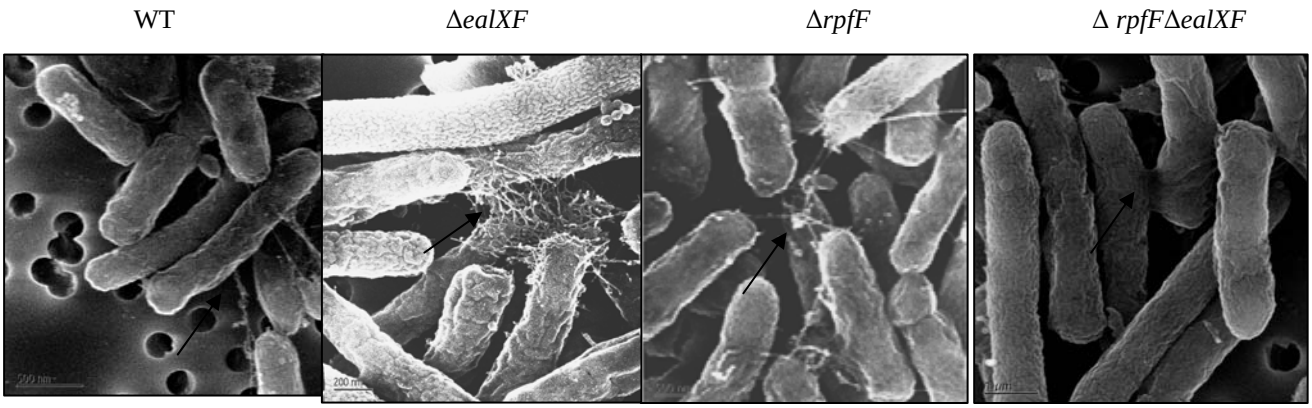

Figure 3. Scanning electron micrography of wild-type and mutant cells. Red arrows indicate the polar pili on WT, *ΔealXF*, *ΔrpfF* and connected cells on *ΔrpfFΔealXF* double mutant.

The double mutant show aggregative cells behavior

Bacterial cells grown for five days at 28°C in PWG were scraped and suspended in 1mL of 10mM KPO_4 buffer. The suspension was vortexed for 30s to thoroughly disperse the cells and an aliquot was used to microscopy analyses and the samples were left for 10 min in room temperature to verify the aggregative behavior of the cells. Unlike the other strains, the double mutant showed obvious visible cellular aggregates in the buffer (**Figure 4**). Transmission microscopy demonstrated the presence of more planktonic cells of *ΔealXF* and WT than the *ΔrpfFΔealXF* double mutant. In the *ΔrpfF* mutant only small aggregates and planktonic cells were observed. In the *ΔrpfFΔealXF* double mutant there mainly only huge cell aggregates were observed (**Figure 5**). A similar phenotype of huge cell aggregation was observed in the *Xathomonas campestris* double mutant *ΔrpfFΔclp* (He et al., 2007). However the authors had no explanation for this phenotype. Clp is a conserved global regulator that may be able to bind cGMP or c-di-GMP and activate the expression of different sets of genes. In *Xanthomonas*, Clp was identified as the downstream global regulator in the DSF signaling pathway. So the similar phenotype observed in the *ΔrpfFΔealXF* double mutant from *Xf* and the *ΔrpfFΔclp* mutant from *X. campestris* may be explained by a higher imbalance of intracellular c-di-GMP and thus probably a corresponding effect on the activity of the Clp protein. The higher imbalance in *Xf* double mutant could be explained due to the additive effect of the lack of activity of two different enzymes that degrade c-di-GMP, EalXF and RpfG . RpfG has a HD-GYP domain and is apparently activated by phosphorylation by RrpfC which, in turn, is activated by DSF binding). Thus, in the absence of DSF, RpfG may have less or no activity in c-di-GMP degradation. However this hypothesis remains to be further investigated.

WT **Δ *ealXF-*** **Δ *rpfF*** **Δ *rpfF* Δ *ealXF-***

Figure 4. Phenotypic analyses of wild type and mutant strains of *Xf* for DSF production, c-di-GMP degradation and double mutant to both characteristics. Aggregative cells are quickly formed in the double mutant.

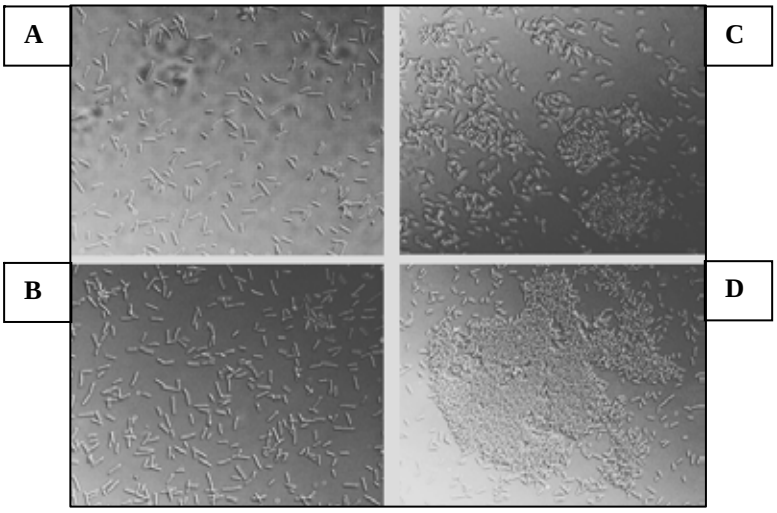

Figure 5. Microscopic analysis of mutants cells of *Xf* showing single cells of wild-type (A), which were similar to the ***Δ*** *ealXF* deletion mutant (B), to small scale cell aggregates of the **Δ** *rpfF* deletion mutant (C) and extensive cell aggregates formed by the double **Δ***rpfF* **Δ** *eaXF* deletion mutant (D).

ΔealXF mutants produce less biofilm

Mutants were evaluated for biofilm formation in XFM medium in both glass tubes and 12-well polystyrene culture plates. The tubes were incubated for seven days at 28ºC with agitation at 200 rpm. The plates were maintained under the same conditions without agitation. The biomass attached at the liquid-air interfaces (glass tubes) and adhered onto the wells in the culture plates were quantified by Crystal violet 0.1% staining. Both mutant *ΔrpfF* and *ΔealXF* accumulated less biofilm than the wild-type strain (**Figure 6**), but this reduction was significantly different only for the *ΔealXF* mutant. These results suggest that degradation of c-di-GMP can positively regulate genes associated with biofilm formation. The phenotype of the *ΔrpfFΔealXF* double mutant suggests that c-di-GMP and DSF may both be involved in the regulatory pathway that converge to the same gene regulation, but in absence of both, an imbalance of c-di-GMP level occurs, thereby affecting gene regulation.

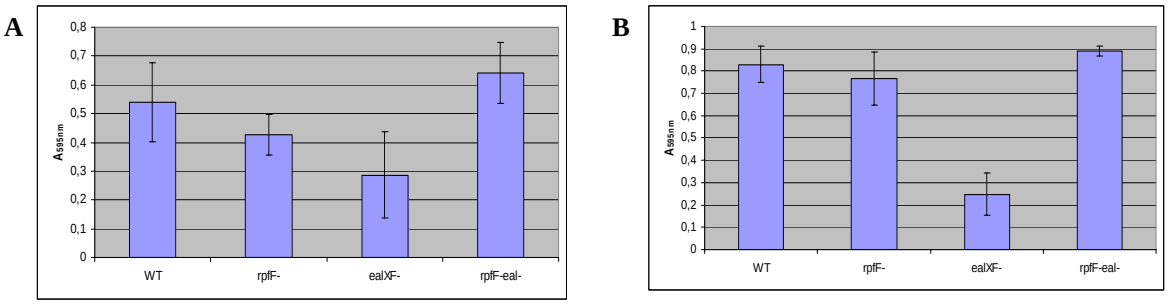

Figure 6. Biofilm formation by the *Xf* wild type and *ΔrpfF*, *ΔealXF* and *ΔrpfFΔealXF* mutants after 7-day of growth at 28°C on glass tube (A) and polystyrene plates. Biofilms were stained with 0.1% crystal violet. The results shown are average of four or three repetitions.

Mutants with altered c-di-GMP levels show different level of virulence in plants

To access the pathogenicity of the mutants generated in this work and to also compare their virulence with a mutant deficient in c-di-GMP synthesis, we included in our experiment a PD0279 mutant that has a GGDEF domain (made by Chatterjee et al., not published). Each strain was mechanically inoculated into 15 Thompson seedless grapevines. The number of symptomatic leaves was counted weekly. Symptomatic leaves were scored for the presence of typical PD symptoms (loss of chlorophyll and drying of the leaf margins). For cell migration assays, petioles of leaves were harvested at six different distances (30 cm apart) at 11 weeks after inoculation. The petioles were surface sterilized and droplets of petiole macerate was placed on PWG plates to determine the presence or absence of the bacteria. As previously demonstrated by Chatterjee et al (2008) plants inoculated with Δ*rpfF* mutants show disease symptoms at about the same time as plants inoculated with the wild type (**Figure 7**). However, subsequently the number of symptomatic leaves was higher in plants inoculated with the *rpfF* mutant than the WT strain (**Figure 7**). The plants inoculated with *ΔealXF* also showed higher number of symptomatic leaves at all sampling times. The amount of the symptomatic leaves for this mutant was significantly higher than for the *Δggdef* mutant. Almost no symptoms were observed in plants inoculated with the double mutant. Some symptomatic leaves were observed only in some plants near the point of inoculation with the *ΔrpfFΔealXF* double mutant, and no increase of the disease was subsequently observed (**Figure 7**). The movement of the *Xf* strains was determined by estimating the presence of cells in leaf petioles at different distances away from the point of inoculation. At 11 weeks after inoculation, cells of the WT, Δ*rpfF*, Δ*ealXF* and *Δggdef* were detected at 170, 180, 150 and 110 cm respectively. No cells were observed for the *ΔrpfFΔealXF* double mutant (data not shown). The leaf symptoms for each mutant and WT are shown in **Figure 8**. These results indicate that the Δ*rpfF* and *ΔealXF* mutants are highly virulent and apparently much more efficient in migration than the *ΔrpfFΔealXF* double mutant that exhibited an avirulent phenotype. Also, the *ΔealXF* mutant was more virulent than the *Δggdef* mutant suggesting that the low level of c-di-GMP suppresses virulence in *Xf*.

Taken together, our results, along with others, suggest a signal regulatory pathway, where c-di-GMP levels are essential for regulation of bacterial virulence factors in *Xf* (**Figure 9**).

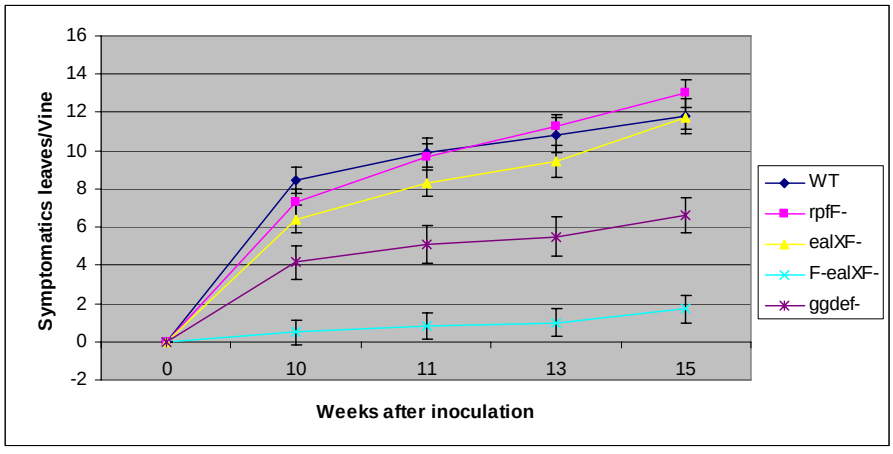

Figure 7. Progression and severity of Pierce's disease of grape incited by a wild type and mutants of *Xylella fastidiosa*. The vertical bars represent the standard error of the mean of the number of infected leaves per vine determined from 15 replicate plants for each treatment.

WT *ΔrpfF* *ΔealXF* *ΔrpfFΔealXF* *Δggdef*

Figure 8. Severity of Thonpson Symptomatic leaf inoculated with WT Temecula strains and the mutants after 11 weeks. The *Δggdef* and *ΔrpfFΔealXF* show less severity.

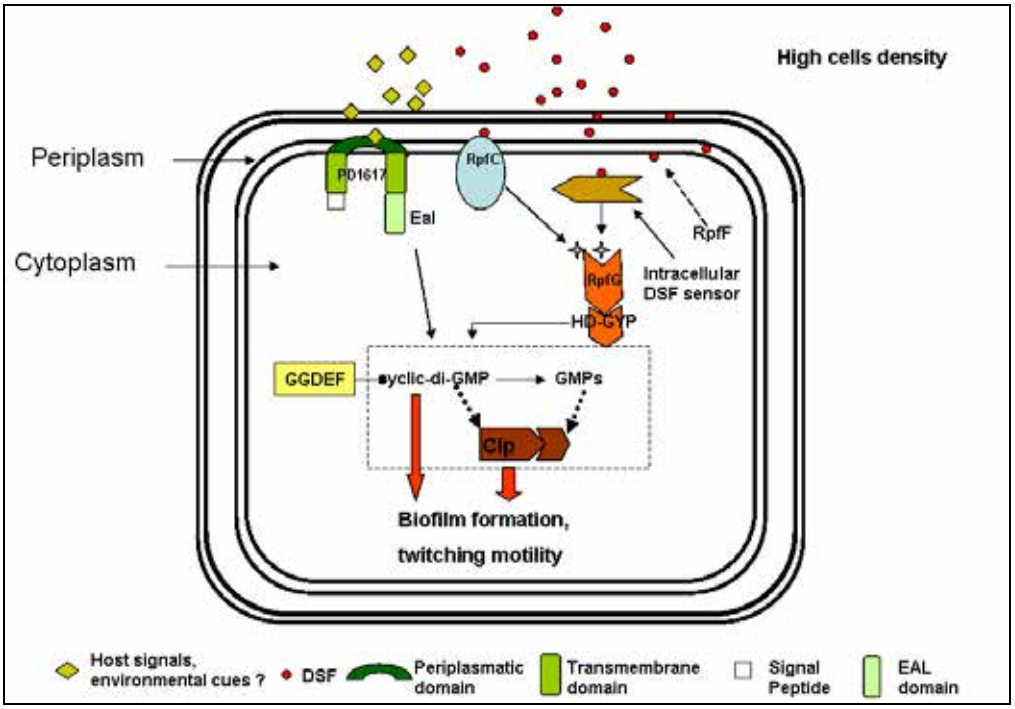

Figure 9. Hypothetical model for the DSF and c-di-GMP network in *Xf*. Proteins with c-di-GMP metabolizing domains such as GGDEF, EAL and HD-GYP contribute to the balance of c-di-GMP level, and as a consequence alter the transcriptional expression of genes regulated by DSF. DSF (produced by RpfF) is recognized by rpfC or another unknown intracellular DSF sensor that activates RpfG. RpfG degrades c-di-GMP to GMP decreasing its levels. Similar function is observed for the EAL domain protein, which has a transmembrane domain that could also sense environmental signals and change the levels of the second massager c-di-GMP. c-di-GMP level could directly or indirectly (Clp protein?) modulate the expression of genes associated with biofilm and motility in *Xf. a*.

CONCLUSIONS

The results from this work demonstrate that the levels of c-di-GMP in *Xf* regulate genes involved with biofilm formation, and that virulence of this pathogen to plants is low when c-di-GMP levels are low favoring gene expression leading to biofilm formation. The regulation of these genes is also mediated by DSF levels (Charteejee et al., 2008).

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GRAPE RECOGNITION OF *XYLELLA* SURFACE PROTEINS AND THEIR RELATIONSHIP TO PIERCE'S DISEASE SYMPTOM DEVELOPMENT

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Reporting Period
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ABSTRACT
The aim of this project is to understand the role played by surface proteins, especially the elongation factor "temperature unstable" (EF-Tu) in recognition of and disease induction by *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease (PD) of grapevine, with the goal of interfering with this recognition and, if possible, disease induction. 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. Expression of a *Xf* EF-Tu fusion protein in *Escherichia coli* altered the cell morphology and seems sickening to the cell if targeted to the extracellular space. 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. Further analysis showed that purified intact *E. coli* EF-Tu and an N-terminal 18 amino acid peptide of *E. coli* EF-Tu were also capable of inducing reporter gene expression. These results suggest that, in addition to its role in protein synthesis, EF-Tu may be a signal in grapevine of *Xf* infection and that its recognition may be mediated by a known receptor, EFR.

LAYPERSON SUMMARY
The elongation factor "temperature unstable," EF-Tu, is a protein found on the outside of *Xylella fastidiosa* (*Xf*) bacteria. It has been shown that the presence of *Xf* in grape turns on certain grape genes. Purified *E. coli* EF-Tu protein, which is a very close match to the *Xf* EF-Tu protein, is capable of turning on at least one of these same genes. This suggests that the EF-Tu found on the outside of the *Xf* bacteria may be recognized by the grape plant. Release of the *Xf* EF-Tu from the bacteria may explain the distance between the bacteria replication sites in the xylem and the Pierce's disease sites at the leaf margins. Interference with grape recognition of the released EF-Tu may lead to a reduction in the disease symptoms.

INTRODUCTION
Long term, economical and sustainable control of Pierce's disease (PD) is likely to be achieved most effectively by deploying grapevine cultivars resistant to or tolerant of *Xylella fastidiosa* (*Xf*). Interference with symptom development (i.e., creation of tolerance) is conceivable by preventing the full functioning of *Xf* virulence factors. The mechanisms by which *Xf* induces symptoms in infected grapevine have not been established. *Xf* surface proteins are candidates for symptom-inducing factors. Examples of *Xf* surface proteins are a major outer membrane protein MopB (Bruening and Civerolo 2004), the hemagglutinin-like minor outer membrane proteins H*Xf*A and H*Xf*B (Guilhabert and Kirkpatrick 2005), a protein that is recognized by a single chain, monoclonal antibody (Bruening et al. 2008), and possibly a form of the protein synthesis elongation factor "temperature-unstable" (EF-Tu) (Bruening et al. 2008). We reported earlier 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 that have been categorized as "microbe-associated molecular patterns" = MAMPs because of their ability to induce defense responses in specific 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 serve as 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 µM by pressure-infiltration into Arabidopsis leaves, induced resistance to *Pseudomonas syringae* and caused Arabidopsis to accumulate defense gene mRNAs (Kunze et al. 2004). *E. coli* EF-Tu and *Xf* EF-Tu gene sequences show 77% identity and 88% similarity in amino acid sequence, and both proteins induce chlorosis when pressure infiltrated into C. chenopodium leaves (Bruening et al. 2007). Those regions that show identity between the *E. coli* and *Xf* EF-Tu gene sequences also showed >90% identity with >100 eubacterial EF-Tu sequences (Kunze et al. 2004). Some

bacteria have evolved an EF-Tu protein with at least one 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). Therefore, it will not be surprising if *Xf* EF-Tu is found to be capable of inducing reactions in grapevine, including reactions that lead to symptom development. This work is an extension of our previous grant entitled "Exploiting *Xylella fastidiosa* Proteins For Pierce's Disease Control." The objectives for the current project are given below.

OBJECTIVES

1. Test *Xf* EF-Tu for its ability to induce scorching in grapevine.
2. Identify a grapevine receptor for *Xf* EF-Tu.
3. Interfere with *Xf* EF-Tu-induction of scorching using RNAi or by expression of alternative receptor.
4. Characterization of *Xf* EF-Tu and its immobilization and localization.

RESULTS AND DISCUSSION

Grape 9353 promoter activation by purified E. coli EF-Tu

The insoluble character of *Xf* EF-Tu, although interesting as an indicator of possible non-protein-synthesis functions of this protein, has prevented us from purifying *Xf* EF-Tu and therefore from having it available for direct injection into grapevine petiole. As is indicated below, all attempts at expressing intact *Xf* EF-Tu on its own directly in *E. coli* also were not successful. However, *E. coli* transformed to express *Xf* EF-Tu as part of a fusion protein was successful and resulted in possibly biologically active material, as indicated below.

In the previous reporting period, we described the effect of expression of a fusion protein with *Xf* EF-Tu as its amino end and the bacteriophage fd adhesin protein P3 as its carboxyl end. During bacteriophage fd infections or when the P3 gene is expressed in *E. coli*, P3 is targeted to the outside of *E. coli* cells, so we expect the fusion protein to be similarly localized. The fusion protein construction was placed under the control of the *lac* promoter and has the usual P3 signal peptide for extracellular targeting replaced by another signal peptide, dsbA, that should enhance folding for the *Xf* EF-Tu-P3 fusion protein (Steiner et al. 2006).

Work by the laboratory of Prof. Douglas R. Cook resulted in the discovery of a few genes whose expression is associated with *Xf* infection but not with, for example, abiotic stress. The laboratory of Prof. David Gilchrist prepared transgenic Thompson's Seedless grapevine lines bearing a green fluorescent protein (GFP)-encoding sequence under the control of two of the *Xf*-infection specific promoters, one of them being designated here as 9353 (Cook et al., 2005, da Silva et al., 2005, Gilchrist et al., 2007, 2008). The specificity of the 9353 promoter to *Xf* infection was demonstrated by observations of GFP expression after inoculation of *Xf* but not after inoculation of the xylem-invading bacterium *Xanthomonas campestris* (Gilchrist et al., 2008). Last year we reported that a set of four *E. coli* cell suspensions were petiole-injected into 9353:GFP grapevine lines. One suspension was of *E. coli* cells bearing the gene for the *Xf*-EF-Tu-P3 fusion protein and stimulated by exposure to the gratuitous inducer of the *lac* promoter, IPTG. This cell suspension induced accumulation of GFP, whereas the same *E. coli* strain not exposed to IPTG and a control *E. coli* strain bearing a P3 construction (no EF-Tu), and exposed or not to IPTG, failed to mediate GFP accumulation (Bruening et al., 2008). These results suggest that *Xf* EF-Tu alone may be sufficient to trigger transcription under control of the *Xf*-infection-specific 9353 promoter, a conclusion consistent with *Xf* EF-Tu having MAMP activity in grapevine. It is possible that the *Xf* EF-Tu protein represents a signal that is released in some way by the *Xf* bacteria and then accumulates in the leaf margins, leading to PD symptoms at a distance from known sites of bacterial accumulation (Gambetta et al., 2007).

As described previously, changes were noted in *E. coli* cells expressing the *Xf* EF-Tu-P3 fusion protein, compared to control cells. Growth was greatly slowed and cells became elongated and more fragile. An alternative hypothesis, to the hypothesis that the *Xf* EF-Tu-P3 fusion protein acts as a MAMP, is that the *Xf* EF-Tu-P3-expressing cells ruptured after injection into the petiole, thereby releasing *E. coli* EF-Tu. That is, the stimulator of 9353 promoter-controlled GFP expression could be *E. coli* EF-Tu rather than the *Xf* EF-Tu-P3 fusion protein, serving as a surrogate for *Xf* EF-Tu. It also is possible that both the *E. coli* and the *Xf* EF-Tu proteins are MAMPs in grapevine.

To test *E. coli* EF-Tu for MAMP activity in grapevine, we injected *E. coli* EF-Tu protein, purified by covalent chromatography (Caldas et al., 1998), into the petioles of 9353:GFP grapevine and examined them for GFP expression after various times post injection. As **Figure 1** reveals, GFP accumulation in pith cells, which is the characteristic response of the 9353:GFP grapevine petioles to infection by *Xf* cells, was observed in petioles that had been injected days earlier with *E. coli* EF-Tu (right hand three panels).

Controls not injected (leftmost panels) or injected with bovine serum albumin (BSA, middle panels) did not show fluorescent pith cells. This result shows that expression from the 9353 promoter of grapevine is stimulated by the presence of purified *E. coli* EF-Tu. The EF-Tu amino acid sequences of *Xf* and *E. coli* are 77% identical and 88% similar. Therefore, it is entirely

possible that *Xf* EF-Tu, could it be obtained in pure and injectable form, also would induce GFP accumulation in 9353:GFP grapevine.

Full length EF-Tu protein expression extracellularly appears to be toxic to expressing E. coli cells

As mentioned above, expression of *Xf* EF-Tu extracellularly from *E. coli* cells seems to have a toxic effect on the cells. Our initial construction produced the *Xf* EF-Tu protein either as untethered *Xf* EF-Tu with a small addition of amino acids at the C-terminus, which should accumulate in the extracellular space, or as EF-Tu fused to the N-terminus of the bacteriophage fd p3 protein, which should anchor the entire fusion protein to the outside of the *E. coli* . These two forms of the *Xf* EF-Tu are produced from the same gene construction by the action of a suppressible stop codon that separates the *Xf* EF-Tu from the bacteriophage fd P3. Previous experiments showed a marked decrease in growth of *E. coli* expressing this initial construct containing the suppressible stop codon
within two hours of induction.

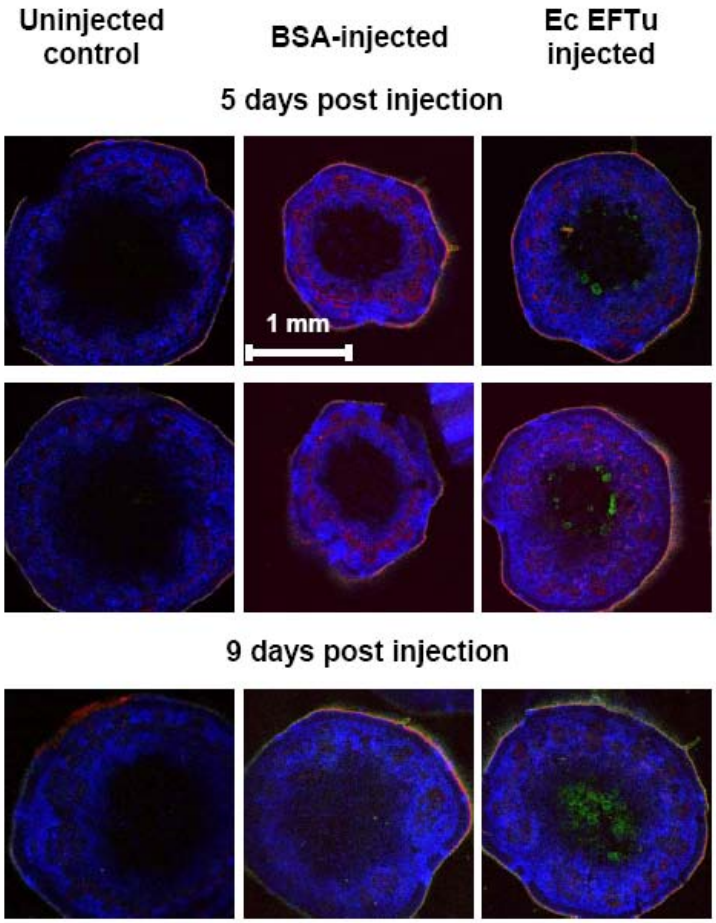

Figure 1. Either chromatographically purified *E. coli* EF-Tu or bovine serum albumin (BSA) (each at about ~0.9 µM in water) was injected into petioles of transgenic grapevine containing a green fluorescent protein (GFP) open reading frame under control of the grapevine *Xf*-infection-specific 9353 promoter. Each petiole was injected twice, ~0.5 cm apart and near the stem. At five and nine days after injection, leaves were removed and cross-sections were prepared at a site ~1 cm towards the lamina from the injection site. *E. coli* EF-Tu (Ec EFTu) was far more effective than BSA at inducing GFP accumulation We have attempted in numerous experiments to express intact *Xf* EF-Tu in a pure extracellular form from *E. coli* . No clones have been obtained, suggesting that extracellular EF-Tu may be very sickening to *E. coli* . The fusion protein construction was modified by insertion of a frameshift downstream of the *Xf* EF-Tu coding region. This should cause the *Xf* EF-Tu protein produced and exported to be free of the cell for easy purification. We have attempted to produce this construct several times, being careful to ensure that all of the steps are working appropriately, but have been unsuccessful each time. We have recovered only the starting material or constructions that have been substantially deleted in the *Xf* EF-Tu region. Given that even the fusion protein described above has substantial adverse effects, perhaps it is not surprising that we have not been able to generate *Xf* EF-Tu as an export from *E. coli* .

Our interest in the idea that *Xf* EF-Tu has a detrimental effect on the cells relates to results from the Gilchrist laboratory that suggests that high density cultures of *Xf* bacteria contain substantial numbers of non-viable cells. To investigate the

possibility that there is a role for the free form of the *Xf* EF-Tu in the deaths of the *E. coli* cells that were expressing it, we made constructs that deleted portions of the p3 region including the suppressible stop but maintained the reading frame so the C-terminal anchor sequence would hold the *Xf* EF-Tu to the cells themselves. If the free form of *Xf* EF-Tu is required to generate this phenotype, removing the suppressible stop should relieve this phenotype. An experiment with *E. coli* bearing this deleted construct without the suppressible stop codon showed a decrease in growth with similar kinetics compared to that seen with induced cells containing the initial construction suggesting that this aspect of the phenotype does not depend on the presence of a free form of the *Xf* EF-Tu. However, we cannot be sure that the presence of the free form of *Xf* EF-Tu would not generate a more extreme phenotype. We are currently extending this experiment by adding back the deleted region without the suppressible stop codon to more closely match the original construct.

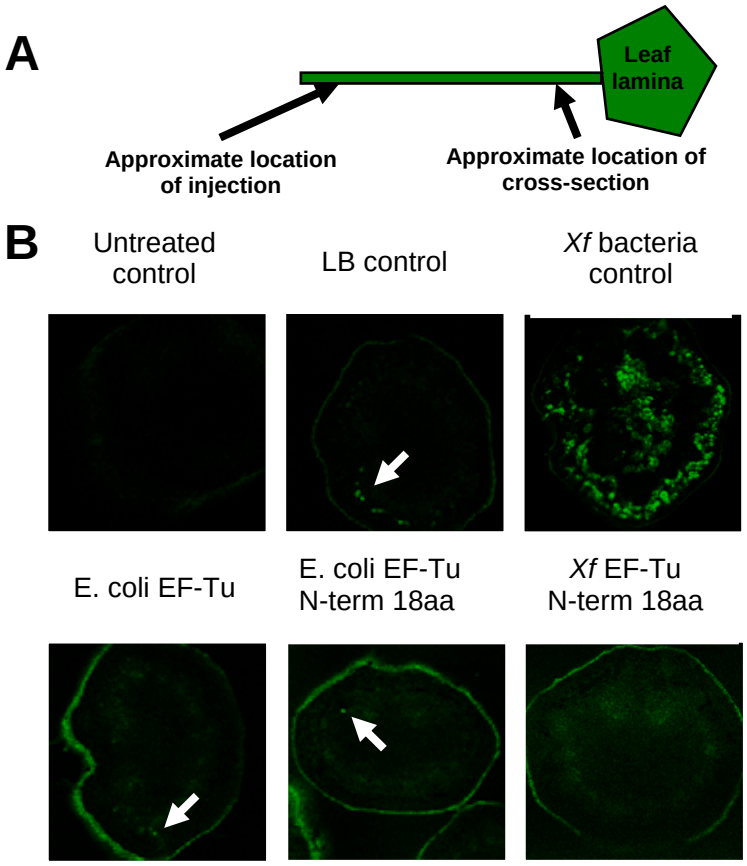

Figure 2. Full length *E. coli* EF-Tu protein solution was prepared at 0.9 µM and injected as in **Figure 1**. Peptides corresponding to the amino-end 18 amino acid residues of *Xf*-EF-Tu and *E. coli* EF-Tu, both amino-end acetylated, were prepared at 200 µM in water. **A**. Injection sites and cross-section sites in the petiole are shown relative to the leaf lamina. The petiole injections were as described in figure 1. **B**. At 17 days post-injection, leaves with petioles were collected and cross-sections both near the injection sites and near the leaf lamina were examined. No GFP expression was seen near the injection site. The three upper panels show results for an uninjected control, a control petiole exposed to bacteria-free LB microbiological media [known to activate GFP expression from the 9353 promoter (Lincoln, unpublished observation)], and a control petiole exposed to *Xf* bacteria (image provided by Lincoln from previous experiments). The lower three panels show three experimental injections: full length *E. coli* EF-Tu protein and amino-end peptides of *E. coli* and *Xf* EF-Tu proteins, respectively. Images were captured and provided by James Lincoln, Gilchrist laboratory

Grape 9353 promoter stimulation by E. coli and a Xf EF-Tu-amino-end-sequence-derived peptide

We obtained a commercially synthesized peptide having a sequence corresponding to the 18 amino acid sequence of the amino end of *E. coli* EF-Tu, followed by tyrosine and cysteine residues for convenient labeling or immobilization. A second peptide of similar design corresponds to the *Xf* EF-Tu amino end. The two 20-mer peptides are amino-end acetylated, as is *E. coli* EF-Tu. Kunze et al. (2004) demonstrated that an *E. coli* EF-Tu peptide of this design was recognized by, and induced defense responses in, Arabidopsis.

Neither of our synthetic peptides induced development of chlorosis when solutions were pressure-infiltrated in to leaves of *C. quinoa*, suggesting that recognition of EF-Tu in *C. quinoa* operates under a different mechanism from EF-Tu recognition by

brassica species. To test for the possible activation of the 9353:GFP gene of grapevine by the peptides, peptide solutions were petiole-injected.

Even though the GFP expression seen after injection of either full length *E. coli* EF-Tu protein or an amino-end 18 amino acid EF-Tu peptides, the peptide signal was weak compared to that seen for *Xf* bacteria (compare **Figure 2**, lower left and middle with upper right), it is in the same region of grapevine petiole seen when the cross-sections from near the leaf lamina of positive samples are examined. The amino end peptide has been shown to invoke response from Arabidopsis that is dependent on the EFR receptor (Kunze et al., 2004). Hence, grapevine may have an EFR-like receptor that could be responsible for signaling involving the 9353 promoter.

The response to injection of the *Xf* EF-Tu amino-end 18-mer peptide, although also weak, was more generalized spatially (**Figure 2**, lower right panel). It is known that the strength of the response by the Arabidopsis EFR receptor to an amino-end 18-mer peptide from *Xf* EF-Tu is weaker than that seen with the *E. coli* EF-Tu peptide (Kunze et al., 2004). It is also possible that the overall weak responses seen when compared to previous experiments (compare **Figure 1** and **Figure 2**) may be due to the reaction of the plant itself at the time of this assay. It has been suggested that the overall reaction of the plant to the *Xf* bacteria might be reduced during the winter months (Gilchrist and Lincoln, personal communication). To determine which is the case, we will repeat this experiment when the weather improves. (The green margins seen in the lower images of **Figure 2** represent overflow of the red autofluorescence seen by the confocal microscope into the green photomultiplier tube. The white arrows indicate sites of GFP expression which did not correspond to sites with strong red autofluorescence) follow up experiment was performed using the purified intact *E. coli* EF-Tu and a peptide corresponding to the amino-end 18 amino acid sequence of *E. coli* EF-Tu at the same concentrations that were used above. Similar levels and locales for GFP expression were seen using the intact EF-Tu protein and the N-terminal peptide (compare **Figure 3** left and right panels).

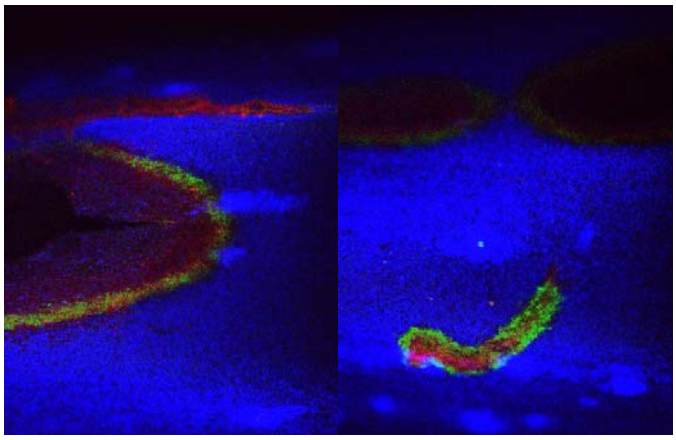

Figure 3. Confocal micrograph of 9353 promoter-GFP transgenic Thompson Seedless grapes infiltrated with intact *E. coli* EF-Tu (left panel) and *E. coli* EF-Tu N-terminal 18 amino acid peptide (right panel) in water. Images are of intact petioles with their long axis aligned left to right. Confocal microscopy kindly performed by Dr. Jim Lincoln.Both samples show a region of GFP expression near the site of infiltration, although the GFP response is higher in the peptide injected petiole. This could be due to the higher molar concentration of the peptide used. To confirm this result and to allow assays without consideration for the visibility of the GFP signal, we are currently checking these results by quantitative reverse transcription PCR using the actin mRNA as a control and both the GFP mRNA and the native 9353 mRNA as a more sensitive assay.

CONCLUSIONS

Xf EF-Tu, when expressed extracellularly on *E. coli* cells, leads to induction of a known *Xf*-responsive promoter as well as conferring a deleterious phenotype on the *E. coli* cells. Injection of either purified *E. coli* EF-Tu or its N-terminal 18 amino acids leads to qualitatively similar responses from this same promoter. The known EF-Tu receptor from Arabidopsis, EFR, recognizes both EF-Tu and the amino end peptide, suggesting that the grapevine response may be due to the presence of a grapevine EFR homologue. This recognition could be involved in disease symptom development and may explain the apparent separation of *Xf* bacterial accumulation site and PD symptoms sites. If so, it may be possible to interfere with this recognition and block Pierce's disease symptoms.

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

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ABSTRACT

Previously we demonstrated that twitching motility (TM) in *Xylella fastidiosa (Xf)* is dependent on an operon encoding signal transduction pathway proteins (*pilG, pilI, pilJ, pilL, chpB* and *chpC*), which is related to the system that controls flagella movement in *Escherichia coli*. We report four advances in examining this system. First, the gene *pilJ*, putatively coding for a methyl-accepting chemotaxis protein (MCP), is shown to be essential for TM as an insertional mutation in this gene resulted in a twitching-minus phenotype on agar. Further characterization of such mutant is underway. Second, we have results on Pierce's disease (PD) reduction with previous mutants in the operon gene *pilL*. Third, we have made advances on identifying the *Xf* chemotaxis attractant using a chimera protein fusing the *Xf* MCP, *pilJ*, to an *E. coli* MCP. Fourth, we have begun exploring the localization of PilJ in relation to the *Xf* pili.

LAYPERSON SUMMARY

This project involves studies targeting the regulation of chemical sensing pathways by which *Xylella fastidiosa* is able to control its movement within the plant environment. Several genes and gene products of this chemical sensing pathway are being examined. We found that one gene in particular is essential for cell movement (twitching motility), and we found differences in grapevine saps that may influence the sensing pathway.

INTRODUCTION

Bacteria sense and respond to changes in their environment, integrating the signals to produce a balanced response. *Xylella fastidiosa* (*Xf)* is a non-flagellated xylem-restricted Gram-negative bacterium that moves within grapevines via TM that employs type I and type IV pili (Meng et al. 2005). Movement appears to be controlled by a chemosensory system similar to that first reported in *E. coli* where a group of *che* genes regulated the rotation movement of flagella. These proteins work by means of a phosphorylation cascade to ultimately control the direction of flagella rotation (Hazelbauer, 2008). We previously described the new operon involved in TM and likely to be responsible for the chemosensory regulation of type IV pili in *Xf* (**Figure 1**). Herein, we further characterize this operon creating a mutation in *pilJ* and analyzing the effect of a *pilL* mutant *in planta* and describing our advances in understanding the role of *pilJ* signaling in *Xf*.

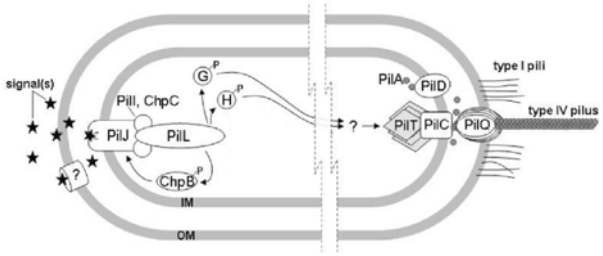

Figure 1. Model for chemosensory regulation of twitching motility in *Xf*. PilJ, the single polar methyl-accepting chemotaxis protein senses environmental signal(s). PilL phosphorylates , PilG, PilH and ChpB. ChpC and PilI couples PilL to PilJ. ChpB might mediate adaptation to a constant chemical concentration 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 PD and, in particular, 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:
   a. Express PilJ or a chimeric form of PilJ in a strain of *E. coli* previously deleted of all methyl-accepting chemotaxis protein genes.
   b. 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 operon. The construction of an allelic exchange mutant for *pilJ* gene in *Xf* was performed according to Chatterjee *et al*. 2008 with slight modifications. The disruption of the *pilJ* locus in marker-exchange mutants was confirmed by PCR (not shown).

Twitching motility. Examination of *pilJ* on PW agar surfaces revealed colony morphologies with smooth margins consistent with loss of type IV pili twitching motility function (**Figure 2**)

Virulence and movement on grapevines. The *pilL* mutants were inoculated into Cabernet sauvigon grapevines. Symptom expression was assessed 12 weeks following inoculation (**Figure 3**). *pilJ* mutant will be assayed in the Spring of 2010.

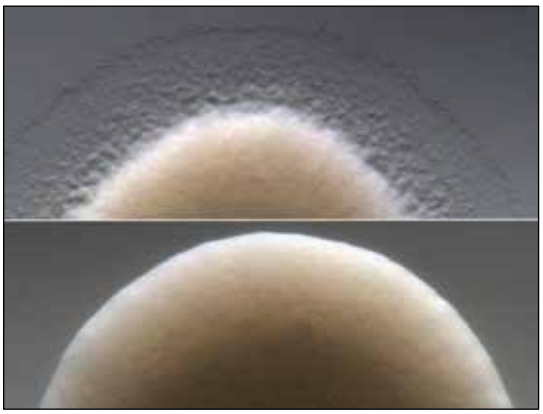

Figure 2. Example of colony morphologies of *pilJ* mutant (lower) and Wild-type Temecula isolate (upper) grown on PW agar for 5 days.

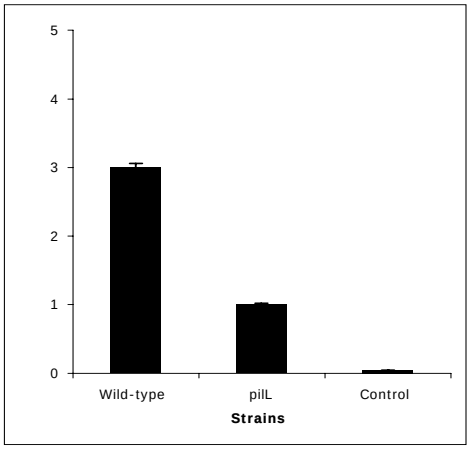

Figure 3. PD expression in greenhouse grown Cabernet sauvignon vines 12 weeks following inoculation.

Complementation of *Xf* chemoreceptor in *E. coli*. Chemoreceptors are transmembrane proteins that bind ligand in the periplasmic domain and associate with a kinase, CheA, in their cytoplasmic portion. Changes in ligand binding result in a phosphorylation cascade: CheA autophorphorylation, CheA phosphorylates a shuttle protein (CheY), CheY modifies flagella proteins, and the flagella alter their rotation and the bacteria moves from random tumbling ("tumble") to smooth swimming ("run") (Hazelbauer, 2008). The putative *Xf* chemoreceptor, *pilJ*, was cloned and expressed in an *E. coli* strain lacking chemoreceptors. To determine if PilJ complemented the *E. coli* system and activated the *E. coli* CheA, we used the ligand-independent pseudotaxis assay (Wolfe, 1989; Ames, 1996). In this assay, cells lacking both chemoreceptors and the normal ligand adaptation system are locked in a smooth swimming response (Bibikov, 2004). In agar at levels to support swimming (0.25%), these cells become trapped in the agar pores, unable to tumble and leave agar dead ends. When *E. coli* chemoreceptors are expressed in these cells, the receptors interact with the CheA kinase and allow the cells to alternate between smooth swimming and tumbling. As a result, the cells can maneuver through the agar alleyways and form a swarm on the plate. PilJ failed to form a swarm, indicating that PilJ does not activate the CheA and complement the *E. coli* chemotaxis system (**Figure 4**).

Construction of a chimeric chemoreceptor. To facilitate PilJ functioning in an *E. coli* system, we constructed chimeric chemoreceptors that contain the periplasmic ligand binding domain of the *Xf* PilJ fused to the cytoplasmic signaling domain of the *E. coli* serine chemoreceptor, Tsr (**Figure 5**). The chimeras should only detect the PilJ ligand as we swapped the periplasmic serine binding site of Tsr with the proposed PilJ ligand binding site. The chimera also should successfully activate the *E. coli* CheA kinase as the CheA binding site is maintained in the Tsr cytoplasmic portion. We constructed two chimeras; one chimera linked PilJ and Tsr in the transmembrane domain and the other, based on published MCP chimeras (Kristich, 2004), linked in the HAMP domain (Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases). The two fusion proteins activate the *E. coli* signaling pathway as measured in the pseudotaxis assay (Wolfe, 1989; Ames, 1996) (**Figure 5**). Western blot analysis, using antibodies to the *E. coli* chemoreceptor portion (Ames, 1994), suggest that the chimeric chemoreceptors are produced at 10-15 times lower levels than wild-type *E. coli* Tsr (data not shown). PilJ contains codons rare to *E. coli*, which presumably affects protein production. We are currently modifying the promoter region to enhance protein production.

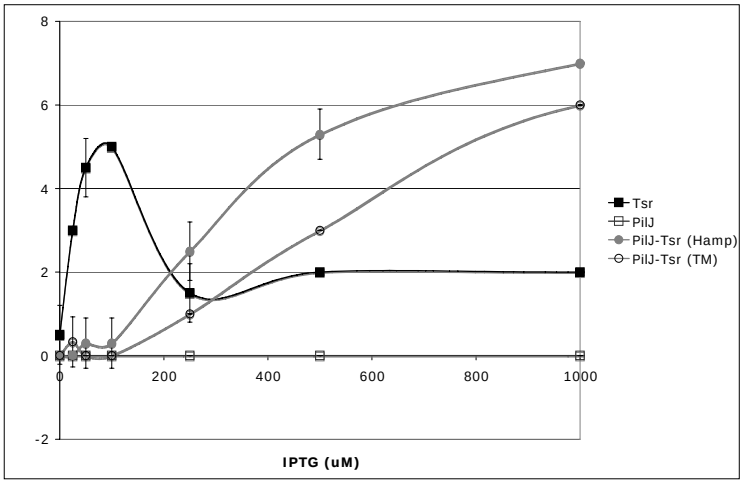

Figure 4. Pseudotaxis of cells expressing PilJ constructs. Constructs were expressed in *E. coli* strain UU1535 (*Δmcp, Δaer, ΔcheR/B*) and patched onto 0.25% tryptone agar plates with 50ug/mL ampicillin and varying concentrations of IPTG inducer. Cells grew for 20 hours at 30°C.

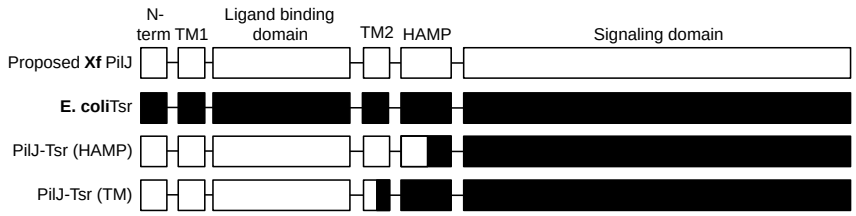

Figure 5. *E. coli* chemoreceptors (including serine receptor, Tsr) contain a periplasmic ligand binding domain and a cytoplasmic HAMP (Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases) domain and signaling domain. *Xf* PilJ is proposed to have similar domains. We constructed chimeras that connect the PilJ to Tsr through the proposed HAMP domain or transmembrane (TM) domain. These chimeras should bind *Xf* PilJ ligand but successfully interact with *E. coli* cytoplasmic proteins involved in chemoreceptor signaling.

Identifying the attractant. In order to confirm that PilJ binds to a molecule in grape sap, we tested the chimeras using a standard swarm plate assay (Adler, 1966). In this qualitative assay, cells are patched onto soft agar plates with a uniform amount of potential attractant. Cells expressing the ligand adaptation system (as oppose to the pseudotaxis assay) alternate between running and tumbling (Eisenbach, 2007). When chemoreceptors bind attractant the cells run smoothly until Brownian motions force them to tumble and reorient. As the cells "sense" ligand via the chemoreceptors, consume the attractant, and migrate through the plate they create a visible smooth ring at the interface between metabolized ligand and available attractant. Behind the ring, the attractant is depleted because the cells have consumed it; in front of the ring the attractant is abundant. Cells lacking chemoreceptors or cells patched onto plates without a chemoreceptor ligand are "blind"; do not experience extended runs, do not progress as far from the original patch, and fail to create a smooth ring even though the media may contain desired nutrients.

When we tested the PilJ-Tsr chimeras in the swarm plate assay, they produced a noticeable ring on soft agar plates containing *Vitis riparia* sap harvested in New York (**Figure 6**). The *E. coli* serine chemoreceptor, Tsr, also formed a ring indicating that *V. riparia* contains detectable levels of serine. Tsr supported a larger swarm ring than the chimeras, which may reflect the different levels of a) PilJ-Tsr chimera attractant compared to serine and/or b) PilJ-Tsr chimera and Tsr proteins as discussed above. As expected, PilJ did not support a swarm as the earlier tests showed that it failed to interact with the *E. coli* kinase CheA. Based on these results, we are currently testing various known components of sap (Anderson, 2007) to identify the PilJ ligand/s. As chemoreceptors can have multiple ligands (Kondoh, 1979) and attractants may not be metabolized (Topp, 2007), we are also testing the PilJ chimeras in established chemotaxis assays that do not require consumption of the ligand (Adler, 1973; Grimm, 1997; Yu, 1997).

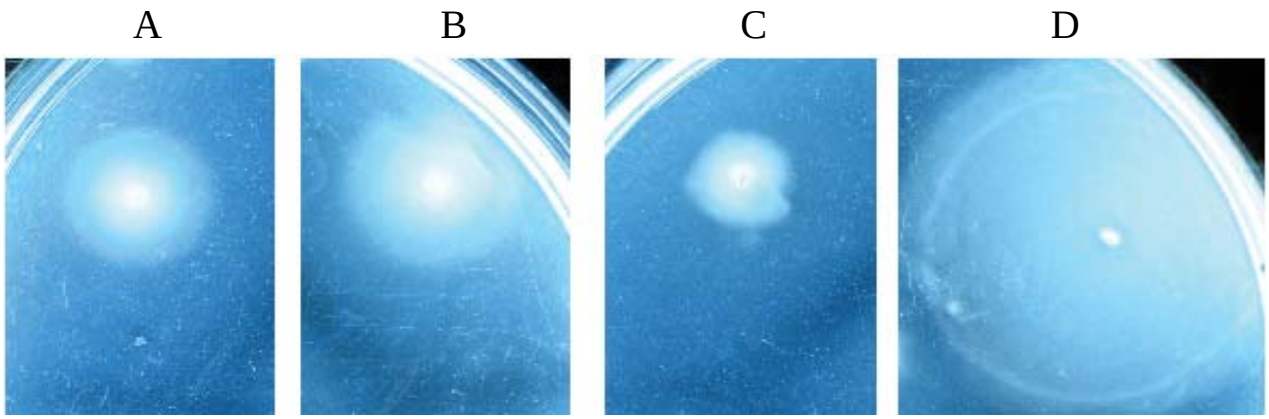

Figure 6. PilJ induces chemotaxis response to *V. riparia* sap. Constructs were expressed in *E. coli* strain, UU1250 (*Δmcp*, *Δaer*), patched onto swarm plates containing 80% sap, and grown for 18 hours at 30°C. Constructs were A) chimera PilJ-Tsr (TM), B) chimera PilJ-Tsr (HAMP), C) *Xf* PilJ, and D) *E. coli* serine chemoreceptor Tsr.

Localization of the chemoreceptor. Chemoreceptor localization has been studied in only a handful of organisms and found to be polar or cytoplasmic (Maddock, 1993; Harrison, 1999; Bardy, 2005; DeLange, 2007). In *E. coli*, chemoreceptors cluster into a polar lattice that presumably allows the receptors to work in concert and amplify the signal (Parkinson, 2005). As a result, the chemoreceptors are physically at a distance from the flagella. Likewise in *Pseudomonas aeruginosa* the chemoreceptor PilJ is polar localized (DeLange, 2007). We wish to learn if the *Xf* PilJ is a) polar localized, b) at one or both poles, and c) if it co-localizes with the pili. To answer these questions, we are taking two approaches. First, we are expressing PilJ protein for antibody production, which can then be used to label the chemoreceptor as previously reported (Maddock, 1993; Harrison, 1999; Bardy, 2005; Lamanna, 2005). Additionally, similar to earlier chemotaxis studies (Wadhams, 2002; Homma, 2004; DeLange, 2007) we will visualize the physical location of PilJ using a GFP-tagged PilJ protein. From these studies, we hope that determining the physical organization of the chemoreceptor system will help elucidate the nature of the *Xf* chemosensory signaling system.

CONCLUSIONS

Our results with a construction of a mutation in *pilJ*, and the previously reported *pilL*, show that these genes are required for twitching motility in *Xf*. They also play a role in virulence as vines inoculated with *pilL* showed less disease. Currently we are studying other phenotypes of the *pilJ* mutant including growth and biofilm formation. We are attempting to find the chemoattractant in grape sap and the localization of PilJ in *Xf*. We are also creating three new mutants *(pilG, pilI* and *chpC*) to further characterize the chemosensory operon.

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

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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. Members of this protein family have been identified as rational targets for the design of novel vaccines directed against Gram-negative pathogens (Wells et al., 2007). Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xylella fastidiosa* Temecula 1 (*Xf*-PD). During the period under review, we have completed our construction of strains containing single or multiple mutations in these genes and are examining how the absence of one or more autotransporters affect *Xf* cell physiology and virulence. We have shown that two of the autotransporters (PD0528 and PD1379) are present on the bacterial cell surface and are involved in autoaggregation and biofilm formation. Studies in grapevines indicate that the absence of either PD0528 or PD1379 reduces the virulence of *Xf* in grapevines. We have also initiated experiments to characterize PD0218, PD0313, and PD0950, the three autotransporters predicted to have proteolytic activity. Strains carrying a mutation in either PD0218 or PD0950 exhibit a hypervirulent phenotype in grapevines. In contrast, grapevines infected with a strain missing all three proteases exhibit symptoms much later than wildtype. Comparisons of the secreted proteins from the three single mutants suggest that each protease has a different set of target proteins. Experiments are currently underway to identify the protein targets of the individual proteases and the virulence factors that require these proteases for their maturation. The ultimate goal is to develop methods for interfering with this maturation, thereby reducing the virulence of this important plant pathogen.

LAYPERSON SUMMARY
Autotransporters are virulence proteins that are found on the surface of many bacterial pathogens. These proteins have been identified as rational targets for the design of novel vaccines and control strategies. The goal of this project is to characterize the six autotransporters of *Xylella fastidiosa (Xf).* Four of these proteins have enzymatic activity. It seems likely that strategies developed to disrupt the function of these proteins in other systems will also be effective against the *Xf* proteins. The remaining two proteins are unique to *Xf* and appear to be involved in the attachment of the bacterium to solid surfaces. Treatments designed to prevent this attachment could have a profound impact on the ability of *Xf* to cause Pierce's Disease.

INTRODUCTION
The causative agent of Pierce's disease (PD) is the Gram-negative bacterium, *Xylella fastidiosa (Xf)* [for a recent review, see (Chatterjee et al., 2008)]. An important feature of the *Xf* infection cycle is the ability of this pathogen to colonize and interact with the xylem tissue of susceptible plants and with the foregut of insect vectors. Successful establishment of the pathogen is dependent on the ability of *Xf* to acquire essential nutrients, to adhere to the host cell surfaces, and to escape any host defense mechanisms. Comparison of the *Xf*-PD genome to other bacterial pathogens has resulted in the identification and characterization of a number of genes that are potential virulence factors. Many of these virulence determinants are proteins that are either secreted to the bacterial cell surface or released into the external environment (Dautin and Bernstein, 2007; Henderson et al., 2004). Our work has focused one category of virulence determinants, the AT-1 autotransporters.

AT-1 autotransporters are dedicated to the secretion of a single specific polypeptide, the passenger domain, across the outer membrane. Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xf*-PD. Functional sequence predictions 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). The goal of this project is to establish the role of these secreted proteins in *Xf* cell physiology and pathogenicity. 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 a role in *Xf* virulence. Thus, characterizing of these proteins and identifying methods for disrupting their function should allow the development of strategies that impact the ability of *Xf* to colonize plant tissue and to initiate the PD disease cycle in susceptible grapevines.

OBJECTIVES
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.

RESULTS AND DISCUSSION

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

Two of the most interesting *Xf*-PD autotransporters are PD0528 and PD1379. The passenger domains of these proteins encode tandem repeats of a 50-60 amino acid motif that is only found in *Xf* species. PD0528 has six repeats, whereas PD1379 has three repeats. In addition, both passenger domains contain WD40 repeats. WD40 repeats are predicted to create a specific structure, a β propeller-like platform (Hudson and Cooley, 2008). In other systems, binding partners associate with this platform either stably or reversibly. Usually, the binding partner is a protein that recognizes a specific consensus binding motif within the β-propeller. Therefore, the presence of WD40 repeats makes it highly likely that the passenger domains of PD0528 and PD1379 are each interacting with a specific protein or set of proteins.

We have created a number of strains and tools that have facilitated our analysis these proteins. Specifically, we have generated strains containing null mutations in either PD0528 or PD1379 and a double mutant that eliminates both proteins. The resulting mutants exhibit decreased autoaggregation and biofilm formation *in vitro*. Interestingly, grapevines infected with either the double mutant or the PD0528::Cm mutant do not exhibit PD symptoms. The major difference is that the double mutant can only be recovered close to the site of inoculation, whereas the PD0528::Cm mutant can be recovered 12 cm above the inoculation site. In contrast, grapevines infected with the PD1379::Gm mutants still exhibit some PD symptoms. However, the symptoms are not as severe as those observed with the wildtype Temecula1 control. To confirm these phenotypes, we are in the process of constructing a series of strains for complementation analysis. We have also generated antibodies against the passenger domains of PD0528 and PD1379 and have used these antibodies in localization studies. Based on immunofluorescence microscopy, the passenger domains of both proteins are exposed on the *Xf* cell surface. Furthermore, fractionation studies indicate that PD0528 is an extremely abundant protein in the *Xf* outer membrane and can also be found in membrane vesicles and in the supernatant. In contrast, PD1379 is present at much lower levels and is only found in the outer membrane. The difference in the localization patterns of PD0528 and PD1379 suggest that these autotransporters may be involved in different aspects of the PD infection cycle.

We have also expressed both PD0528 and PD1379 on the surface of the *E. coli* strain UT5600. UT5600, which is deficient in the outer membrane proteases OmpT and OmpP, is commonly used for autodisplay (also known as live-cell surface display) (Jose and Meyer, 2007). Although the expression levels were low, both proteins were localized to the *E. coli* outer membrane and conferred the ability to autoaggregate and to form a biofilm. The ability of PD0528 and PD1379 to confer new phenotypic properties to *E. coli* indicates that these proteins are directly responsible for the observed traits. Moreover, the fact that these proteins are functional and present in the *E. coli* outer membrane has allowed us to perform preliminary tests concerning how these autotransporters are secreted. Genetic analysis in *E. coli* has established that the secretion and correct folding of most outer membrane proteins occurs through the BAM (β-barrel assembly machine) complex, which is composed of five proteins BamA-BamE (Knowles et al., 2009). There are orthologs to four of these proteins in *Xf*: PD0326 (BamA), PD1620 (BamB), PD1756 (BamD), and PD1375 (BamE). Our studies using the *E. coli* system suggest that the translocation and correct insertion of autotransporters into the *Xf* outer membrane is mediated by the *Xf* BAM complex. As shown in **Figure 1**, a mutation that disrupts the BAM complex (*bamB*::Km^R) interferes with the autoaggregation phenotype conferred to *E. coli* by the PD0528 protein (--■--vs --□--). A simple explanation for this result is that the PD0528 requires BamB for its localization to the *E. coli* outer membrane. It also implies that the mechanism for outer membrane protein localization is conserved between *E. coli* and *Xf*.

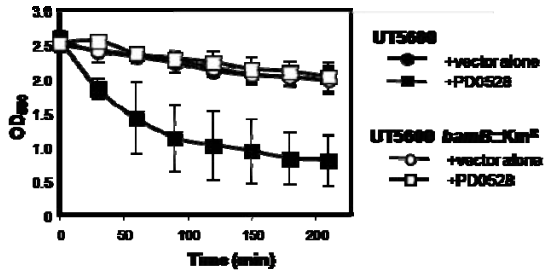

Figure 1: The autoaggregation phenotype conferred by PD0528 requires a function BAM complex.
At the beginning of the experiment, the *E. coli* cultures were adjusted to approximately the same optical density (OD_{550}) and vigorously shaken for 10 s. Samples were then taken ~0.5 cm from the top of the culture tube at the indicated times and the OD_{550} was measured.

The serine protease AT-1 autotransporters:

PD0218, PD0313, and PD0950 are members of the phylogenetic clade containing the S8 subtilisin-like serine protease autotransporters (Tripathi and Sowdhamini, 2008). Members of this family have been implicated in defense, growth on proteinaceous compounds, and the proteolytic maturation of virulence factors. Although many serine proteases have broad

specificities, some are very specialized. One of the best studied members of this clade is the SphB1 autotransporter protein of *Bordetella pertussis* (Coutte et al., 2001). SphB1 serves as a specialized maturation protease, responsible for the timely maturation and extracellular release of the filamentous haemagglutinin FHA. One of the goals of this project is to determine the specificity and targets of PD0218, PD0313 and PD0950.

As a first step in this analysis, we have generated strains containing mutations in one, two, or all three of the AT-1 serine proteases.
These strains and some of their properties are listed in the following table (**Table 1**):

Table 1: The properties of the AT-1 serine proteases mutants.

Strain	AT-1 Mutation(s)	Biofilm formation in vitro	Week PD symptoms first appear*
Temecula	Wildtype	0.688 ±0.12	11
TAM147	PD0218::Cm^R	0.536 ±0.08	8
TAM152	PD0313::Gm^R	0.248 ±0.02	10
TAM146	PD0950::Em^R	0.469 ±0.02	8
TAM148	PD0218::Cm^R, PD0950::Em^R	0.531 ±0.07	8
TAM150	PD0218::Cm^R, PD0313::Gm^R	0.479 ±0.09	8
TAM151	PD0313::Gm^R, PD0950::Em^R	0.580 ±0.07	10
TAM153	PD0218::Cm^R, PD0313::Gm^R, PD0950::Em^R	0.633 ±0.11	14

* Three plants were inoculated for each mutant on 6/22/09. Disease severity was assessed weekly using the visual scale (0 to 5) described by Guilhabert and Kirkpatrick (2005). On this scale, healthy plants receive a score of 0. The table lists the week when the infected grapevines first receive a score of 1 (only one or two leaves with scorching symptoms starting on the margins of the leaves).

Our characterization of the single mutants suggests that the three serine proteases are involved in different aspects of *Xf* cell physiology and pathogenicity. The mutations in PD0218 and PD0950 result in reduced clumping in liquid and a slight decrease in biofilm formation. In contrast, the mutation in PD0313 eliminates clumping in liquid and has a more severe impact on biofilm formation. The PD0313 mutant also forms a confluent lawn on solid medium. When introduced into grapevines, all three mutants produced symptoms earlier than Temecula1. The phenotype observed for our PD0218 mutant is similar to the phenotype reported by Guilhabert and Kirkpatrick (2005) for a strain carrying a Tn5 insertion in this locus. In contrast, grapevines inoculated with the triple mutant TAM153 exhibited symptoms much later than Temecula1. We plan to continue monitoring disease progression every two weeks for a total of 32 weeks after inoculation. Then, experiments will be conducted to determine the bacterial population at various points above the inoculation site.

We have also conducted preliminary experiments testing the feasibility of two approaches to determine the targets of the proteases. One approach is to compare the protein composition of the outer membrane, the membrane vesicles, and the secretome of the single mutants to wildtype on SDS-PAGE gels stained with Syphro Ruby. An example of this type of analysis is shown in **Figure 2**. Based on MALD-TOF-MS analysis of the proteins in indicated band, the PD0218 secretome is missing bacteriocin, which is encoded by PD1427. Bacteriocins are known to contribute to the competitiveness of the producing organisms and have been identified as potential targets for alternative approaches for plant disease control (Holtsmark et al., 2008). The PD1427 bacteriocin resembles the *Rhizobium leguminosarum* RTX (repeats in toxin) protein, a bacteriocin that is similar to hemolysin and leukotoxin (Machado et al., 2001). Subtilisin-like serine proteases are known to function as the maturation enzyme for the bacteriocin-like lantibiotics produced by some Gram-positive bacteria (Tripathi and Sowdhamini, 2008). Therefore, although more experiments are needed, the simplest explanation for our results is that PD0218 is required for the maturation of PD1427.

The first approach works well for abundant proteins, but is of limited usefulness for less highly expressed proteins. Our second approach will be to compare the protein composition of the outer membrane, the membrane vesicles, and the secretome of the single mutants to wildtype by Western analysis. The rate-limiting step for this approach will be the availability of suitable antibodies. In our initial experiments, we used the Anti-PD0528 and Anti-PD1379 antibodies. Although we did not detect any differences using Anti-PD0528 antibody, we obtained an extremely interesting result using the Anti-PD1379 antibody. As shown in **Figure 3**, we are unable to detect PD1379 in the outer membrane of the PD0313 mutant using Anti-PD1379 antibody. It is not clear why the absence of the PD0313 protease is impacting PD1379 localization to the outer membrane. Although we are considering a number of models, further experiments are needed to establish whether or not this is a direct or an indirect effect.

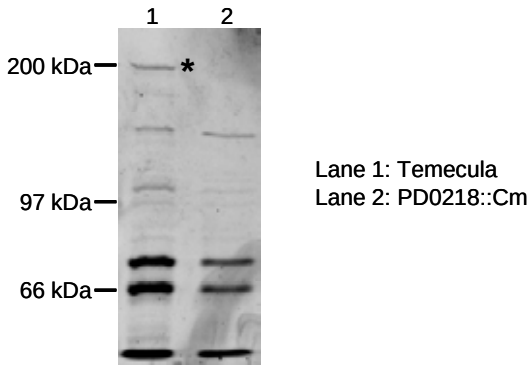

Figure 2: Proteins secreted by the PD0218 mutant. The secreted proteins from PD3-grown wildtype (lane 1) and PD0218 mutant (Lane 2) were concentrated using an Amicon centricon filter. The proteins were separated on a 6% SDS-PAGE gel and stained with Syphro Ruby. The band indicated by the star was excised and then analyzed by MALDI-TOF-MS at the UC Davis Molecular Structure Facility.

Figure 3: Western analysis of the outer membrane proteins using Anti-PD1379 antibody. The outer membrane proteins from wildtype and the protease mutants were separated on a 8% SDS-PAGE gel and then visualized by Western

Finally, we plan to examine how the protease mutants affect the maturation of hemagglutinin using antibodies prepared by Dr. Bruce Kirkpatrick (UC Davis). Based on analogy to SphB1 and its role in the maturation of filamentous hemagglutinin in *B. pertussis*, it is tempting to speculate that one of the three serine protease autotransporters will serve as a specialized maturation protease for *Xf-PD* hemagglutinin.

CONCLUSION

Autotransporters have been identified as rational targets for the design of novel vaccines and control strategies. The goal of this project is to characterize the six autotransporters of *Xf*. During the period under review, we have completed our construction of strains containing single or multiple mutations in these genes and are examining how the absence of one or more autotransporters affect *Xf* cell physiology and virulence. We have shown that two of the autotransporters (PD0528 and PD1379) are involved in autoaggregation and biofilm formation. Studies in grapevines indicate that the absence of either PD0528 or PD1379 reduces the virulence of *Xf* in grapevines. We have also initiated experiments to characterize the three autotransporters predicted to have proteolytic activity. Strains carrying a mutation in either PD0218 or PD0950 exhibit a hypervirulent phenotype in grapevines, whereas strain lacking all three proteases is less virulent than wildtype. Comparisons of the secreted proteins from the three single mutants suggest that each protease has a different set of target proteins. Experiments are currently underway to identify the protein targets of the individual proteases and the virulence factors that require these proteases for their maturation. The ultimate goal is to develop methods for interfering with this maturation, thereby reducing the virulence of this important plant pathogen.

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XYLELLA FASTIDIOSA EXTRACELLULAR GENOMIC DNA ENHANCES BIOFILM FORMATION *IN VITRO*

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Reporting Period: The results reported here are from work conducted October 2008 to September 2009.

ABSTRACT
Xylella fastidiosa (*Xf*) produces extracellular DNA in PD3 liquid medium. This extracellular DNA may play a role in enhancing biofilm formation, a factor that is required by *Xf* to establish infection in host plants. Amounts of extracellular DNA generated by *Xf in vitro* were positively correlated with planktonic cell growth and biofilm formation, but were negatively correlated with cell viability. DNase I treatment of actively growing *Xf* cultures in PD3 medium resulted in decrease or inhibition of biofilm formation. In contrast, addition of *Xf* genomic DNA to *Xf* cultures promoted biofilm formation. These results support the hypothesis that biogenesis of extracellular DNA may play a role in *Xf* biofilm formation leading to successful host plant infection.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) generates extracellular DNA in PD3 culture medium. This extracellular DNA may enhance biofilm formation, the process of which the matrix of extracellular polymeric substance is formed, which facilitates establishment of *Xf* infection in plants. The planktonic *Xf* cell growth and its biofilm formation *in vitro* culture were positively correlated with extracellular DNA produced by *Xf*, but negatively correlated with *Xf* cell viability. Biofilm formation was decreased or inhibited when growing cells were treated with DNase I, an enzyme which degrades DNA. In contrast, addition of *Xf* genomic DNA to cultures promoted biofilm formation. These results suggest that biogenesis of extracellular DNA may play a role in *Xf* biofilm formation and, therefore, contribute to development of Pierce's disease.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a Gram negative, xylem-limited bacterium that causes Pierce's disease of grapevine, as well as other diseases of economically important crops and landscape plants (Hopkins, 1989). *Xf* is transmitted by xylem-feeding insects, including the polyphagous and invasive glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis* (Germar)) (Almeida and Purcell, 2003). The mechanism of *Xf* pathogenicity in host plants is not fully understood. It has been reported that a functional relationship exists among *Xf* planktonic growth, aggregation, biofilm formation and pathogenesis in *Vitis* species (Leite *et al*., 2004, Andersen *et al*., 2007). Previously, we reported that differences in xylem sap composition and cell wall properties among PD- resistant and -susceptible grapes may play a role in affecting PD development (Cheng *et al*., 2009). Bacterial biofilms are structured communities of cells enclosed in self-produced hydrated polymatrixes that adhere to inert or living surfaces (Costerton *et al*., 1999). The matrix, which holds bacterial biofilms together, is a complex mixture of macromolecules including exopolysaccharides, proteins and nucleic acids (Sutherland, 2001). A diffusible signal molecule is reportedly required for biofilm formation by *Xf* in the vector(s) and for vector transmission of *Xf* to plants (Newman *et al*., 2004). In addition, cell density-dependent exopolysaccharide synthesis (EPS) is required for virulent biofilm formation *in planta* (Koutsoudis *et al*., 2006). The objective of this study was to determine if extracellular genomic DNA is involved in the *Xf* biofilm formation *in vitro*.

OBJECTIVE
Determine if extracellular genomic DNA is involved in the *Xf* biofilm formation *in vitro*.

RESULTS AND DISCUSSION
Bacteria produce substantial quantities of extracellular DNA through a mechanism that is thought to be independent of cellular lysis and that appears to involve the release of small vesicles from the outer membrane (Muto and Goto 1986, Kadurugamuwa and Beveridge 1995). We assayed cell-free PD3 liquid culture medium following growth of *Xf in vitro* for extracellular DNA. As shown in **Figure 1A**, extracellular genomic DNA was present in cell-free PD3 liquid medium of *Xf* cultures with fragment sizes ranging from less than 100 bp to 10 kb approximately. The most abundant *Xf* extracellular DNA size was ~100 bp detected by agarose gels and was readily distinguished from the intact *Xf* genomic DNA.

We investigated the effect(s) of extracellular genomic DNA produced by *Xf* in PD3 liquid culture medium on *Xf* planktonic growth, biofilm formation and cell viability *in vitro*. As shown in **Figure 1B**, the production of extracellular DNA in the *Xf* culture medium at different time periods of growth was positively correlated with *Xf* planktonic growth and biofilm formation, but was negatively correlated with the *Xf* cell viability (R^2 = - 0.9947). *Xf* cell viability decreased with the increase of Xf planktonic growth and biofilm formation (R^2 = - 0.9967 and R^2 = - 0.9997, respectively). In contrast, *Xf* planktonic growth and biofilm formation increased during growth. To confirm that there was no contamination, 96 cloned DNAs were randomly picked for sequencing. BLAST reports showed that all DNA sequences matched *Xf* genome sequences in the NCBI *Xf* database, indicating that there was no contamination in the *Xf* culture by other bacteria. In addition, no specific DNA sequence may be required for the enhancement of *Xf* biofilm formation *in vitro*. This result is consistent with a previous study in which no specific DNA sequences were found in the population of extracellular DNAs (Allesen-Holm *et al*., 2006). The occurrence of extracellular DNA in the *Xf* growing medium may result from *Xf* cell death during growth *in vitro* and biofilm formation. However, it is not clear if there are other mechanisms also involving secretion of extracellular DNA by *Xf*. Whitchurch *et al* (2002) reported that extracellular DNA derived from membrane vesicles promotes biofilm formation by *Pseudomonas aeruginosa*. Characterization of DNA release in *P. aeruginosa* cultures and biofilms provided evidence that extracellular DNA was generated via lysis of a subpopulation of the bacteria (Allesen-Holm *et al*., 2006). The results of a more recent study also suggested that extracellular DNA was generated in *Staphylococcus epidermidis* cultures through autolysin AtlE-mediated lysis of a subpopulation of the bacteria (Qin *et al*., 2007).

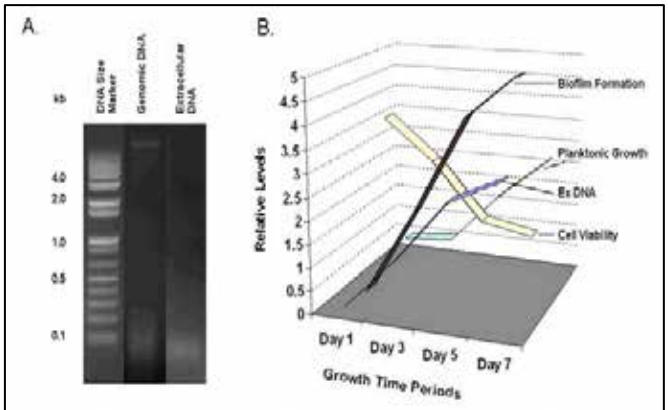

Fig. 1. A. Comparison between *Xf* (strain Temecula-1) genomic DNA and extracellular DNA extracted from PD3 liquid culture medium; B. Relationships of *Xf* growth, biofilm formation, cell viability and extracellular DNA biogenesis in PD3 liquid medium *in vitro*.

In this study, while there was no direct evidence to determine whether the extractcellular DNA in liquid PD3 *Xf* culture was derived mainly from membrane vesicles rather than by cell lysis, *Xf* genomic DNA added into culture medium to mimic extracellular *Xf* DNA resulted in enhancement of biofilm formation. Moscoso *et al*. reported that simultaneous inactivation of *Streptococcus pneumoniae*'s LytA amidase and LytC lysozyme abolished DNA release in liquid culture (Moscoso and Claverys, 2004, Moscoso *et al*., 2006). A choline-binding protein D (CbpD) is essential for competence-induced cell lysis in *S. pneumoniae*, but DNA release is also strongly attenuated in its (cbpD) mutant (Kausmally *et al*., 2005). It is, therefore, possible that biogenesis of extracellular DNA could be a genetically regulated process in bacteria including *Xf*. It is not clear if extracellular DNAs released from host cells could also function in regulating bacterial biofilm formation *in vivo*. In this regard, it would be interesting to evaluate whether the host extracellular DNA released in PD-resistant and -susceptible grapevines could differentially affect cell attachment, aggregation and biofilm formation *in planta*. This may provide insight into understanding the role of extracellular DNA in regulation of host-pathogen interactions toward genetic resistance or susceptibility *in planta*.

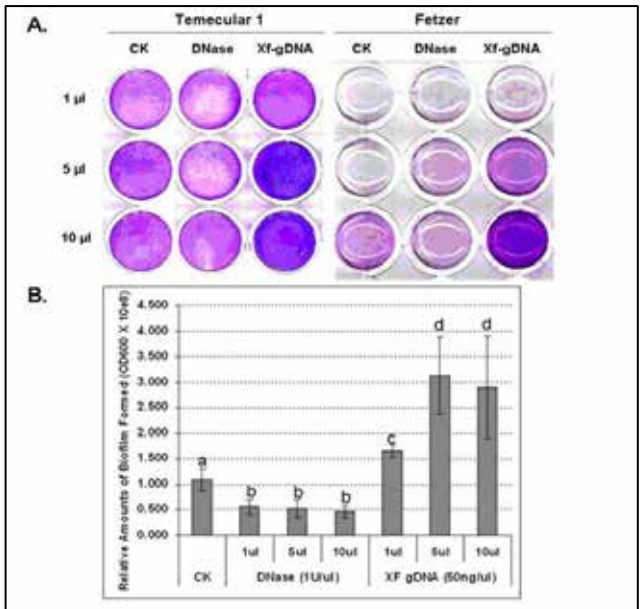

Fig. 2. Effects of DNase I and *Xf* (strain Temecula-1) genomic DNA on *Xf* biofim formation. A. Bioassay for *Xf* biofilm formation in the presence of various amounts of DNase I and *Xf* genomic DNA; B. Quantitative analysis of the effects of DNAse I and *Xf* genomic DNA treatment on *Xf* biofilm formation. The statistic significances (a, b, c and d) indicate that the differences are very significant among different groups, where no significant statistic difference exists within the same groups (b and d).

Xf bacterial planktonic growth, biofilm formation and cellular aggregation are dependent on the chemistry of xylem sap and can be manipulated by altering xylem chemistry (Andersen *et al*, 2007, Leite *et al*., 2004). *Xf* biofilm formation likely plays a key role in xylem vessel occlusion and is a key virulence factor probably required for *Xf* pathogenicity (de Souza *et al*., 2005, Marques *et al*., 2002, Newman *et al*., 2004). We examined a potential role of extracellular DNA as an additional factor in *Xf* biofilm formation *in vitro* by adding different amounts of DNase I and *Xf* genomic DNA into the pre-cultured *Xf* growing medium. DNase I treatments

diminished the effect of *Xf* biofilm formation in both *Xf* strains, Temecula-1 and Fetzer (kindly provided by Dr. Bruce Kirkpatrick) (**Figure 2A**). In contrast, addition of Xf genomic DNA (Temecula-1) greatly enhanced biofilm formation by both *Xf* strains. Quantitative analyses of the effects of DNase I and *Xf* genomic DNA on biofilm formation revealed that all DNase I treatments decreased the cell density of *Xf* biofilm by nearly 50%, although there were no significant differences between the DNase I concentrations used. As expected, addition of *Xf* genomic DNA significantly increased the cell density of *Xf* biofilms 1.5- to 3-fold depending on the concentrations of *Xf* genomic DNA added (**Figure 2B**).

Extracellular DNA plays a role in the maintenance of biofilms formed by gram-positive and gram-negative bacteria (Tetz *et al*., 2009). Digestion of *P. aeruginosa* and *S. pneumoniae* extracellular DNA changed the properties of the biofilms formed by these bacteria (Whitchurch *et al*., 2002, Izano *et al*., 2008, Moscoso *et al*., 2006). However, the mechanism(s) of how extracellular DNA functions in *Xf* biofilm formation is not clear. A functional DNA binding and uptake system was suggested to be involved in the biofilm formation by *S. mutans*, where the presence of synthetic competence-stimulating peptide significantly promoted the release of DNA and enhancement of biofilm formation (Petersen *et al*., 2005). The DNA binding-uptake system is a multi-protein complex that is required for the assembly of type IV pili and for the secretion of certain proteins in gram-negative bacteria (Chung and Dubnau 1998). In addition, pseudopili cross the cell wall and allow the extracellular DNA to access a membrane-bound receptor in *B. subtilis* (Chen and Dubnau, 2004). Type IV pili, flagellum-mediated motility and quorum sensing-controlled DNA releases are involved in the formation of mature multicellular structures in *P. aeruginosa* biofilms (Barken *et al*., 2008). Based on a biophysical study of the bacterial organization in a model extracellular DNA matrix, bacteria can spontaneously become ordered in a matrix of aligned concentrated DNA, in which rod-shaped cells of *P. aeruginosa* follow the orientation of extended DNA chains (Smalyukh, 2008). It is likely, therefore, that extracellular DNA may function through interaction with specific proteins on the bacterial membrane that would favor or facilitate biofilm formation. This process may be coordinately regulated by bacterial pili and/or flagellum-mediated motility and/or quorum sensing systems.

CONCLUSIONS

The present study suggests that biogenesis of extracellular DNA may be a result of autolysis of *Xf* cells or other cellular mechanism(s) or both during the planktonic growth that was associated with enhanced biofilm formation *in vitro*. Further research is needed to assess the role of extracellular *Xf* DNA in biofilm formation and pathogenesis *in planta.*

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THE ROLE OF LIPOPOLYSACCHARIDES IN VIRULENCE, BIOFILM FORMATION, AND HOST SPECIFICITY OF *XYLELLA FASTIDIOSA*

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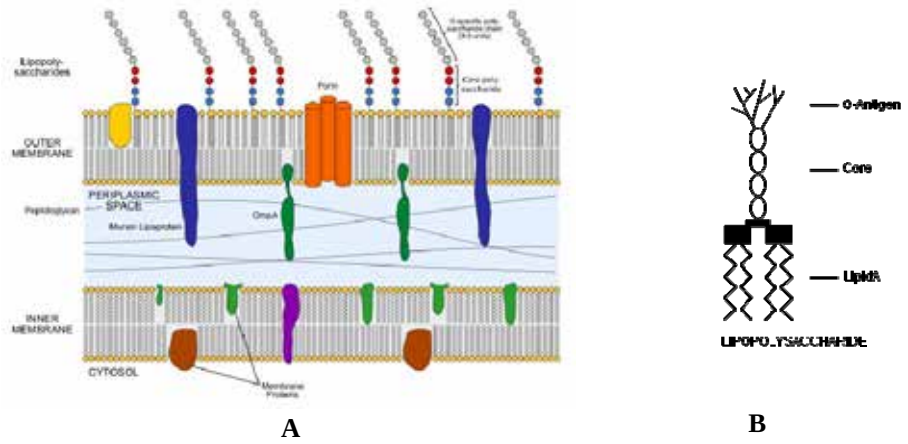
Reporting Period: This project has just been funded. Therefore, we are only reporting preliminary results.

ABSTRACT
This project focuses on the lipopolysaccharide (LPS) component of the outer membrane of *Xylella fastidiosa (Xf)*. In particular, we are investigating if the O-antigen portion of this molecule contributes to *Xf* surface attachment and biofilm formation. More importantly, by targeting genes involved in O-antigen biosynthesis, we will determine if LPS is an important virulence factor for *Xf* infection of grape. Additionally, we will determine if LPS contributes to the high level of host specificity observed for this pathogen.

LAYPERSON SUMMARY
Xylella fastidiosa (*Xf*) is a bacterium that has the ability to infect many different plant species. In some plants, this bacterium can cause serious disease. In grapevine, this disease is known as Pierce's disease (PD) and has caused millions of dollars of damage to the California grape industry alone. *Xf* species also infect other economically important crops such as almond, oleander and citrus. Interestingly, while all *Xf* isolates belong to the same group or species, some isolates can cause disease in one host but not another. For example, oleander strains cannot cause disease in grapevine and vice versa. One major goal of this project is to understand the bacterial mechanisms that dictate this high level of host specificity. We are focusing on a key component of the bacterial cell membrane, called lipopolysaccharide (LPS), and how certain parts of this molecule may be important in dictating host specificity. We are also investigating how the LPS molecule is related to bacterial virulence and other key aspects of the disease process, like attachment to the plant cell wall. This molecule makes up more than 70% of the bacterial membrane and if LPS does prove to be an important factor during *Xf* plant infection, its abundance in the bacterial cell membrane makes it a logical target for disease control. Furthermore, antimicrobial compounds that weaken the LPS molecule generally make the bacterium more sensitive to other stresses. Therefore, compounds targeted towards LPS synthesis could increase the efficacy of other anti-*Xylella* compounds currently being developed when both are used in conjunction.

INTRODUCTION
Xylella fastidiosa (Xf) is a gram negative, xylem-limited bacteria with a broad host range encompassing at least 28 families of monocots and dicots, and causes disease in hosts such as grape, almond, peach, plum, alfalfa, elm, sycamore, coffee, oleander, maple, and citrus (Hopkins and Purcell, 2002). The molecular mechanisms that determine this host specificity are poorly understood. This project aims to explore the role of lipopolysaccharides (LPS) as both a virulence factor and host specificity determinant of *Xf*. We will focus on the O-antigen portion of the LPS molecule in three strains of *Xf*, the PD strain (Temecula 1), the almond leaf scorch strain (Dixon-ALS), and oleander leaf scorch strain (Ann-1). These three strains were chosen because either full or draft sequences of the genome are available. This will allow us to easily identify genes that are putatively involved in O-antigen biosynthesis and to construct targeted deletion knockouts in these genes. Additionally, these three strains infect different hosts, grape, almond and oleander. Interestingly, while grape and almond isolates are considered to be separate subspecies or pathovars, grape strains can cross colonize and cause both PD and ALS symptoms. Whereas almond strains do not cause PD symptoms in grape but can sometimes colonize grape, albeit at low titer (Almeida and Purcell, 2003). Furthermore, the oleander strain cannot infect grape or almond and both the almond and grape strains cannot infect oleander (Almeida and Purcell, 2003). This provides an opportunity to study the role of the O-antigen moiety of the LPS molecule as a potential host specificity determinant for *Xf*.

LPS comprises approximately 70% of the outer membrane of gram-negative bacteria and is therefore essential for growth and viability (**Figure 1A**). Because LPS is what is largely displayed on the cell surface it mediates interactions between the bacterial cell and its surrounding environment. LPS (sometimes called "endotoxin") has been implicated as a major virulence factor in both plant and animal pathogens, such as *Escherichia coli*, *Xanthomonas campestris* pv. *campestris*, and *Ralstonia solanacearum* just to name a few (Muhldhorfer and Hacker 1994, Dow *et al*. 1995; Hendrick *et al*. 1984). Because of its location in the outer membrane, LPS can also contribute to the initial adhesion of the bacterial cell to a surface or host cell (Genevaux *et al*. 1999, Nesper *et al*. 2001). Additionally, host perception of LPS is well documented and occurs in both plants and animals (Newman *et al.* 2000). The immune system can recognize several regions of the LPS structure and can mount a defense response in response to bacterial invasion based on this recognition. Bacteria can also circumvent the host's immune system by altering the structure of their LPS molecule or by masking it with capsular or exopolysaccharides.



A **B**

Figure 1. **A**. Schematic of a gram-negative bacterial cell wall indicating the location of LPS in the outer membrane. **B**. Schematic of a single LPS molecule composed of lipid A, core oligosaccharide and O-

LPS is composed of three parts: 1) lipid A, 2) core oligosaccharide and 3) O-antigen polysaccharide (**Figure 1 B**). Lipid A is anchored in the membrane and core oligosaccharides are assembled on the preformed lipid A molecule. O-antigen is assembled elsewhere and ligated onto the core oligosaccharide-lipid A complex. Both lipid A and core oligosaccharide are relatively conserved among bacterial species. O-antigen is highly variable even amongst strains of the same species. O-antigen is the immunodominant portion of the LPS molecule and contributes to serotype designation of different strains within the same species. O-antigen is not required for bacterial viability but is often implicated in virulence and host specificity. Even small changes in the type and order of the sugars comprising the O-antigen can result in major changes in virulence.

The lifestyle of *Xf* requires attachment to diverse carbohydrate substrates such as the plant xylem wall and chitin in the mouthparts and foregut of the sharpshooter insect vector. In both environments, *Xf* forms biofilms or biofilm-like structures. Previous studies show that *Xf* produces an extracellular exopolysaccharide (EPS) that is present in minute quantities during initial surface attachment and early biofilm formation. However, in mature biofilms this EPS (termed fastidian gum) is a major component of the three-dimensional *Xf* biofilm both *in vitro* and *in planta* (Roper *et al*. 2008). Other studies have demonstrated that proteinacous adhesins such as type I pili and hemagluttinins contribute to surface adhesion and cell-cell aggregation (Li et al. 2007, Guilhabert and Kirkpatrick, 2005). Because of the location and abundance of LPS in the outer membrane we hypothesize LPS also plays a key role in mediating initial attachment to the carbohydrate substrates *Xf* encounters in the plant and insect.

In this project, we will construct mutants in the O-antigen biosynthesis pathway that will either be completely devoid of O-antigen or produce truncated versions of O-antigen. We will assay these mutants for virulence in their respective host plants as well as their ability to infect non-hosts. Furthermore, because of its abundance in the outer membrane (70%) of *Xf*, LPS is a logical target for developing novel therapeutics against *Xf* for control of PD. In fact, a bacterium with defective LPS is often more susceptible to oxidative stress, antimicrobial peptides, and other stresses bacteria encounter *in planta*. Therefore, treating PD infected grapevines with antimicrobial compounds designed to inhibit or truncate LPS synthesis may be one way to control PD.

OBJECTIVES

1. a. Characterization and comparison of the LPS profiles from the grape, almond and oleander strains of *Xf* grown in PW broth and PW solid media
   b. Investigate the possibility of phase variation in *Xf* LPS
2. Construct *Xf* mutants in O-antigen biosynthetic genes
3. Test virulence and host specificity of the O-antigen mutants *in planta*
4. Test attachment and biofilm formation phenotypes of *Xf* O-antigen mutants

PRELIMINARY RESULTS AND FUTURE STUDIES

Objective 1: Characterize the LPS profiles from the grape, almond and oleander strains of *Xf*.

A. Comparison of the LPS profiles from *Xf* grown *in vitro*:

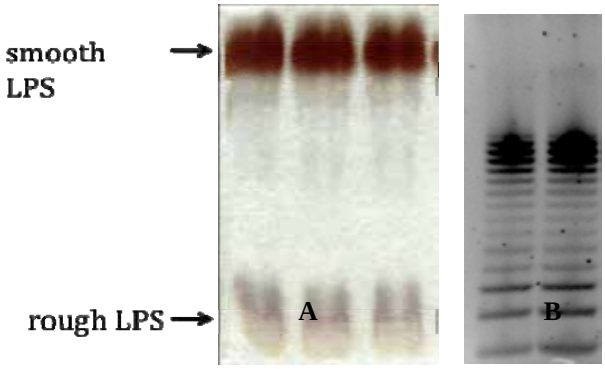


Figure 3. A. Sodium deoxycholate PAGE gel of *Xf* LPS grown on PW medium indicating the presence of both smooth (O-antigen) and rough (core) LPS. All lanes contain LPS from the grape strain of *Xf*. **B.** Tricine-SDS PAGE gel of *P. stewartii* LPS grown on nutrient indicating the presence of both smooth (O-antigen) and rough (core) LPS. All lanes contain LPS from wild type *P. stewartii*. **Note the enhanced resolving power of the Tricine-SDS PAGE gel compared to the Sodium deoxycholate gel.**

A bacterium with only lipid A and core oligosaccharide is said to have "rough" LPS and one with lipid A, core oligosaccharide and O-antigen is said to have "smooth" LPS. There can be both smooth and rough variants of the same bacterial species. Preliminary data indicate that the LPS extracted from *Xf* Temecula 1 grown on solid medium has both rough (core LPS) and smooth (O-antigen) components of the LPS molecule (**Figure 3A**). In this study we will compare the LPS profiles of *Xf* Temecula 1, *Xf* Ann1 and *Xf* Dixon by Sodium deoxycholate PAGE electrophoresis to determine if all three strains have smooth and rough forms of LPS and if there are any variations in the bandings patterns in the O-antigen fraction. Furthermore, we will analyze the *Xf* LPS on Tricine-SDS PAGE gels to provide better resolution of the individual LPS bands. **Figure 3B** is a Tricine SDS PAGE gel of LPS extracted from *Pantoea stewartii* subsp. *stewartii*, another xylem dwelling phytopathogen. This figure is included merely to demonstrate that this technique is capable of resolving individual bands in both the core and O-antigen portions of the LPS molecule. By using this technique we will be able to detect even subtle differences in the LPS banding patterns of the three strains of *Xf* as well as the mutants we will construct in Objective 2.

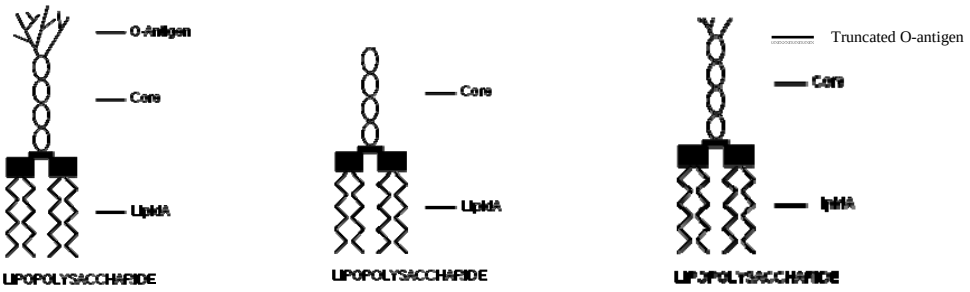
B. Comparison of the *Xf* Temecula 1 (grape strain) LPS profile in PW medium vs. grapevine xylem sap. The outer membrane of a bacterial cell is strongly influenced by its surrounding environment. Gram-negative bacterial pathosystems can undergo a process called phase variation, which is defined as a reversible change in the antigenic determinants in response to environmental conditions. It is well documented that this phenomenon occurs in the extracellular and membrane bound polysaccharide portion of the bacterial cell surface (Bergman *et al*. 2006; Lerouge *et al*. 2002). This includes exopolysaccharide, capsular polysaccharide and LPS. Specifically in the LPS fraction, bacteria can display different O-antigen profiles by varying the degree of polymerization or by completely abolishing synthesis of the O-antigen. The cells can alternate from smooth to rough or semi-rough (truncated O-antigen) LPS variants depending on the developmental phase of the cell. The structure of the O-antigen expressed can be a key factor in how the bacterium interacts with its host or vector. A bacterium may display different O-antigen sugars on its surface that essentially changes its external appearance to the host (Bergman et al. 2006, Guerry *et al*. 2002). This putative masking effect may be important when *Xf* cells are initially introduced into the plant by the sharpshooter vector and presumably not covered or protected in large amounts of EPS.

The aim of this objective is to determine if *Xf* LPS undergoes phase variation in different growth conditions. We will assay this by growing the *Xf* Temecula 1 grape strain of *Xf* in PW broth vs. PW broth amended with increasing concentrations of grapevine xylem sap vs. pure xylem sap.

Objective 2: Construct *Xf* mutants with altered LPS profiles. The goal of this objective is to construct *Xf* mutants that either 1) completely lack O-antigen ("rough" LPS mutants) or 2) produce truncated forms of O-antigen ("semi-rough" mutants). Genomic analyses reveal approximately 30 *Xf* genes involved in LPS biosynthesis. In this objective we will focus on two of these genes that are involved in the biosynthesis of O-antigen as well as the attachment of O-antigen to core LPS. While the mechanism of assembly is likely the same for O-antigen assembly and ligation for all *Xf* strains, the composition of the O-antigen could be markedly different depending what sugars are incorporated into the O-antigen chain.

The first gene of interest is a *waaL* ortholog, designated PD0077 in the genome of the Temecula 1 grape strain. All three strains (oleander, grape and almond) have a single copy of this gene. We will construct deletion mutants in this gene in all three strains by site-directed mutagenesis. In other bacterial systems, mutations in *waaL* prevent the ligation of O-antigen (Perez *et al*. 2008). Therefore, a *Xf* mutant in *waaL* would produce rough LPS composed only of lipid A + core and be completely devoid of O-antigen as depicted in **Figure 4B**. The LPS phenotype of the *waaL* mutants will be confirmed by Tricine-SDS PAGE gel electrophoresis described in Objective 1. As documented in other bacterial species, we expect *Xf* Δ*waaL* mutants to be decreased in virulence if the O-antigen expressed *in planta* is indeed providing some kind of masking effect from the host defense responses (Berry *et al*. 2009; Carroll *et al*. 2004; Moran 2008). However, a mutation in *waaL*

could very well have the opposite effect. These mutants could potentially be hypervirulent if the host has evolved to recognize *Xf* O-antigen and absence of the molecule leaves the plant partially blind to *Xf* invasion.



A. wild type *Xf* LPS **B**. putative *Xf* Δ*waaL* LPS **C**. putative *Xf* Δ*wzy* LPS

Figure 4. A. Model of wild type *Xf* LPS molecule containing all three components: lipid A, core polysaccharide, and O-antigen **B**. Model of hypothetical Δ*waaL Xf* LPS molecule containing no O-antigen. **C.** Model of hypothetical Δ*wzy Xf* LPS molecule with truncated O-antigen. Images adapted from http://www.wikipedia.org/

The second gene we will target is the *wzy* ortholog, designated PD0814 in the Temecula 1 grape genome. This gene is also present in single copy in all three strains. Wzy is an O-antigen polymerase responsible for the assembly of O-antigen prior to its ligation to the core. Wzy acts in concert with Wzz, a chain length regulator or "molecular ruler" to polymerize LPS O-antigen subunits to a certain chain length. We predict that *Xf* mutants in *wzy* will produce an O-antigen that look likes Figure 4C.

*All mutants constructed in this study will be complemented with the wild type copy of the gene. We will use the complementation vectors now available for *Xf* or introduce a wild type copy of the gene into a neutral part of the chromosome as reported by the Igo lab at UC-Davis.

Objective 3: **Test *Xf* O-antigen mutants for virulence and host specificity**. For all experiments described below, we will mechanically inoculate plants using the pin-prick method originally described by Hill and Purcell 1995. All plants will be rated on a disease scale of 0-5 with 0 being healthy and 5 being dead (Guilhabert and Kirkpatrick, 2005). First, for the virulence assays, each of the LPS mutants we constructed for each strain will be tested for virulence in their respective susceptible host plants. If a particular mutant is no longer pathogenic or hypervirulent, we will quantify *Xf* populations/gram of plant tissue by performing isolations on petioles harvested nearest the point of inoculation. The results of these experiments will indicate if the O-antigen portion of the LPS molecule is indeed a virulence factor for *Xf*. Secondly, for the host specificity assays, we will inoculate all strains into "non-host" plants and determine if an alteration in O-antigen correlates with a shift in host range or specificity. It is well documented that different pathovars or serotype groups display different O-antigens on their surface (Benedict et al., 1990; Lerouge et al. 2002). It is assumed that presence/absence and carbohydrate composition of the O-antigen correlates with the ability of a particular bacterial strain to infect one host but not another. However, there are very few studies actually documenting this. In one study, a spontaneous mutant of *Xanthomonas campestris* pv. *citrumelo* was altered in core LPS and lost O-antigen displayed an altered host range (Kingsley et al. 1993). Normally, this pathogen has a wide host range that includes citrus and bean. The spontaneous mutant lost the ability to infect citrus but retained its ability to infect bean. The authors attributed this difference to the change in the LPS profile although other polysaccharides were affected. It will be interesting to see if by removing O-antigen or altering its chain length if the Temecula 1 strain will retain its ability to infect both grape and almond and if Dixon-ALS can gain the ability to cause disease in grape. Furthermore, can these changes in O-antigen allow the oleander *Xf* Ann1 strain to become a pathogen of grape or almond and the *Xf* Dixon and *Xf* Temecula1 strains become pathogens of oleander?

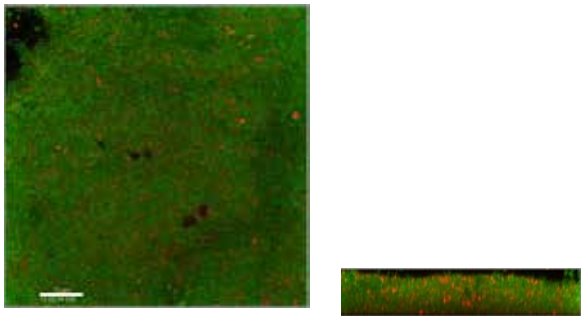
For these experiments, *Xf* Temecula 1 grape strain mutants will be inoculated into almond and oleander. The *Xf* Ann1 oleander strain mutants will be inoculated into grape and almond and the *Xf* Dixon almond strain mutants will be inoculated into grape and oleander. This is outlined in **Table 1**. All mutants and wild type will also be inoculated in their respective host plants in order to compare symptoms; *WT=wild type

Objective 4: **Test surface attachment and biofilm formation of *Xf* O-antigen mutants.** *Xf* is known to attach to glass surfaces and form a biofilm at the air/liquid interface when grown in liquid culture (**Figure 5**). In this objective we will further characterize the *Xf* Temecula 1 Δ*waaL* and Δ*wzy* mutants by assaying 2 different behaviors: surface attachment and biofilm formation. In order to quantify surface attachment, *Xf* wild type Temecula 1, Δ*waaL* and Δ*wzy* mutants will be grown in PD3 medium in 10 ml borosilicate glass tubes and attachment on the surface walls of the tubes will be assessed by a

crystal violet staining method (Espinosa-Urgel *et al*. 2000). We will assess the capability of the *Xf* Temecula 1 Δ*waaL* and Δ*wzy* O-antigen mutants to form biofilms on glass surfaces. Biofilms will be imaged using a confocal laser scanning microscope available in the UCR core microcopy facility.

Table 1. Inoculations for *Xf* host specificity tests. *Xf* Temecula 1 grape strain mutants will be inoculated into almond and oleander. The *Xf* Ann1 oleander strain mutants will be inoculated into grape and almond and the *Xf* Dixon almond strain mutants will be inoculated into grape and oleander. All mutants and wild type (WT) will also be inoculated in their respective host plants in order to compare symptoms.

	Host Inoculations		
***Xf* mutant**	**Grape**	**Almond**	**Oleander**
WT Temecula 1		x	x
Δ*waaL* Temecula 1		x	x
Δ*wzy* Temecula 1		x	x
WT Dixon	x		x
Δ*waaL* Dixon	x		x
Δwzy Dixon	x		x
WT Ann1	x	x	
Δ*waaL* Ann1	x	x	
Δ*wzy* Ann1	x	x	



A B

Figure 5. Confocal laser scanning microscope images of *Xf* biofilms formed at the air/liquid interface after 10 days growth in PD3 medium. *Xf* cells are depicted in green and *Xf* EPS is depicted in red. **A**. Overhead view of the *Xf* biofilm. **B**. Sagittal view of the *Xf* biofilm.

CONCLUSIONS

This project aims to further elucidate the molecular mechanisms of *Xf* virulence. At the same time, we will investigate the possibility of O-antigen as a host specificity determinant for this pathogen. While there are likely several factors that contribute to host specificity of *Xf*, we are investigating if O-antigen presence and composition is involved. We feel that the wide host range and stringent host specificity of different *Xf* strains affords a unique opportunity to study the molecular mechanisms underlying the host specificity observed for this pathogen.

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THE SEARCH FOR GENETIC DIFFERENCES BETWEEN GRAPE STRAINS OF *XYLELLA FASTIDIOSA*

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ABSTRACT
To create a reliable genetic fingerprint of the Texas Pierce's disease (PD) strains of *Xylella fastidiosa* we have been evaluating both highly variable sequences (small sequence repeats) and genes that are either highly conserved (gyraseB) or moderately conserved (ZOT). To speed the hunt for genes with fingerprinting potential we performed a whole genome comparison (Nimblegen microarray) between the Temecula strain (ATCC) and a Texas strain (Gil Bec 514). Gene regions with potential single nucleotide polymorphisms (SNPs) were further analyzed by individual gene sequencing. We sequenced four genes (pilY, a multi-drug efflux transporter, a phage-related tail protein and a periplasmic protease) for five Texas PD strains in triplicate. On one gene all Texas strains showed one SNP difference from the Temecula strain, two genes showed variable SNPs with only some Texas strains giving a complete match with Temecula. Finally, one gene showed one or more SNP difference between all Texas PD strains and Temecula. These SNP differences will be compared to other genetic analyses to determine the most reliable method for identifying genetic variability within Texas.

Section 4: Pathogen and Disease Management

GRAPE ROOTSTOCK VARIETY INFLUENCE ON PIERCE'S DISEASE SYMPTOMS IN CHARDONNAY

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ABSTRACT
Chardonnay is a *Vitis vinifera* scion variety that is susceptible to Pierce's disease (PD). We are evaluating the effect of rootstock variety on PD symptom expression in Chardonnay grown in an experimental vineyard at Weslaco, Texas with high natural PD pressure and abundant vectors, including glassy-winged sharpshooter. The rootstocks under evaluation are Dog Ridge, Florilush, Tampa, Lenoir, and Freedom. Natural *Xylella fastidiosa* (*Xf*) infection is permitted to test the effect of rootstock variety on PD in the Chardonnay scions. Vines grafted on Dog Ridge are much larger than on other rootstocks, which are similar in size; PD symptoms in vines grafted on Dog Ridge were the lowest observed amongst the rootstocks.

LAYPERSON SUMMARY
Rootstocks that reduce Pierce's disease (PD) symptoms of the scion could be one way to reduce damage from PD without changing scion varieties or clones. Rootstocks have been reported to reduce PD symptoms, but have not definitely been shown to provide or confer sufficient protection against PD to be recommended as a PD management tool. We are evaluating several rootstocks in a PD prone area, the Lower Rio Grande Valley of Texas. Chardonnay (PD susceptible) vines grafted on Dog Ridge rootstock were the largest in the trial and had the fewest PD symptoms.

INTRODUCTION
Rootstocks are widely in use in viticulture to manage damage from soil-borne pests and provide adaptation to soils. In citrus (He et al. 2000) and peach (Gould et al. 1991), rootstock variety has been reported to impact expression of *Xylella fastidiosa* (*Xf*) diseases in scions. Pierce (1905) reported that rootstock variety affected expression of "California vine disease" (Pierce's disease; PD) in grape. Grape rootstock trials in Mississippi showed a large effect of rootstock trial on vine longevity in a region recognized for high PD pressure (Loomis 1952, 1965, Magoon and Magness 1937). If grape rootstocks could contribute PD resistance or tolerance to their scions, this would be a major benefit to viticulture in PD prone areas. Elite wine, juice, and table grape varieties could be grown in areas where viticulture is currently restricted to PD resistant and tolerant varieties whose consumer appeal is low.

The Rio Grande Valley is an excellent location for the field evaluation of PD resistant plant germplasm and PD management techniques. Many insect vectors of *Xf* are native to the region, including the glassy-winged sharpshooter. Susceptible grapevine varieties are infected naturally with *Xf* in the vineyard and demonstrate characteristic PD symptoms and decline. The Rio Grande Valley is similar to many viticultural regions in California; the region is flat, irrigated, and supports multiple types of crops (citrus, grains, vegetables) in close proximity. The Rio Grande Valley is an ideal test environment due to heavy PD pressure, with abundant vectors and inoculum, in contrast to many other locations, especially California, which demonstrate substantial cycling of PD incidence. The USDA Agricultural Research Service Kika de la Garza Subtropical Agricultural Research Center in Weslaco, Texas is located in the heart of the Rio Grande Valley and provides an ideal experimental location for the evaluation of PD management practices, including rootstock evaluation.

Five rootstocks are being evaluated in this project. Freedom is a complex interspecific hybrid developed as a root-knot nematode resistant rootstock by the USDA ARS, Fresno, California (Clark 1997); its parentage includes *Vitis vinifera, V. labrusca, V. x champinii, V. solonis,* and *V. riparia* (Garris et al. 2009). Freedom is widely used in California viticulture. Dog Ridge is a *V. x champinii* selection recognized for its nematode resistance and resistance to PD, but it is rarely used as a rootstock. Lenoir, most probably a *V. aestivalis*/*V. vinifera* hybrid, was used historically as a rootstock and presently is cultivated as a wine grape in PD prone regions (including some parts of Texas) (Galet 1988). Tampa (Mortensen and Stover 1982) includes a *V. aestivalis* selection and the juice grape Niagara (a *V. labrusca* hybrid) in its parentage. Florilush (Mortensen et al. 1994) is a selection from the cross Dog Ridge x Tampa. Both Florilush and Tampa were selected by the University of Florida as PD resistant rootstocks for bunch grapes. PD resistance is necessary for rootstock mothervines to thrive in Florida, so the PD resistance of Florilush and Tampa should not be construed necessarily as contributing to the PD response of the scions.

OBJECTIVE

To evaluate the impact of rootstock variety on expression of PD symptoms in naturally infected PD susceptible *Vitis vinifera* scion varieties Chardonnay.

RESULTS AND CONCLUSIONS

Grafted vines of Chardonnay on five rootstocks (Freedom, Tampa, Dog Ridge, Florilush, and Lenoir) were planted at the Kika de la Garza Subtropical Agricultural Research Center in Weslaco, Texas in July, 2006. Evaluation of PD response of the vines began in 2007. Experimental vineyard establishment was good and several vines flowered and fruited in 2007 and 2008.

Symptoms on leaves were assessed in October 2008. Percent leaves with marginal necrosis symptom of PD were determined for each vine. Dormant pruning was conducted in January, 2009, and the weight of prunings of each vine, head trained and spur pruned, was collected.

Table 1. Marginal leaf necrosis and dormant pruning weight of vines by rootstock

Rootstock	Number of vines	Mean % leaves with marginal necrosis, Oct. 2008	Weight of dormant prunings, Jan. 2009, kg
Florilush	7	7.0	0.41
Freedom	9	9.0	0.41
Lenoir	10	4.4	0.46
Tampa	10	5.0	0.48
Dog Ridge	10	3.2	1.08

Preliminary results (**Table 1**) indicate that Chardonnay vines grafted on Dog Ridge were the largest and had the least PD symptoms. Additional years of vineyard observations will be necessary before making rootstock recommendations based on vineyard performance. In the Lower Rio Grande Valley at the USDA ARS research vineyard, vines are not demonstrating downy mildew or black rot or more than slight powdery mildew infection; PD remains the chief disease in the research vineyard.

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

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ABSTRACT

Xylella fastidiosa (Xf), a xylem-limited Gram-negative bacterium, is the causative agent of Pierce's disease (PD). A key feature of *Xf* resides in its ability to digest pectin containing pit pore membranes inside the xylem elements permitting its long distance movement enhancing its virulence and vector transmission. In this project we are analyzing the efficacy of xylem targeted effector proteins like polygalacturonase inhibiting protein (PGIP) and a chimeric antimicrobial protein to restrict the movement and to clear *Xf*. The expectation is that expression of these proteins will prevent *Xf* movement and reduce its inoculum leading to a reduction of the spread of PD. Transgenic grapevine plants expressing either PGIP, the human neutrophil elastase-cecropin B (HNE-CECB) chimeric antimicrobial protein and pgip-HNE-CECB have been obtained and the first batches have been tested to validate their efficacy against PD.

Plants expressing pear PGIP have five different modifications to better understand its ability to restrict disease spread. Four of the PGIPs contain different signal peptide sequences (to identify which most efficiently localizes PGIP to xylem tissues and which provides the best distribution through the graft union into untransformed scion tissues) and one without a signal peptide which serves as a control. Based on PGIP activity eight of 10 mPGIP, two of five Ramy, three of 11 XPS, eight of 11 ChiPGIP and six of 10 NtPGIP *in vitro* lines have been transferred to the greenhouse. Fifteen of 27 PGIP transgenic lines (one mPGIP, eight ChiPGIP and six NtPGIP) have been manually inoculated with *Xf* and are in early stages of evaluation for tolerance to PD and movement of PGIP protein. The remaining mPGIP, Ramy, XPS and Chi lines are in the process of multiplication for future *Xylella* infection challenge experiments.

Transgenic grapevine plants expressing a chimeric anti-microbial protein HNE-CECB with its own signal peptide and pgip-HNE-CECB expressed with the signal peptide from pear PGIP have been obtained. The expressed chimeric anti-microbial protein have two functional domains, one (the surface recognition domain, SRD) that specifically binds to the *Xf* outer-membrane protein MopB and the other domain inserts into the membrane causing pore formation that results in the lyses of *Xf* causing its mortality. Twenty-one of 36 HNE-CECB transgenic grapevine lines have been manually inoculated with *Xf* in the greenhouse**.** Observations from the first two rounds are very promising - five of these transgenic lines had low, and six lines had moderate symptoms when compared with wild type Thompson seedless control plants whose symptoms were severe. Magnetic resonance imaging (MRI) of stem sections revealed a variation in number of vessels clogged between negative control and transgenic lines. The remaining HNE-CECB and pgip-HNE-CECB are in the process of greenhouse multiplication to conduct future *Xylella* challenge infections.

LAYPERSON SUMMARY

Transgenic grapevines are being evaluated as rootstocks to mobilize two types of effector proteins to control Pierce's disease (PD) in wild type scion cultivars grafted to such rootstocks. The growth and productivity of grapevines is compromised by growth and movement of *Xylella fastidiosa (Xf)*, its invasion of individual xylem elements and its ability to colonize and occlude the water-conducting vessels which stresses the plant leading to its death. In this project we are analyzing the efficacy of xylem targeted effector proteins like polygalacturonase inhibiting protein (PGIP) and a chimeric antimicrobial protein, the former to restrict the movement *Xf* across xylem elements reducing its pathogenicity and the latter to clear *Xf*

preventing its ability to colonize. Plants expressing PGIP have five different modifications to better understand its ability to restrict disease spread. These plants are being evaluated in the greenhouse for resistance to PD and in grafted plants to evaluate the long distance movement of PGIP. We have also evaluated 21of 36 HNE-CECB that we have in the greenhouse for clearance of *Xf*. We have obtained good evidence that at least four of the 11 evaluated lines show good tolerance to *Xf* infection and magnetic resonance imaging (MRI) of infected stem sections further revealed less number of vessels clogged in the transgenic as compared to control grapevine plants indicating clearance of the infected bacteria. Further experiments with these transgenic lines will confirm the efficacy of these two effector proteins in controlling this important disease of grapevines.

INTRODUCTION

Pierce's disease (PD) in grapevines is a vector transmitted disease where the causative agent a Gram-negative bacterium *Xylella fastidiosa* (*Xf*) is deposited into the xylem tissue by the feeding action of the glassy-winged sharpshooter (GWSS), the insect vector that efficiently transmits the disease and is of greatest concern to growers in California. The virulence of the bacterium is associated with its ability to colonize xylem and its ability to move through pit pore membranes into adjacent water conducting elements (Roper et al. 2007). The growth and productivity of grapevines is compromised by growth and movement of this bacteria that limits itself to xylem tissues and its ability to occlude the water-conducting vessels. The University of California reported that the disease destroyed over 1,000 acres of 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. Our strategy is based on developing and testing proteins that will limit movement of *Xf* and proteins that will clear the bacteria. 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. We are developing a transgenic rootstocks that will produce proteins that can migrate through the graft union into the xylem of the scion to immobilize and clear infecting *Xf* bacteria (Aguero et al. 2006). We have previously shown that grapevine plants expressing a polygalacturonase inhibitory protein (PGIP) are able to protect the plant presumably by limiting the movement of *Xf* (Aguero et al. 2005). Recently, it has been shown that *Xf* expresses a polygalacturonase (PG) a virulence factor that it uses to degrade the pectin containing pit pore membranes in grapevines allowing it to move from one xylem vessel to another (Roper 2007). We also showed that expression of PGIP in grapevine rootstocks is associated with secretion of this protein into the xylem and it movement through the graft union and its presence in xylem sap of the grafted wild type scion grapevine (Aguero et al. 2005). Because *Xf* is xylem-limited, xylem-targeted expression of transgenic therapeutic proteins, such as PGIP and potential antimicrobial proteins needs to occur to prevent and control PD infestations. 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 endoplasmatic recticulum membrane (Nielsen 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 (Vitale and Denecke 1999). We and others have characterized proteins naturally secreted to the xylem of grapevines as they are excellent sources of potential signal peptides (Aguero et al. 2008; Jain and Basha 2003). 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. Additionally we have taken a structure-based approach to develop a chimeric anti-microbial protein for rapid destruction of *Xf* (Kunkel et al. 2007). The designed chimeric anti-microbial protein has two functional domains, one a surface recognition domain, SRD that specifically targets the bacterium's outer membrane and the other domain contains a lytic protein to lyse the membrane and kill *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 (Kunkel et al. 2007). We have combined HNE and cecropinB using a flexible linker so both components can bind simultaneously to their respective targets. 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. The strategy is based upon the fundamental principle of innate immunity in which pathogen clearance occurs in three sequential steps: pathogen recognition, activation of anti-microbial processes, and finally pathogen destruction (Pieters 2001, Baquero and Blazquez 1997).

OBJECTIVES

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

12 mPGIP, 5 Ramy, 11 XSP, 11 ChiPGIP and 10 NtPGIP, plants were assayed for polygalacturonase inhibiting activity in transgenic tissue extracts to validate the introduced transgene expression and were found to display a range of PG inhibitory activity from 0-22%, 0-44%, 0-28%, 0-57 % and 0-45 %, respectively corresponding to the source of the indicated signal

peptide (**Table 1**). The ChiPGIP expressing plants displayed the greater number of lines with strong inhibition than the other lines and all lines assayed showed some level of polygalacturonase inhibiting activity. Also, compared to ChiPGIP there were more lines, three Ramy, five NtPGIP vs one ChiPGIP, which had barely detectable inhibitory activity (**Table 1**). Based on PGIP activity eight mPGIP lines with none (expected) to medium, two Ramy with strong, three XPS with medium, six ChiPGIP with medium to strong and eight NtPGIP with medium to strong PGIP activity have been transferred to the greenhouse and acclimated (**Figure 1 A, B**).

Table 1. Current status of testing of transgenic *Vitis vinifera* var Thompson Seedless grapevines lines expressing PGIP fused with different signal peptides

No.	Signal peptide	Binary Vector	Plant Lines	(+) PCR for PGIP	(+) PGIP Activity	Moved to Greenhouse	*Xf* inoculated	Lines grafted
1	none	pDU05.1002	12	10	9	8	1	1
2	Ramy	pDU05.0401	5	5	4	2	0	0
3	XSP	pDA05.XSP	11	11	5	3	0	0
4	Chi	pDU06.0201	11	11	10	8	8	1
5	Nt	pDU05.1910	10	10	5	6	6	1

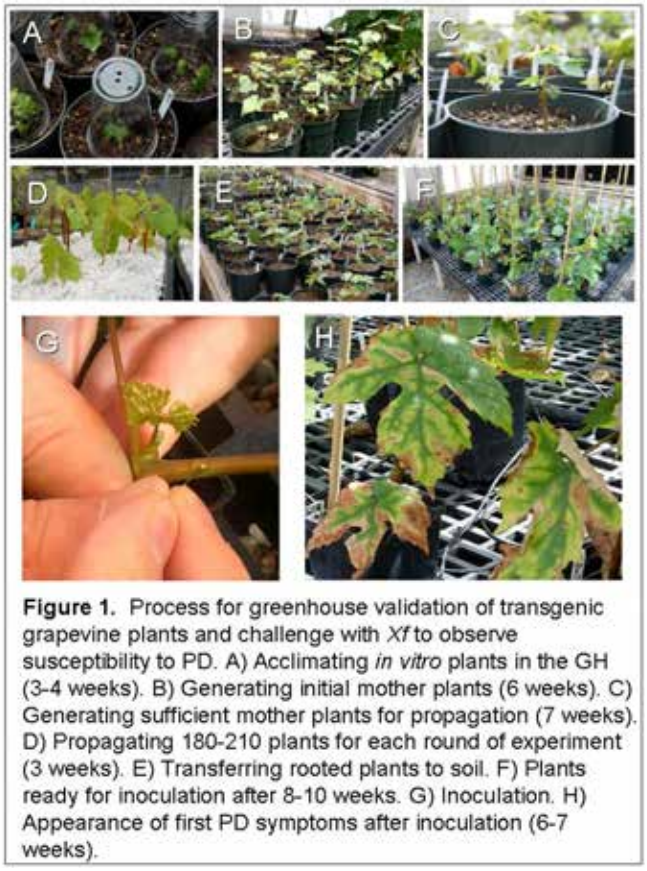

Figure 1. Process for greenhouse validation of transgenic grapevine plants and challenge with *Xf* to observe susceptibility to PD. A) Acclimating *in vitro* plants in the GH (3-4 weeks). B) Generating initial mother plants (6 weeks). C) Generating sufficient mother plants for propagation (7 weeks). D) Propagating 180-210 plants for each round of experiment (3 weeks). E) Transferring rooted plants to soil. F) Plants ready for inoculation after 8-10 weeks. G) Inoculation. H) Appearance of first PD symptoms after inoculation (6-7 weeks).

Each acclimated transgenic line was propagated to obtain four-six plants (**Figure 1C**) that are used as mother plants for further propagation to provide cuttings for *Xylella* infection and grafting experiments. From each line, 25-35 plants are propagated (from cuttings) at the same time (**Figure 1D-F**). *Xylella infection* experiments are done in multiple rounds. Each round consists of five-six transgenic lines and two controls, wild type Thompson (TS) and TS50 as negative and positive control, respectively. Each round of experiments includes 30 plants from each transgenic line, 15 of these are inoculated and the remaining 15 are non-inoculated controls. The positive control, T50 is a transgenic PGIP expressing grapevine previously described (Aguero et al. 2005).

Transgenic TS and controls (wild type TS and TS50) plants are inoculated with 20µl of the GFP expressing *Xf* 3A2 (Newman et al. 2003) containing ~20,000,000 cells. The plants are inoculated with 10 µl the first day and re-inoculated with 10µl the second day; for each inoculation an independently grown *Xylella* culture was used. The *Xylella* is introduced to each plant approximately three-four inches above the soil using an insect pin number zero as shown in the **Figure 1G**. Plants are pruned regularly and kept approximately 90-100cm tall until PD symptoms appear. The time required to conduct each round of *Xylella* challenge is 33 to 37 weeks, starting from *in vitro* plants transferred to greenhouse until the appearance of the first PD symptoms (**Figure 2**).

Fifteen of 27 PGIP transgenic lines (one mPGIP, eight ChiPGIP and six NtPGIP) have been manually inoculated (Almeida and Purcell 2003a) with *Xf* and they are in early stages of evaluation for tolerance against PD. Inoculated grapevines will be evaluated for symptoms of PD after three months. The remaining mPGIP, Ramy, XPS and Chi lines are in the process of multiplication for *Xylella* challenge in the greenhouse. Those lines that show low or moderate PD symptoms after manual inoculation will be tested by insect inoculation of *Xylella* (Almeida and Purcell 2003b). Transgenic grapevines after inoculation with *Xf* are scored for PD symptoms at regular intervals after infection using a standardized score based on percentage of leaf area scorching , a characteristic of PD (Krivanek et al. 2005a, 2005b).

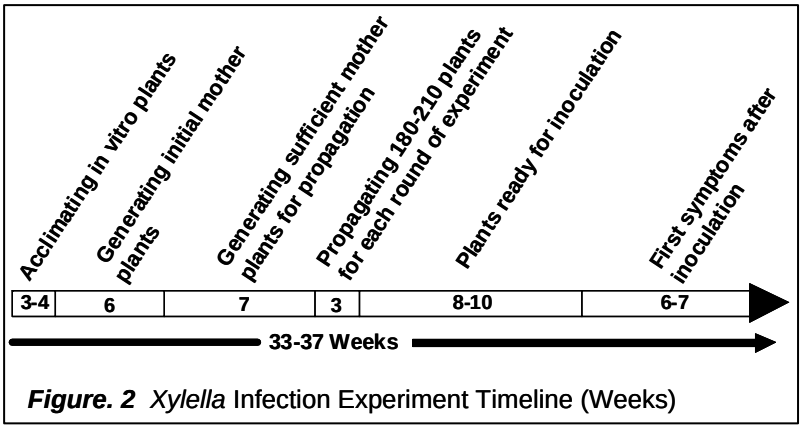

Figure. 2 *Xylella* Infection Experiment Timeline (Weeks)

To evaluate the efficiency of secretion each transgenic line expressing each of the signal sequences fused to PGIP will be used as transgenic rootstocks grafted to wild type scion. After growth xylem sap will be extracted from the stem and leaves of the wild type scion to evaluate the amount of PGIP that is translocated via the xylem into the wild type tissues. We have initiated grafting experiments where selected transformed lines (rootstocks) were grafted with wild type TS (scion). The movement of the PGIP protein from the rootstock up into the xylem of the wild type scion was evaluated using the radial assay (Aguero et al. 2005). Preliminary testing of PGIP activity using leaf extracts and xylem sap from non-grafted TS50 (positive control), ChiPGIP 45-35 and ChiPGIP 45-83 showed PG inhibiting activity. The same lines when grafted also showed inhibiting activity from leaf extract and xylem sap. TS50 showed the highest activity in grafted and non-grafted leaf and non-grafted xylem sap. Interestingly xylem sap from Chi45-35 and Chi45-83 showed a greater inhibition when they were grafted with wild type TS as compared to non-grafted, indicating that the PGIP was moving quite efficiently from the rootstock to the scion with these particular signal peptides (**Figure 3**).

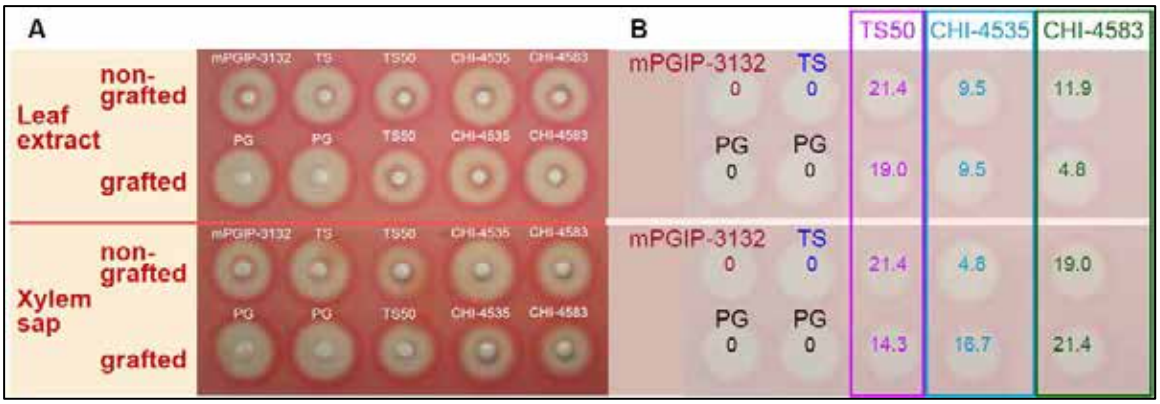

Figure 3. Zone inhibition assay to evaluate PG inhibition activity. **A,** assay plate image; **B,** percent inhibition measured in the assay. Leaf extracts and xylem sap from non-grafted and grafted transformed TS50, CHI 45-35 and CHI 45-83 lines were positive for PGIP activity. Transgenic mPGIP 31-32 that has no signal peptide and wild type TS that has no PGIP show no inhibitory activity. PG is the negative control and TS50 is a positive control.

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

Transgenic grapevine plants were obtained as described in earlier reports with the two constructs, pDU04.6105 (Elastase-Cecropin = HNE-CECB) and pDA05.0525 (pgipSP-Elastase-Cecropin= pgipHNE-CECB). Sixteen of 21 HNE-CECB lines are currently being evaluated for resistance/tolerance to PD. The first two rounds of infection have been completed for the testing of 11 transgenic lines. First PD associated leaf scorch symptoms were visible on control TS grapevines within six-seven weeks post inoculation which consists of cane color change to red and scorching around outer edge of the lower leaves. Most transgenic HNE-CECB expressing lines showed less or delayed disease symptom compared to non-transgenic control and five lines were substantially more resistant than the rest (**Figure 4**). PD symptoms on each of the infected plants were numerically scored based on percentage scorch (**Table 2**).

Figure 4. Leaf number 8 above point of inoculation harvested 10 -11 weeks post-inoculation

Table 2. Disease phenotypic scoring[a] for transgenic grapevines infected with *Xf*.

Round 1	Mean 7 weeks post-inoculation	Mean 11 weeks post-inoculation	Round 2	Mean 10 weeks post-inoculation	Mean 14 weeks post-inoculation
TS	0.73	4.90	TS	4.15	4.40
40-39	0.80	4.18	40-36	2.30	3.00
40-41	0.80	3.34[b]	40-74	2.80	3.10
40-151	0.14[b]	2.70[b]	40-89	2.00[b]	2.50[b]
40-168	0.50	3.74	40-92	1.50[b]	2.50[b]
40-179	0.60	4.78	40-146	2.12[b]	2.50[b]
			40-157	2.30	3.10

[a]Scoring system is base on scale of 0 to 5, 0 = 0% and 5 = 100% scorch (leaf dropped). [b]P value is less than 0.001.

MRI images from stem sections from approximately 15-20cm above point of inoculation reveal clearance of bacterial inoculums in transgenic lines expressing less PD symptoms correlated to a variation in number of vessels clogged between negative control and transgenic lines (**Figure 5**). To obtain MRI xylem vessel cross section images an Avance 400 instrument was used. Instrument setting was: TR: 110.7, TE: 4.5ms, FA: 30.0deg, TA: 1:25NEx4, FOV: 1.2cm, MTX 256/192, Pos-0.80mmF.

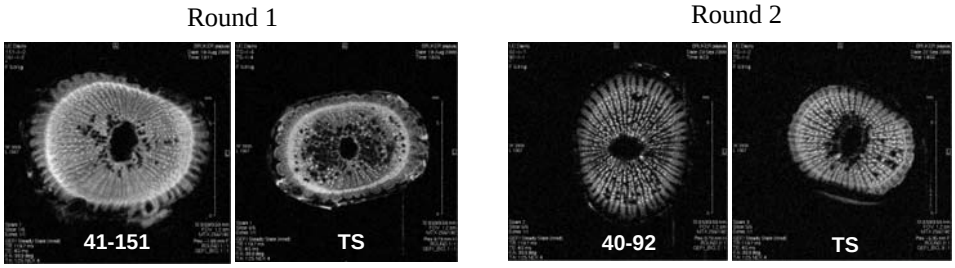

Figure 5. MRI images from experimental and non-transgenic control (TS).

CONCLUSIONS

The main objective of this project is to validate the efficacy of xylem targeted effector proteins like polygalacturonase inhibiting protein (PGIP) to limit movement and a chimeric antimicrobial protein to clear *Xf*.

PGIP transgenic grapevines lines that are secreted with four different signal peptides are being evaluated for their improved ability to secrete PGIP long distance through the graft union. These plants are also being manually inoculated with *Xf* to evaluate increased tolerance against PD associated with increased secretion efficiency associated with specific signal peptide sequence. Initial grafting experiments showed that Chi signal peptide mobilized PGIP efficiently when Chi transgenic plant was used as rootstock to the TS wild plant used as scion.

Eleven HNE-CECB transgenic grapevine lines have been evaluated for PD resistance by inoculating with *Xf*. Several promising transgenic lines showed low or moderate symptoms of PD. MRI stem sectioned images revealed a variation in number of vessels clogged between negative control and HNE-CECB transgenic lines indicating that clearance of the bacteria may be occurring in some of the transgenic lines.

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SYSTEMIC RESISTANCE TO PIERCE'S DISEASE BY TRANSGENIC EXPRESSION OF PLANT-DERIVED ANTI-APOPTOTIC GENES

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Reporting Period: The results reported here are from work conducted October 2008 through September 2009.

ABSTRACT
Xylella fastidiosa (*Xf*) can persist as an endophyte or a pathogen depending on the host with which it is associated. We established that cell death symptoms in susceptible grapes result from the activation of programmed cell death (PCD) pathways with morphological markers of apoptosis. 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 expressed constitutively as transgenes. Using a functional cDNA screen, we identified several novel genes from grape and heterologous plants that suppressed PCD when expressed as transgenes. In 2007, we reported transgenes PR1 and CB456, expressed in the root stock cultivar Freedom, suppressed PD symptoms. In 2008, we reported comparable suppression of PD symptoms and a 4-6 fold reduction in the amount of bacteria present in inoculated transgenic Thompson Seedless plants expressing PR1 and CB456 fused to the 35S promoter when compared with untransformed control plants. All of the untransformed control plants died within two-three months after inoculation while the transgenic plants were asymptomatic up to 12 months, after which they were pruned, and cuttings made for a second inoculation. Current results from 2009 inoculations confirm the protection observed in 2008 and indicate a positive relationship between message level of CB456, a reduction in PD symptoms and a several fold reduction in bacteria titre in the inoculated plants. From the perspective of the grape-bacterial interaction, it appears that the anti-PCD genes tested to date suppress PD symptoms and functionally confine the bacteria to an endophytic ecology in the xylem equivalent to that seen in the related asymptomatic host *Vitis californica*. Experiments underway will determine if the protective effect of these genes is capable of being transferred across a graft union to protect a susceptible scion. In total, eight commercial wine varieties will be evaluated under controlled greenhouse conditions for susceptibility to PD when grafted to transgenic and to untransformed rootstocks as controls.

LAYPERSON SUMMARY
The mechanism by which *Xylella fastidiosa* (*Xf*) leads to death of susceptible grape plants is by activation of a genetically regulated form of programmed cell death (PCD), also known as apoptosis, which is functionally conserved in both animals and plants. Altered expression of known apoptosis-blocking animal and animal virus genes is a widely sought strategy for suppressing disease in animals where cell death is a basis for disease. We developed a functional screen for anti-PCD plant genes, identified six potential anti-PCD genes from cDNA libraries of grape, and transformed them into Pierce's disease (PD) susceptible plants. Two of these grape sequences (PR1 and CB456), when constitutively expressed as transgenes in susceptible grape lines, suppressed PD symptoms and reduced bacterial titer in the inoculated plants. These protective sequences are capable of being secreted outside the cell and will be evaluated for their ability to protect untransformed winegrape scions across a graft union. The current efforts will move the proof-of-concept protection strategy to potential application and quantify the effect of the transgenes on the bacteria resident in the plants. The eight inoculated untransformed winegrape varieties used as controls will simultaneously provide quantitative data on their relative susceptibility of to PD, a data set which at the present does not exist.

INTRODUCTION
Published information from our laboratory and others 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 genetically determined pathways leading to apoptosis or programmed cell death (PCD) (1,2,3,4,9). 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 in this manner they can serve as nutrients for microbial cells when they are present in the immediate environment (1, 7). Hence, bacteria, like *Xylella fastidiosa (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. 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. Hence, this research on Pierce's disease (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. Altered expression of known apoptosis-blocking animal and animal virus genes, or treatment with anti-apoptotic pharmacologically active peptides, and regulatory RNAs have been

shown to block PCD and suppress disease in plants where cell death is a symptom of disease, as is the case of PD (2,3,4,5,6,10).

In the case of *Xf* and many other plant pathogenic bacteria, the bacteria live predominantly as endophytes or epiphytes but occasionally as pathogens. The relative susceptibility of the individual plant species is determined by unknown genetic factors. Presumably, sensitivity to the presence of the bacteria expressed as cell death-dependent symptoms is the result of signals expressed by the bacteria that lead to activation of PCD as appears to be the case with PD. With past funding we 1) developed a functional screen that enabled us to identify six novel genes (out of ~200,000 screened) from grape that suppressed programmed cell death (PCD) in laboratory studies (7, 11). We reported in 2008 (8) that the first of these genes tested involving two different anti-PCD DNA sequences (P14LD and CB456) were very effective in suppressing PD symptoms when introduced into the fruited PD-susceptible cultivar Thompson Seedless. Of equal importance to control of the disease was data indicating that the bacterial titer in the protected plants were reduced in amount by up to two to four orders of magnitude below that reached in untransformed Thompson Seedless vines that are killed within two months after inoculation. Analysis of the disease level in transgenic and non-transformed control plants was based on a five point visual rating scale, measurement of the bacterial titer by quantitative qPCR, and visualization of the GFP-expressing *Xf* by confocal microscopy (8). These results established that protection is related to suppression of symptoms (cell death) and suppression in bacterial multiplication. We have extended the analysis to include additional transgenes, quantitatively measured the amount of bacteria present by qPCR in plants expressing different transgenes, and measured the movement of bacterial in transgenic and control plants. Initial data indicates a positive correlation between the qPCR determined bacterial titre and the message level of CB456; high message, low bacteria titre. Lastly, the protective sequences are active in suppression of PD only when secreted outside the cells suggesting they may function across a graft union. This latter possibility is being tested with eight commercial grape varieties grafted to two different rootstocks expressing PR1 and CB456.

OBJECTIVES

1. Continue to evaluate recently obtained Thompson Seedless transgenic grape plants expressing the six candidate anti-apoptotic genes for blocking of PD symptoms (**Table 1 and Figure 1**).
2. Determine the bacterial movement within an inoculated susceptible grape stem by qPCR over time following inoculation (**Figure 1**).
3. Assess relationships between message level of CB456 and PR1 and the level of bacterial titre in transgenic plants (**Figure 2**).
4. 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* . (Figures 2 and 2A)
5. Assess efficacy of protection against PD across a graft union by PR1 and CB456, first with Thompson Seedless. (**Figure 3**).
6. Perform parallel inoculations of the suite of eight winegrape varieties both on their own rootstocks and on untransformed Freedom and Thompson Seedless rootstocks. This objective addresses the research priority in the RFP regarding short term collection of quantitative data of the relative resistance (susceptibility) of commercial winegrape varieties.
7. Secure patent protection as intellectual property for those genes that prove to be capable of blocking PD in grape.
8. Collaborate with PIPRA to obtain permits to enable field evaluation of transgenic PR1 and CB456 in a location providing for controlled inoculation.

RESULTS AND DISCUSSION

Genes identified as potential anti-PCD genes from the conditional life-death screens and cross graft protection potential.

The protective genes or DNA sequences, isolated by a functional anti-PCD screen, have been described in earlier reports to this symposium and the results of inoculation of the first set of transgenic plants of Cv Freedom and Thompson Seedless were reported in 2007 and 2008 (7,8). In summary of the previous results, resistance against PD was observed in the susceptible grape rootstock by the first two anti-apoptotic transgenes tested, P14LD and 350 bp DNA sequence associated with a plant pathogenic nematode up-regulated gene designated p23. The expression of these two sequences, not only protected the transgenic plants against PD symptoms and plant death but maintained the population of *Xf* at four or more orders of magnitude below the level observed in untransformed plants that died within 2 months (10^7 to 10^8 bacteria per gram of stem tissue) compared with the asymptomatic transgenic plants that carried a level of 10^2 to 10^4 cells/gm stem tissue. We are continuing to extensively evaluate the remaining four potential anti-PCD genes using the easily transformable susceptible test variety Thompson Seedless. These results have been extended to experiments designed to determine if the protection afforded by a rootstock expressing these transgenes can protect the scion of susceptible commercial winegrape varieties. Selected asymptomatic P14LD and CB456 transgenic Thompson Seedless and Freedom (commercial rootstock) plants were propagated and are being grafted to eight commercial winegrape varieties; Chardonnay, Pinot Gris, Sauvignon Blanc, Cabernet Sauvignon, Pinot Noir, Zinfandel, Syrah and Merlot. These combinations are being tested first under controlled greenhouse conditions. Plans are made to for field testing if the greenhouse results are positive. The first set of grafted plants have been inoculated with *Xf.* Data collection is scheduled to begin in November (**Figure 3**; example of micro-propagated grafted plant).

Controlled inoculation of Thompson Seedless grape plants expressing anti-apoptotic genes.
Clonal populations of transgenic lines bearing the genes indicated in **Table 1** and additional copies of transgenic PR1 and CB456, the plants were trained to grow as two or three canes and maintained by periodic pruning of side and top branches as illustrated in **Figure 1**. The transformed plants were individually inoculated April 29 through May 1 of 2009. The inoculation method was by needle puncture of the stem to allow uptake of 20 µl of GFP-tagged Xf at 2 x 10^7 cfu/ml. The plants were monitored visually for symptoms with each plant being photographed at four-five months post-inoculation (**Figure 1**). The level of *Xf* bacteria was monitored by qPCR for bacterial movement and multiplication. Plants were initially scored for disease severity in October 2009, using a five point scale (1=dead and 5= asymptomatic). Representative control (scored as 1) and transgenics (scored as 5) are shown in **Figure 1**. Data collection and qPCR analysis of all the plants in **Table 1** is continuing. We anticipate completion of this extensive data set by the end of 2009.

The effect of anti-apoptotic transgenes on *Xf* bacterial populations measured by qPCR
The effect of blocking PCD-based symptoms in the transgenic PR1 and CB456 plants on the bacterial multiplication and spread is not known but is critical to establish the limiting parameters of protection against PD. The first experiment in this series was to stem-inoculate control and transgenic CB456 plants with *Xf*. Inoculated canes were sampled at 1 cm sections from the point of inoculation to 20 cm above the inoculation site (**Figures 2 and 2A**). Bacterial movement in the susceptible control plant was limited to the first 10 cm at three weeks post inoculation with an average titre of 500 to 1,000 cells per cm of stem but by three months had progressed to the 20 cm distance with a relatively uniform distribution in all sections of 10^6 to 10^7 cells per cm of stem (**Figure 2**). In contrast, the CB456 had no detectable bacteria (<100 cells) in any of the stem sections at the three week interval but cell numbers increased by the three month while the transgenic plants remained asymptomatic. At three months, the distribution of cells in the CB456 transformed plants was uniform over the 20 cm length at a titre of 5x10^3 to 10^4 cells per cm. The net effect of the CB456 transgene under these conditions is to limit bacterial titre but not distribution/movement of bacteria in the asymptomatic plants.

Relationship between the level of *Xf* bacteria in the grape stem to the quantitative expression level of transcript CB456. A question of biological importance is the relationship between the amount of message, bacterial titre in the stem and PD symptoms. **Figure 4** shows the results of the first experiment using CB456 as the target wherein there is a strong positive relationship between high message level and suppression of Xf bacterial titre in the inoculated transgenic CB456. The transcript level and bacterial titre in the stem were measured by simultaneous qPCR (**Figure 4** with representative plants). Another point is apparent from these data; there is a wide range of expression levels of the CB456 transcript seen in independent transformants. This underscores the need to assay multiple transformants as in any genetic screen. The net result is that, given the observed relationship between message level and protection level, it is important to establish the level of transcript expression when selecting individual transformant plants to propagate and carry forward as rootstocks or clonal populations of potential protected plants. These analyses will continue until all current populations of plants bearing the respective transgenes have been characterized.

Assessment of potential protect of untransformed scions from transformed rootstock expressing PR1 and CB456
Micro-grafting is conducted in sterile Magenta GA-7 Plant Culture Boxes (3 x 3 x 4") containing 50 ml media under a 16 h light, eight h dark photoperiod at 25°C. The rootstocks and scions for micro-grafting are selected from established, actively growing plants in agar culture. Rootstock plantlets obtained *in vitro* are allowed to grow until several leaves are produced (six–eight weeks) and divided into three–four explants, each containing a single node. The apex of the rootstock nodal explants arr then cut longitudinally with a new, sharp scalpel blade, producing a small (two–four mm) longitudinal cleft, and placed on the medium. A scion with a single node and a leaf was selected to match the size of the rootstock. The basal part of the stem of the scion was cut into a wedge to match the cleft of the rootstock and was carefully fitted on to the cleft of the rootstock on the medium. After four weeks incubation healing in a magenta box, the rooted plantlet is transferred to sterile soil and covered with an inside out plastic bag to avoid desiccation. The bag is slowly removed over a one week period and the grafted plant is ready to be transferred to the greenhouse for assays. Success rate is greater than 90% using this procedure. The first set of grafted plants was moved to the greenhouse June 2009.

CONCLUSIONS
The experiments and results described herein indicate progress toward identifying DNA transcripts of grape which, if regulation of the natural transcripts is altered in transgenic plants, result in the suppression of symptoms of PD with an associated limitation in bacterial titre to levels generally associated with a benign endophytic association. A natural example of such an asymptomatic endophytic relationship is given by *Vitis californica* where the bacterial titre (~10^2 to 10^4 cells per cm stem) is similar to the asymptotmatic PR1 and CB456 transgenic plants. In contrast, untransformed control plants exhibiting PD death symptoms had bacteria levels four orders of magnitude higher. Further, we observed a positive relationship between high message levels of CB456 with suppressed symptoms of PD and suppressed bacterial titre in the protected plants. To date the most effective of the anti-PCD transgenes are a pathogenesis related gene designated PR1 and a non-coding 350 bp DNA sequence associated with a nematode up-regulated gene designated p23. Rootstocks derived from transgenic PR1 and CB456 plants, shown to be protected against PD symptoms, have been grafted to eight different susceptible commercial winegrape varieties to be tested under controlled greenhouse conditions for the possibility of movement of the protective effect across a graft union. In summary, the current experiments indicate that the effect of the

anti-PCD genes leads to suppression of symptom expression but exert a bacterial growth limiting activity but are not lethal to the bacteria. The symptom suppressive genes do not act as antibiotics and do not affect the non pathogenic endophytic ecology of the bacteria in the xylem.

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FUNDING AGENCIES

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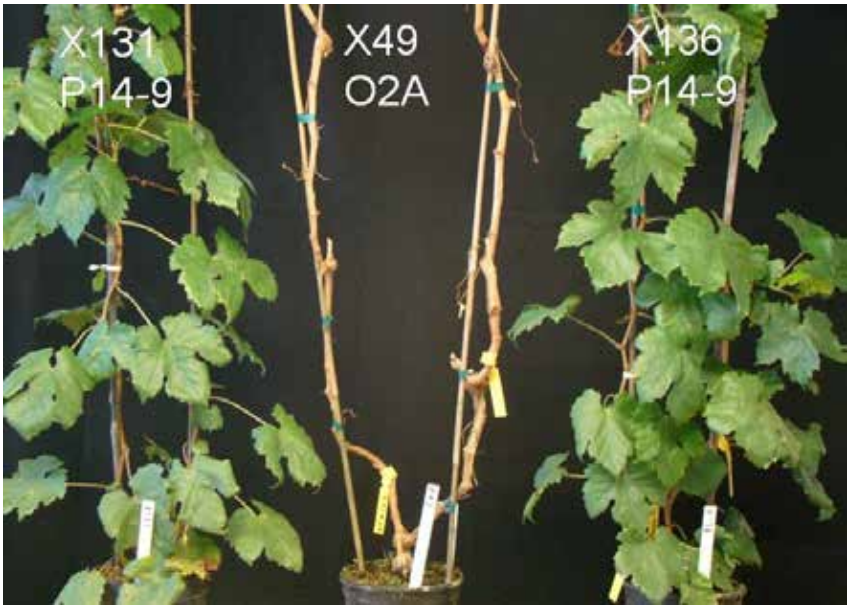

Figure 1. Illustration of two independent transgenic plants expressing the PR1 gene compared with the mock-transformed O2A control. The plants were inoculated with ~2,000 bacterial cells into the stem at the location marked with the yellow tags. This photo was taken 4 months after inoculation.. Bacteria levels determined by qPCR were 10^2 for the PR1 plants vs 10^5 for O2A.

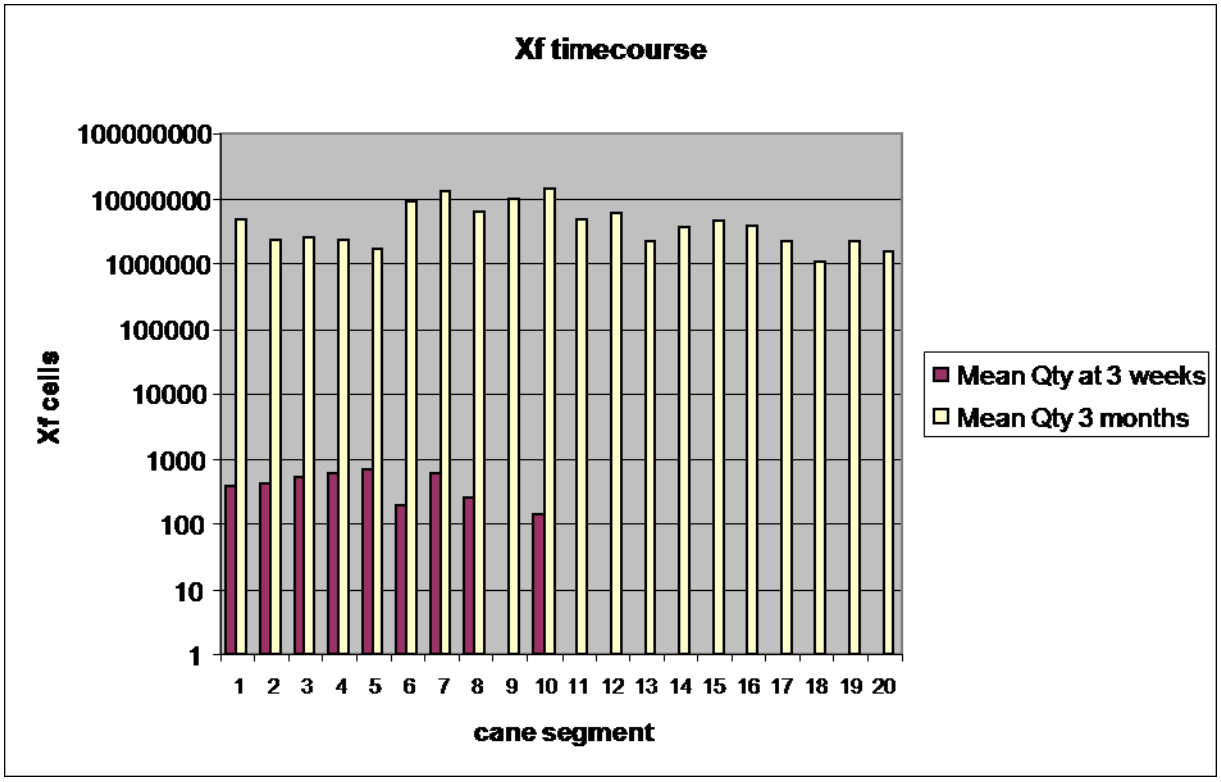

Figure 2. Distribution of bacterial cells in a susceptible grape stem following inoculation of the stem with $2x10^3$ cells of *Xylella fastidiosa*. Sampling of consecutive cane sections was at 3 weeks and 3 months followed by qPCR determination of bacteria concentrations.

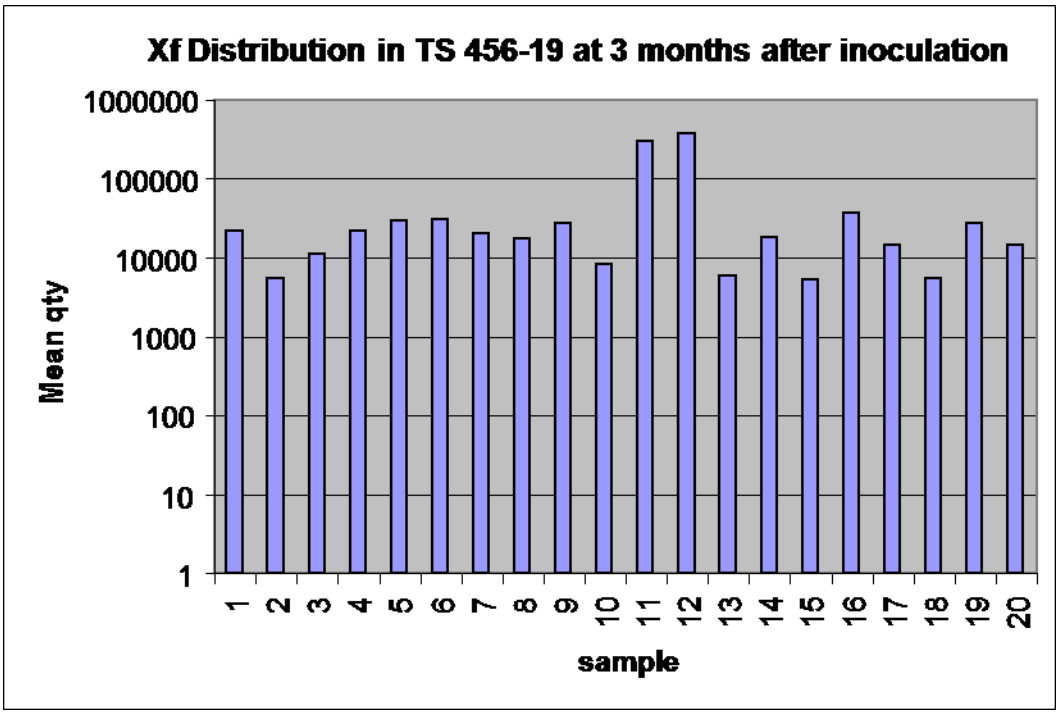

Figure 2A. Distribution of bacterial cells in a CB456 transgenic grape stem following inoculation of the stem with $2x10^3$ cells of *Xylella fastidiosa*. Sampling of consecutive cane sections from the inoculation site (1) in 1 cm sections up to 20 cm (20) was at 3 months followed by qPCR determination of bacteria concentrations. Compare these levels with Figure 2 determination in untransformed Thompson Seedless.

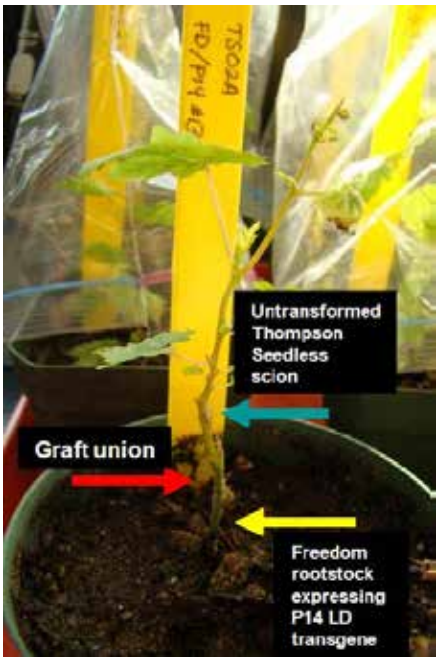

Figure 3. Illustration of grafting of micro-propagated rootstock and scion under sterile conditions

Table 1. Thompson Seedless plants transformed with anti-PCD genes under control of the 35S promoter being tested for susceptibility under controlled greenhouse conditions. Plants, each with two canes, were inoculated in April and May, 2009. First cane assays using qPCR were initiated at 5 months post inoculation (August 2009). © = control plants.

Genotype	# Independent transformants	# of Plants
TS – CBP14B ©	5	5
TS - CBP14LD	18	38
TS - CB376	7	12
TS - CB456	13	28
TS - I35 ©	4	6
TS - CBMT	3	3
TS - CBWG23	5	8
TS - CBWG71	4	6
TS- control ©	7 different genotypes	20
total	66	126

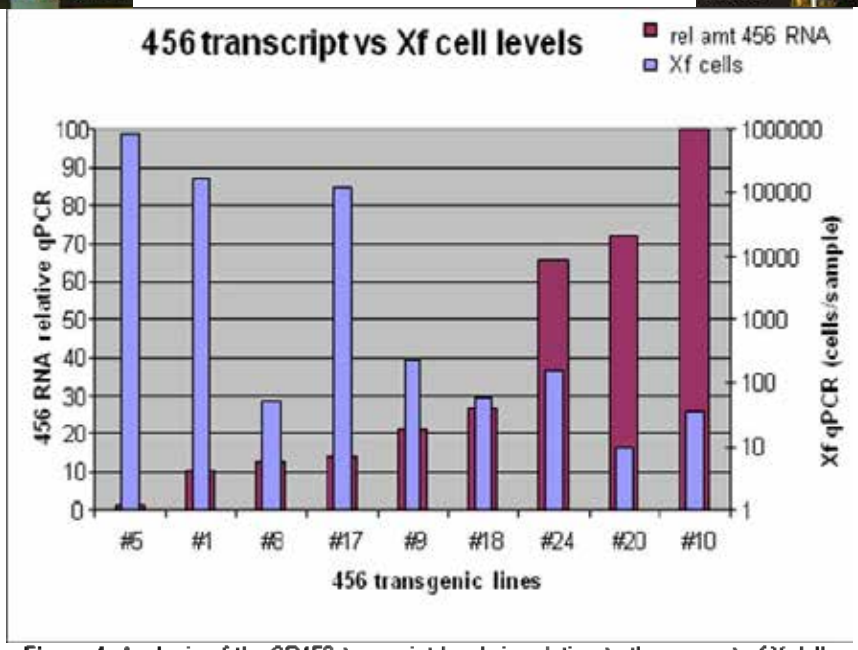

Figure 4. Analysis of the CB456 transcript levels in relation to the amount of Xylella fastidiosa cell in the stems of individual primary transgenic lines stem-inoculated with $2x10^3$ bacterial cells four months prior to sampling. Note there is an inverse relationship between the message level and the amount of bacteria residing in the stem tissues. #1 and #10 are shown as representative plant phenotypes.

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 through September 2009.

ABSTRACT
Fifty Orange Muscat and Cabernet Sauvignon/110R treated with *Xylella fastidiosa (Xf)* strain EB92-1, along with 50 untreated controls of each, were transplanted into plots in the Bella Vista Vineyard in Temecula on July 21-22, 2008. In August 2009, Pierce's disease (PD) symptoms were extensive in the Bella Vista Vineyard, with 35-50% of the vines having symptoms in this first full season in the vineyard. Differences in the incidence of PD between the treated and untreated vines were not significant. Symptoms were generally mild and were less severe in the EB92-1 treated Cabernet Sauvignon vines than in the untreated vines. There were no definitive PD symptoms in the Beringer Vineyard in Napa and only a few possible PD symptoms in the Preston Vineyard in Sonoma. In comparisons of methods of treatment with EB92-1 in 2009, symptoms began to develop in Chardonnay and Merlot that had been injected in different tissues with the biocontrol strain. There were no significant differences in PD among vines injected in the scion, rootstock, or both. In 2009, the incidence of PD symptoms in the UF vineyard with scion from clean Chardonnay was high. Field injection of these clean scion plants with EB92-1 reduced the incidence of PD from 70% to 9%. The scion wood from Chardonnay mother vines infected with EB92-1 had slightly less PD than the uninfected scion wood. This indicates that there could be some transfer of the biological control from the mother plant through scion wood, which would be a preferred treatment method over having to inject every vine by pin pricking.

LAYPERSON SUMMARY
In a test planting of Orange Muscat and Cabernet Sauvignon in Bella Vista Vineyard in Temecula, almost 50% of the vines had developed Pierce's disease (PD) symptoms by August 2009, only 13 months after transplanting. The incidence in the vines treated with EB92-1 was very similar to the vines not treated. Under these kinds of severe PD pressure and high populations of leafhopper vectors in tests in Florida and Georgia, it is often the second year after planting before a beneficial effect from the biocontrol is observed. PD development over the next two years should determine whether the biocontrol strain EB92-1 will be effective under these extreme conditions. In the biocontrol tests in Preston vineyard in Sonoma and Beringer Vineyard in Napa, symptoms that are PD-like have only occurred in a vine or two. There should be enough disease to begin to evaluate the biocontrol in these vineyards next year. Currently, treatment with the biocontrol strain involves needle injection of every grapevine with a suspension of the bacteria. This is a laborious and time-consuming job. We are evaluating the use of mother vines infected with the biocontrol strain EB92-1 as propagation material for scion wood and/or rooted cuttings. If the biocontrol strain can be transmitted through scion wood, it would eliminate the inoculation step. In 2009, vines developed using scion wood from mother vines of Chardonnay infected with EB92-1 had slightly less PD than the uninfected scion wood had after two years in the vineyard in Florida. This indicates that there could be some transfer of the biological control from the mother plant through scion wood. Further observation of these plants will be made next year.

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 (Hopkins and Purcell, 2002). 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 PD is resistance. The results of our 10 years of research on the biological control of PD of grapevine by cross protection with weakly virulent strains of Xf have demonstrated that this is a potential means of controlling this disease (Hopkins, 2005). We have identified at least one strain that was able to control PD in *V. vinifera* for 13 years in Central Florida. We are testing this strain in commercial vineyards on a limited basis and, if these tests are successful, the strain will be ready for commercial use. The overall goal of this project is to develop a biological control system for 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.

In previous research with the biocontrol strain, the bacteria were injected into the grapevines after they were transplanted into the vineyard. This is a labor-intensive procedure. Three methods in order of increasing desirability are vineyard injection, nursery injection, and propagating wood from mother vines that are infected with the biocontrol strain. We are currently evaluating injection of the biocontrol strains into the vines in the nursery, prior to transplanting into the vineyard. The use of scion or rootstock propagating wood from mother vines that are already infected with the biocontrol strain would make this technology less labor-intensive, less costly, and more consistent. It would eliminate any variability in the relative effectiveness of injections into different plants.

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 PD 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 AND DISCUSSION

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

All plants for the vineyard tests in the Bella Vista Vineyard in Temecula, in the Beringer vineyard in the Napa Valley, and in Preston Vineyards in the Sonoma Valley were planted in April, 2008 in greenhouses at UC Davis. 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.

In late fall 2008, PD-like symptoms were observed in most of the vines at Bella Vista, treated or untreated (Observation by Barry Hill). However, it was very hot and dry in 2008 and some of these symptoms may have been due to the weather. In the summer of 2009, PD symptoms were still extensive in the Bella Vista Vineyard, but were observed in only about half of the vines that had symptoms in 2008. Differences in the incidence of PD between the treated and untreated vines were not significant (**Table 1**). Symptoms did appear to be more severe in the untreated Cabernet Sauvignon vines than in the EB92-1 treated vines. The Orange Muscat planting was interspersed with mature vines that were nearly 100% infected with PD. This entire planting, except our experimental vines probably will be removed this year. There have been similar experiments in Georgia and Florida where leafhoppers were abundant and 40% of the vines developed PD in a single year. Under this situation, EB92-1 did not reduce PD incidence during that year, but slowed the spread in the second and third years. The effectiveness of the biocontrol has appeared to increase after the first year in the vineyard. The next two years will indicate whether the biocontrol can be effective under the severe disease pressure in this Temecula vineyard.

Table 1. Effect of EB92-1 on PD incidence in new grape plantings transplanted on July 21-22, 2008 into Bella Vista Vineyard in **Temecula.**

Treatment	% PD[1]	8/25/09 Rating[2]
	Cabernet Sauvignon	
Untreated	35	1.9
EB92-1 treated	40	1.4
	Orange Muscat	
Untreated	50	2.3
EB92-1 treated	44	2.2

[1]Percentage of total vines that have PD symptoms.

[2]Disease rating was an average per symptomatic vine on a scale of: 1 = any symptom of PD, such as marginal necrosis (MN) on a basal leaf or two; 2 = moderate marginal necrosis, more than 5 %; and 3 = severe symptoms.

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. On August 26, 2009, these vines were mapped for symptoms. Most of the Viognier vines had been pruned back to a two bud spur last winter. There were no definite symptoms on August 26. There were a few vines that had minor yellow and/or necrotic leaf margins on the basal leaves of the 2009 growth. Some of these were sampled for the presence of the PD pathogen or the biocontrol strain EB92-1. All of the Barbera vines appeared to be healthy with no PD symptoms. The Viognier block has significant PD incidence in the mature vines and these test vines should begin to develop PD symptoms in 2010. The block of Barbera did not appear to have any PD symptoms, even in the older vines. The disease pressure appears to be very low in this Barbera block.

For transplanting into the Beringer Vineyard in Napa, 50 Reisling/3309 and 50 Chardonnay/3309 were treated with EB92-1 on June 25, 2008 and 50 vines of each were left untreated as controls. The vines were transplanted in Beringer Vineyard in early April 2009. On August 26, these vines had not started to develop PD symptoms. Many of these vines were exhibiting drought stress.

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

On May 29, 2007, Merlot/101-1 plants were injected with EB92-1 in the greenhouse. Treatments were (1) EB92-1 in scion only, (2) EB92-1 in rootstock only, (3) EB92-1 in both rootstock and scion, and (4) Nontreated. On June 21, vines were transplanted into the vineyard in 3 replications of 3 plants per treatment. On June 13, 2007, Chardonnay CL96/3309 were injected with EB92-1 in the greenhouse. Treatments were (1) scion only, (2) rootstock only, (3) rootstock plus scion, (4) nontreated, and (5) scion only after transplanting into vineyard (These injections were done on July 26). On July 3, vines were transplanted into the MREC vineyard.

In 2009, PD began to occur in a number of these vines, especially in the Chardonnay test (**Table 2**). There was no significant difference among treatments, but symptoms were very mild and often do not develop any further in plants treated with EB92-1. There was less PD in the Merlot plants, with two treatments having no symptoms.

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

	% PD incidence in September 2009 in:[1]	
Treatment	**Merlot/101-14**	**Chardonnay/3309**
Scion injection	0	29
Rootstock injection	0	43
Scion & Rootstock injection	25	33
Scion field injection	-	29
Untreated	11	38

[1]%PD is the number of plants with symptoms divided by total number of plants x 100.

Plants of Chardonnay/Salt Creek were obtained by grafting green cuttings from Chardonnay plants from the vineyard onto rooted cuttings of Salt Creek. The grafting was done between May and July in 2007. Grafted plants were transplanted into the vineyard on August 14, 2007. Treatments included (1) Cuttings from Chardonnay not infected with EB92-1 on Salt Creek, (2) Cuttings from EB92-1 inoculated Chardonnay on Salt Creek, and (3) Cuttings from Chardonnay not infected with EB92-1 on Salt Creek, but injected in the vineyard with EB92-1 on August 29. In the first year, there were no significant differences among the Chardonnay/Salt Creek treatments.

In 2009, the incidence of PD symptoms in the scion from clean Chardonnay was high (**Table 3**). As expected, field injection of these clean scion plants with EB92-1 reduced the incidence of PD from 70% to 9%. Plants developed using scion wood from mother vines of Chardonnay infected with EB92-1 had slightly less PD than plants developed with uninfected scion wood. This indicates that there could be some transfer of the biological control from the mother plant through scion wood. Further development of the symptoms will be observed. This evaluation of scion from treated mother vines is especially significant, because scion wood from infected mother vines would be a preferred treatment method over having to inject every vine by pin pricking.

Table 3. Transmission of biocontrol in scion from infected Chardonnay mother plant grafted onto Salt Creek rootstock.

	% PD incidence in September 2009:
Treatment	
Scion from clean Chardonnay	70
Scion from clean Chardonnay injected with EB92-1 in the field	9
Scion from EB92-1 Chardonnay mother plant	55

CONCLUSIONS

The biocontrol strain did not reduce the incidence of PD in the Bella Vista Vineyard in Temecula. Leafhopper vectors are not controlled in this vineyard and PD incidence is extremely high in the vineyard. Plants treated with EB92-1 did appear to have milder symptoms and than the untreated plants. Symptoms are just beginning to develop in the tests in Sonoma and Napa. Vines developed using scion wood from mother vines of Chardonnay infected with EB92-1 had slightly less PD than vines developed with uninfected scion wood after two years in the vineyard in Florida. Development of plants with scion

wood from infected mother vines would be a preferred treatment method over having to inject every vine by pin pricking. The successful completion of the biocontrol tests in Temecula, Sonoma, and Napa would lead to an effective control of PD that is environmentally friendly. This project should yield results within the next two years and if the control is successful, there should be a biological control for PD available for commercial use in vineyards in California.

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IDENTIFICATION AND UTILIZATION OF COLD TEMPERATURE INDUCED GRAPEVINE METABOLITES TO MANAGE PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted July 2009 to October 2009.

ABSTRACT
This work builds on discoveries made in the past six years of research on better understanding the mechanism(s) responsible for the PD(PD) cold curing phenomenon. A thaumatin-like (TLP) grape protein was found in elevated levels in the xylem sap from cold-exposed vines. We have cloned and expressed TLP in *E .coli* and our preliminary finding show that crude TLP protein extract possesses anti-*Xylella fastidiosa (Xf)* activity *in vitro*. Greater amounts of total phenolics were measured in xylem sap extracted from cold-exposed vines. We are beginning to characterize these phenolic compounds, and assess their potential anti-*Xf* activity *in vitro*.

LAYPERSON SUMMARY
Previous work on "cold curing" of Pierce's disease (PD) affected grapevines led to the identification of thaumatin-like protein (TLP) in grapevine xylem sap. TLP is expressed in greater amounts in vines that have been exposed to cold temperatures and may be associated with the cold curing phenomenon. Currently we have cloned and expressed TLP in *E. coli*. Producing TLP in *E. coli* should allow us to produce enough protein to better evaluate the role of TLP in curing of PD. Crude extracts of *E. coli* expressing TLP were applied to PD3 medium plates and initial results showed TLP-amended medium plates greatly inhibited the growth of one strain of *Xylella fastidiosa*. We are currently working on producing purified and biologically active TLP. Previous work also identified polyphenolic compounds as a possible mediator of the "cold curing" phenomenon. Here, we show that a specific polyphenolic compound, resveratrol, is produced in vines that experience cold curing, while it is absent from grapevines grown in warmer environments.

INTRODUCTION
In our previous project we characterized many biological parameters of xylem sap from cold-exposed (freezing temperatures) and "warm", (non-freezing) temperatures in both field grown and cold chamber exposed grapevines. We found that *Xylella fastidiosa (Xf)* infected potted grapevines that were exposed to freezing temperatures at several sites in northern California and vines exposed to -5C in cold chamber emerged pathogen free the following summer (Meyer and Kirkpatrick, 2004-2008). We measured many different biological parameters, such as pH, organic acid, sugar and ion concentrations, and osmolarity in Pierce's disease (PD) susceptible *Vitis vinifera* 'Pinot Noir' and PD-less susceptible *V. vinifera* 'Cabernet Sauvignon' grapevines over three winters.

One of the parameters determined in these previous studies was the protein profiles of cold- and warm-treated xylem sap. One of these proteins, a thaumatin-like protein (TLP), was significantly up regulated in cold exposed vines. We have cloned and expressed the *V. vinifera* TLP protein and showed some inhibition of *Xf* growth when crude protein extracts from TLP-expressing *E. coli* were applied to PD3 medium plates. Work is currently being conducted to purify and demonstrate the biological activity of recombinant TLP protein.

We have also been assessing the potential role that xylem sap phenolic compounds may play in the "cold curing" process. In collaboration with the Waterhouse lab at UC Davis, we have characterized the phenolic compounds in cold and warm xylem sap by HPLC/MS, and identified that the major polyphenol in cold-exposed xylem sap is trans-resveratrol. We have also begun to evaluate the potential toxicity of trans-resveratrol to *Xf*.

OBJECTIVES

1. Over-express the grapevine thaumatin-like protein (TLP) in transgenic grapevines. Prepare anti-TLP antibodies to quantify TLP in transgenic xylem sap using ELISA and western blot analyses..
2. Inoculate TLP-expressing grapevines with *Xf* and determine the incidence and severity of PD in TLP-transgenic versus non-transgenic *V. vinifera*.
3. a. Fractionate and chemically characterize the phenolic compounds that are present in xylem sap from cold-exposed grapevines.
   b. Compare the phenolic compound composition and concentration in xylem sap extracted from cold- and warm-exposed *V. vinifera* grapevines as well as grapevines treated with abscisic acid (ABA) under non-freezing conditions.
   c. Determine if these compounds affect *Xf* growth/survival *in vitro.*
4. Determine if foliar and drench applications of ABA can increase PD-curing rates in field-grown vines under non-freezing conditions.

RESULTS AND DISCUSSION

We have cloned and expressed *Vitis vinifera* thaumatin-like protein in *E.coli* (**Figure 1**). We have used various methods of protein expression and purification to attempt to produce TLP protein that is both relatively pure and biologically active. The presence of disulfide bonds in the protein structure has presented some challenges as much of the recombinant TLP protein is found as inclusion bodies in E. coli. We are continuing to try different approaches to overcome some of the problems we have experienced.

Xylem sap total phenolics from ABA-treated and non-treated controls have been measured. Trends showing that total phenolics were found in higher concentrations in cold-exposed vines were also seen in the ABA-treated vines (Meyer and Kirkpatrick, 2008). In addition, the phenolic content in ABA-treated vines was higher than non-treated vines (**Figure 2**).

Xylem sap was expressed from dormant 'Cabernet Sauvignon' and 'Pinot Noir' grape vine canes obtained from the Chateau Leidigh Estate Winery located in Placer County in February, 2009. Previous work has shown that grape vines infected with *Xf* show significant recovery when exposed to cold winter temperatures, such as those experienced in this Placer county vineyard. As a control, we also collected sap from Davis grown vines where curing, due to warmer temperatures, is significantly less than that observed in Placerville. Sap was expressed by placing canes in a "pressure bomb", allowing one end of the cane to protrude from the cylinder, and then pressurizing the chamber with air to pressures between 300 and 400 psi, to collect the xylem sap exudate. These samples were kept frozen at -80C until they were analyzed by High Performance Liquid Chromatography/Mass Spectrometry by Mauri Anderson of the Waterhouse Lab.

The xylem sap samples were chromatographed using reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with electrospray ionization (ESI) mass spectrometry (MS), which produced good resolution. Phenolic compounds were identified based on retention time, UV spectra from diode array detection, and MS using commercially available reference standards. In the Placerville (cold) Pinot Noir samples, a number of phenolic compounds were identified: B procyanidins, catechin, epicatechin, trans-resveratrol, caftaric acid, and a resveratrol tetramer. Cabernet Sauvignon samples produced an identical polyphenol profile except that the resveratrol tetramer was not present. Interestingly, the warm Pinot Noir sap lacked characteristic peaks for trans-resveratrol as well as the resveratrol tetramer. The fact that resveratrol is present in vines that experience "cold curing" while it is absent in vines that do no undergo "cold curing" suggests that resveratrol may play a role in the curing process.

We decided to analyze trans-resveratrol's potential anti-*Xf* activity *in vitro* because it was the most abundant polyphenol in cold xylem sap based on the RPHPLC/MS analyses. Resveratrol has also been shown to have positive effects on human cardiovascular health, and negative effects on several diverse microbes including certain bacteria (*Chan 2002*, and *Frémont 2000*), life, which further justified our interest in evaluating its effects on *Xf.*

Commercially available Trans-Resveratrol from Sigma-Aldrich (product number R5010) was used in a plate based assay. A 7.5 µg/ml stock solution of Trans-Resveratrol was prepared by dissolving 7.5 mg of trans-resveratrol in 100 ml of water overnight, and in the dark to prevent isomerization to the cis-resveratrol form (*Bonnefont-Rousselot et al. 2009*). Solid PD3 media was autoclaved for 15 min and allowed to cool to approximately 50-60C (comfortable to the touch). 100 µl and 250 µl of the trans-resveratrol stock solution were added to 500 mls of PD3 media to achieve concentrations of 15 ng/ml and 38ng/ml respectively. These values were chosen based on the concentrations of trans-resveratrol detected in xylem sap from cold exposed grapevines. The plates were allowed to solidify in the dark for two hours. After solidification, approximately 10 µl of a 10^8 CFU/ml suspension of *Xf* 'Fetzer' or *Xf* 'Temecula' were spread onto each plate using a plastic inoculating loop. These plates were then placed in a crisper which had been wrapped with aluminum foil, to prevent light from causing isomerization of the trans-resveratrol, and incubated for 10 days at 28C. Both strains grew well on PD3 plates which contained no trans-resveratrol, while there was no apparent growth of *Xf* Temecula on the reserveratrol supplemented PD3

plates. Surprisingly, similar growth inhibition was not observed with the 'Fetzer' strain (**Figure 3**). This experiment has only been performed once and it is now being repeated with the same, as well as additional, strains of *Xf*.

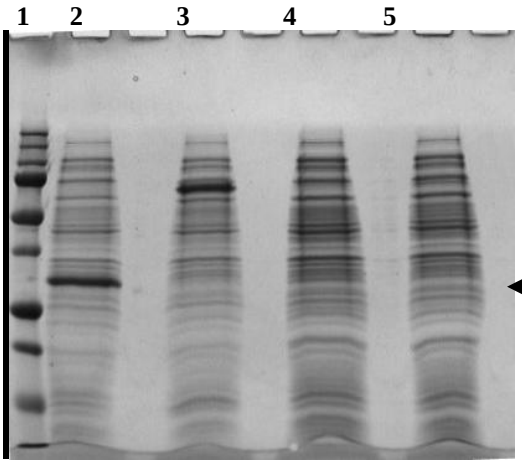

Figure 1: Thaumatin-like Protein (TLP) that was cloned and expressed using a *E. coli* expression vector. Note that arrow points to the correct size of grapevine TLP protein (~35 kD).

Lane1: Dual color SDS ladder.
Lane 2: Cell lysate from IPTG induced *E. coli* with TLP construct.
Lane 3: Cell lysate from IPTG induced *E. coli* with a polygalacturonase (PG) construct (positive control).
Lane 4: Cell lysate from *E. coli* with TLP construct, not induced.
Lane5: Cell lysate from *E. coli* with PG construct, not induced.

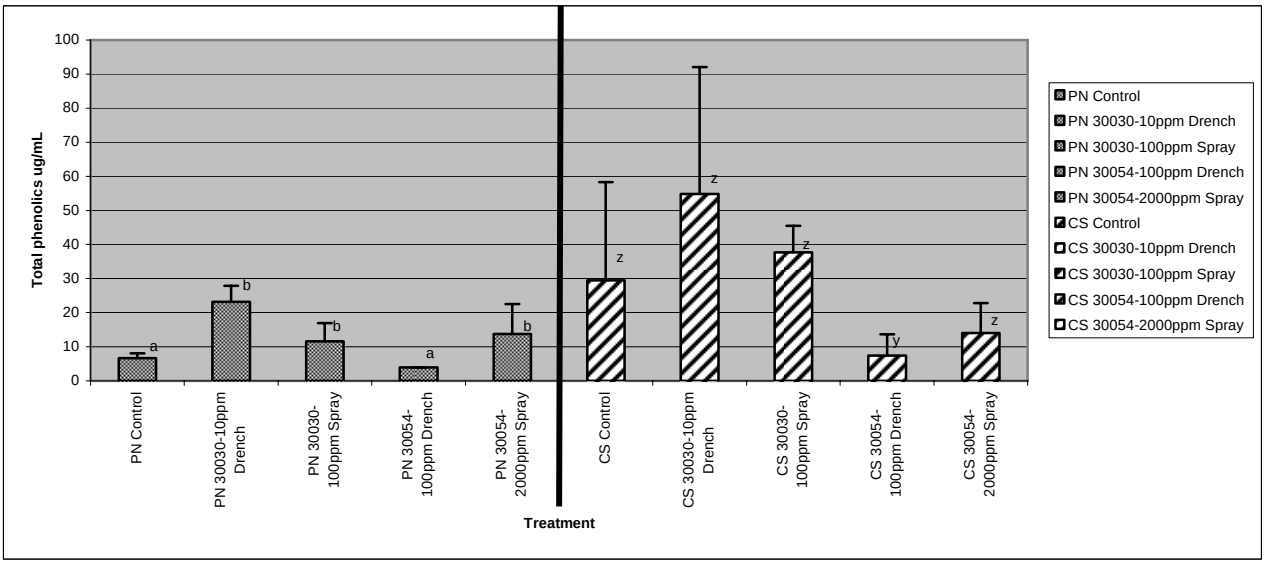

Figure 2. Total phenolic content of xylem sap from ABA treated vines as measured by a gallic acid colorometric assay. PN = Pinot noir xylem; CS = Cabernet sauvignon xylem sap. 30054 = a natural ABA, 30030 a chemically modified ABA. Different letters are significantly different by unpaired t-test with a 2-tailed p-value<=0.05

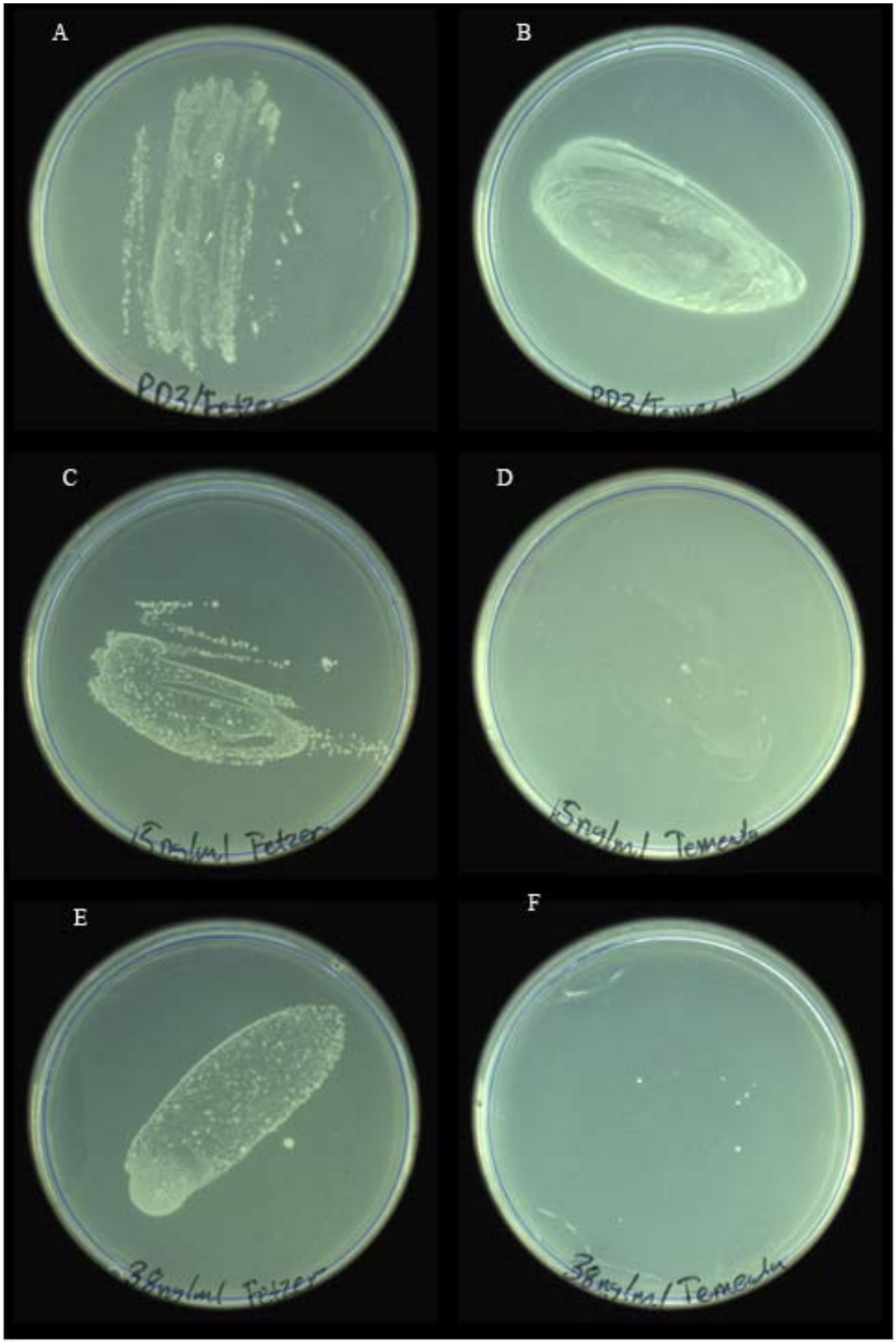

FIGURE 3. Results of plating Xf Fetxer and Xf Temecula on PD3 media (A and B) and PD3 media containing 15 ng/ml Resveratrol (C and D) and 38 ng/ml Resveratrol (E and F). The PD3 plates containing Resveratrol and Xf Temecula showed much less growth than Xf Fetzer at the same Resveratrol concentrations

CONCLUSIONS

Though the production of a purified and biologically active TLP protein has had its challenges due to the formation of TLP inclusion bodies, we believe that TLP protein in the inclusion bodies can still be used in the production of good quality anti-TLP antibodies. Efforts using other expression systems and methods to denature and renature the TLP inclusion bodies are

underway. The anti-TLP antibodies will be used to quantify TLP in xylem sap from cold and ABA-treated grapevines and detect TLP in transgenic grapevines. Assuming that TLP expressed in grapevine sap has increased anti-*Xf* activity, it is possible that TLP-expressing transgenic grapevines could decrease the incidence and/or severity of PD.

Phenolic compounds, specifically trans-resveratrol, show promise as agents that are harmful to the growth of at least one strain of *Xf*. The results of our plate assay are supported by the fact that we detected no resveratrol in warm winter sap collected in Davis, where we observe significantly less overwinter curing than in Placerville. It has been previously reported that resveratrol production in *Vitis vinifera* can be up-regulated by several diverse factors such as plant injury, UV light exposure, and pathogen invasion (*Pryce et al. 1976*, and *Gautheron et al. 1991*). It is possible that cold temperatures may serve as an external stress that increases the production of trans-resveratrol.

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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 2009 to September 2009.

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 result in lost pathogenicity and 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 enzymatically 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 antibody (scFv) libraries 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 root stock to determine if the peptides can provide protection to the plant from Pierce's disease.

LAYPERSON SUMMARY
This period we have again made progress on Objectives 1-3. We have been able to overcome the solubility issues with expression of *Xylella fastidiosa (Xf)* PG in E. coli using FPLC techniques. We now are able to produce large quantities of soluble purified *Xf* PG. We also hope that the *Pichia pastoris* yeast expression system we are currently using will provide us with active *Xf* PG. Additionally; we have completed the panning procedure for peptide 2 and the commercially available *Aspergillus aculeatus* PG. We have shown that the antibodies raised against peptide 2 can bind to full length *Xf* PG, and thus have produced our first candidate monoclonal phage to use in the inhibition assays. We hope to soon have enough active *Xf* PG to test these phages in inhibition assays

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 lost 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 Pierce's disease (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 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 compared to non transgenic plants in PD symptom progression. If significant disease inhibition is shown we will use these transgenic grapevines as rootstock and see if they can also provide resistance to grafted scions.

OBJECTIVES:
1. Isolate a sufficient amount of biologically active *Xf* 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 peptidesspecific 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 PD development with inoculated, non-transgenic control plants.

RESULTS AND DISCUSSION

Objective 1.

The method we described previously for generating active *Xf* PG remains the method that delivers the most protein in active form, however we would still like to obtain greater amounts of active *Xf* PG. The biggest problem we have had with expressing *Xf* PG in *E. coli* is that recombinant *Xf* PG aggregates in inactive, insoluble, inclusion bodies. We have been able to overcome the solubility problem of expressing *Xf* PG in *E. coli* and can now produce large quantities of purified soluble *Xf* PG. We were able to first solubilize recombinant protein in a denaturing buffer. The *Xf* PG was then bound to a FPLC metal affinity column (Ni^{2+}). While the *Xf* PG was bound to the column we slowly renatured it using buffer exchange to remove the denaturant. Once the all of the denaturant was removed we eluted purified soluble *Xf* PG (**Figure 1**). We have not been able to detect polygalacturonase activity in assays of the FPLC solubilized and purified *Xf* PG thus far, however because the protein is soluble we are continuing our efforts to detect activity using this preparation. We are also currently using this solubilized *Xf* PG in panning experiments with the Tomlinson I and J phage libraries.

Additionally, we are using a *Pichia pastoris* yeast expression system to produce active PG. The *Pichia pastoris* expression system has a few advantages; it can form disulfide bonds in the cytoplasm unlike *E. coli* and it is able produce glycosolated proteins which an E. coli expression system cannot. Additionally, *Pichia pastoris* is able to secrete recombinant proteins into the medium making the following protein purification and activity assays steps easier. We are currently screening our *P. pastoris* expression constructs for *Xf* PG production.

As was reported previously 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.

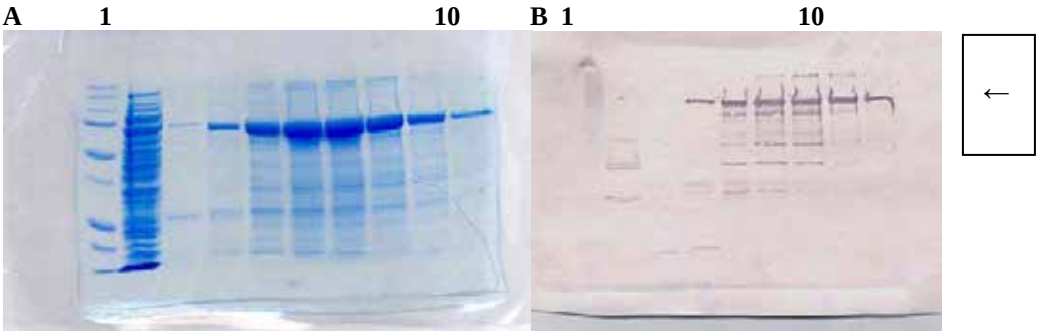

Figure 1. **A:** Coomassie stained Polyacrylamide gel electrophoresis of purified, soluble *Xf* PG fractions from FPLC refolding/ purification experiment, arrow denotes correct *Xf* PG band size. Lane 1 Bio-rad dual color protein ladder, lane 2 column flow through, lanes 3-10 soluble *Xf* PG fractions **B:** Western blot analysis of purified, soluble *Xf* PG fractions from FPLC refolding/purification experiment using *Xf* PG polyclonal antibodies. Lane 1 Bio-rad dual color protein ladder, lane 2 column flow through, lanes 3-10 soluble *Xf* PG fractions

Objective 2.

We have done extensive *in silico* analyses of the enzymatically 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 amino acids, consisting 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 may be located on the protein, and which amino acids are involved in catalysis and substrate binding, we had synthesized two 14mer peptides derived from the *Xf* PG sequence, one which will target the active site directly and a second that will target an area providing entry into the active site. Additionally, these peptides were injected into rabbits to create polyclonal antibodies. These polyclonal antibodies were used in a western blot that confirmed that the antibodies created against each 14-mer peptide could also recognize full length *Xf* PG (**Figure 2**).

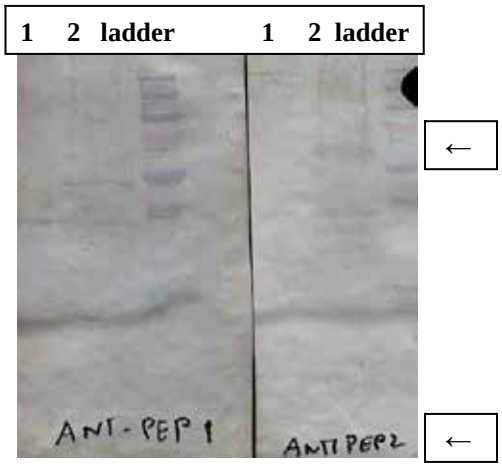

Figure 2. Lane 1 is *E.coli* lysate containing no *Xf* PG. Lane 2 is *E. coli* lysate containing recombinant *Xf* PG. Arrow represents the location of the *Xf* PG band.

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 scFvs showed a higher binding affinity to BSA conjugated peptide 2 than to BSA alone, or to the wells of the plate. With this knowledge 90 individual colonies from each library were picked from the third round phage pool and used in a monoclonal ELISA to determine which monoclonal scFvs had the highest binding efficiencies. The 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 indentify full length recombinant PG (**Figure 3**) (Tanaka et al 2002).

Additionally, we have finished phage panning against the commercially available *Aspergillus aculeatus* PG and are nearly finished panning against the solublized *Xf* PG. We are currently screening the monoclonal phages for specificity to *Aspergillus aculeatus* PG and *Xf* PG. Once we confirm specificity for either target we will use these phages in PG inhibition assays.

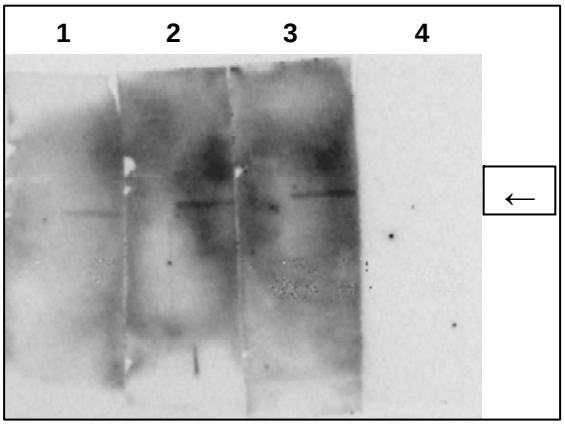

Figure 3. Western blot analysis of three representative monoclonal scFv phages (J-library). Lanes 1-3 are *E. coli* lysate containing recombinant *Xf* PG each membrane strip was reacted with a single monoclonal phage from the third round of panning. Arrow represents location of *Xf* PG band. Molecular 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 finding suitable PG activity assays to use in the PG-inhibition assays. We are currently exploring different expression systems to generate more active PG to use in phage panning and activity assays. We have acquired 16 candidate scFv phage, by panning against peptide 2 conjugated to BSA that are capable of indentifying full length *Xf* PG that we will be using in *Xf* PG inhibition assays, as we have described previously. If one of the candidate phage can inhibit *Xf* PG activity *in vitro* then we can transform grapevines with the peptide and determine if they provide plants with resistance to Pierces 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 2008 to September 2009.

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. In previous work, we showed that *Xf* HA proteins are secreted into the supernatant as soluble proteins, inserted into the outer membrane, and associated with membrane vesicles. Furthermore, the HA proteins are secreted via a two-partner secretion pathway and processed from a predicted molecular weight of 360 kD to a 220 kD mature protein. Based on this information we generated HA-expressing transgenic *Nicotiana tabacum* SR-1 (Petite Havanah) tobacco and *Vitis vinifera* cv. Thompson seedless grapevines. We show that the T-DNA is successfully integrated into the tobacco genome and that HA mRNA is transcribed in the transformed plants. The T_2 tobacco generation is being evaluated for resistance against Pierce's disease (PD). We hope that the expressed HA protein will act as "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.

LAYPERSON SUMMARY
Hemagglutinin (HA) proteins play an important role in adhesion and biofilm formation of *Xylella fastidiosa* (*Xf*). Previous studies by Guilhabert and Kirkpatrick, 2005, showed that mutants in the identified HA genes no longer formed clumps in liquid medium like wt *Xf* cells (1). Clearly the HA proteins play an important role in mediating cell-cell interactions. Research in the Almeida lab has also shown that HA proteins play important roles in attachment processes during vector transmission (2). Research conducted in our lab showed that HA proteins are present in the outer membrane of *Xf* cells and that these proteins are also secreted into culture medium and in vesicles at low concentrations. The 10.5 kb HA genes should theoretically encode a protein of approximately 360 kD, however we showed that the native size of the HA proteins in the outer membranes, culture supernatants and membrane of vesicles is approximately 220 kD (3). The cleavage site lies downstream of the N-terminal 2300aa and approximately one third of the C-terminal part is cleaved off (3). Based on this information, we created two different binary plasmids that we used for transformation of tobacco and grapevines. The first, smaller construct codes for the N-terminal hemagglutination domain that is proposed to mediate clumping of *Xf*. The second, larger construct codes for the mature 220 kD protein that we found to be secreted. Here, we show that the transformation was successful and that the tobacco plants express the HA gene. We are now evaluating the second transgenic tobacco generation for resistance against PD. We believe that the expressed HA proteins may mediate clumping of insect inoculated *Xf* cells. Therefore, the *Xf* infection may stay local at the point of introduction in form of cell clumps, and spreading of *Xf* cells through the plant can potentially be minimized. Regular pruning of grapevines in fall might eliminate the infections, leaving healthy plants behind.

INTRODUCTION
Xylella fastidiosa (*Xf*) hemagglutinins (HAs) are large secreted proteins that play important roles in mediating cell-cell aggregation and plant pathogenicity. Mutations made in either HA gene HxfA (PD2118) or HxfB (PD1792) resulted in strains that did not aggregate in liquid culture and 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 proteins in the xylem of transformed grapevines, the HAs can act as a "molecular glue" to clump *Xf* cells and retard their ability to systemically colonize grapevine and cause Pierce's disease (PD). In our previous work we showed that *Xf* HA proteins contain an N-terminal hemagglutination domain that is putatively responsible for the aggregation of *Xf* cells (3). We also showed that the HA proteins are secreted into the outer membrane and into the supernatant, and that they are processed from a predicted 365 kD pre-protein to a mature 220 kD protein (3).

Here we describe the generation of transformed tobacco and grapevine plants by using two constructs for agrobacterium mediated plant transformation; firstly, plants were transformed to express the N-terminal portion of HxfB protein containing the hemagglutination domains, and secondly, plants were transformed to express the portion of HxfB corresponding to the mature 220 kD protein.

OBJECTIVES

1. a. 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.
2. a. 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.
3. a. 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 1 and 2

The results of these objectives have been reported in the Proceedings, 2008 Pierce's Disease Research Symposium. CDFA, Sacramento, CA.

Objective 3

Transformation of tobacco and grapevines

The 5' part of the HA gene coding for the hemagglutination domain (AD1-3), and the longer part coding for the 220 kD protein (220) were PCR amplified from the gene HxfB (PD1792). The resulting 4000 bp and 6300 bp PCR products were cloned into pCR-2.1-TOPO and sequenced using primers generated every 600 bp on the gene sequence to confirm the integrity of the cloned fragments. To enable secretion of the expressed HA proteins outside the eukaryotic cells of tobacco and grapevines, a signal peptide pGIP (4) was synthesized by the company DNA2.0 (Menlo Park, CA) and fused N-terminally to the cloned HA products. The codon usage of the synthesized signal peptide was optimized for expression in eukaryotes. The pGIP-HA fusions were cloned into vector pDE00.0113 (Dandekar lab) containing the 35S promoter and ocs3' terminator creating pDE00.0113-pGIP-AD1-3 and pDE00.0113-pGIP-220. Upon verification by sequencing, pDE00.0113-pGIP-AD1-3 was digested with AscI and the resulting cassette cloned into the binary vector pDU97.1005 (Dandekar lab) creating pDU08.2407. The plasmid was transformed into Agrobacterium tumefaciens strain EHA101 and the culture submitted to the Ralph M. Parsons foundation transformation facility on the UC Davis campus for transformation of tobacco SR-1. Unfortunately, the pDU97.1005 marker gene nptII confers resistance to kanamycin. In transformation experiments with grapevines using binary plasmids containing the nptII gene, many escapes were observed that prolonged the time needed to generate transformed grapevines. Therefore, we digested pDE00.0113-pGIP-AD1-3 and pDE00.0113-pGIP-220 with EcoRI and ligated the obtained cassette into the binary plasmid pCAMBIA1300 (Canberra, Australia). Vector pCAMBIA confers resistance to hygromycin. This marker gene is more suitable for transformation of grapevines than nptII and is functional in grapevines as well as is tobacco. Binary plasmids pCAMBIA-pGIP-AD1-3 and pCAMBIA-pGIP-220 were transformed into Agrobacterium tumefaciens strain LBA4404 and the culture submitted to the Ralph M. Parsons foundation transformation facility for transformation of Thompson seedless grapevines as well as transformation of tobacco SR-1 for pCAMBIA-pGIP-220.

Analysis of transformed tobacco plants

Four months after submission of the constructs to the transformation facility, 11 transgenic tobacco plants T0 representing single transformation events were obtained for both plasmids pDU-pGIP-AD1-3 (lines 1-11) and pCAMBIA-pGIP-220 (lines A-K). The lines were maintained in a growth chamber at the controlled environmental facility (CEF, UC Davis, CA) at 25°C with a photoperiod of 16 h and 50% relative humidity. Genomic DNA was isolated and PCR with primers that bind to the HA gene was positive for 10 out of 11 tobacco plants for each construct. Untransformed wild type plants were used as negative control **(Figure 1).**

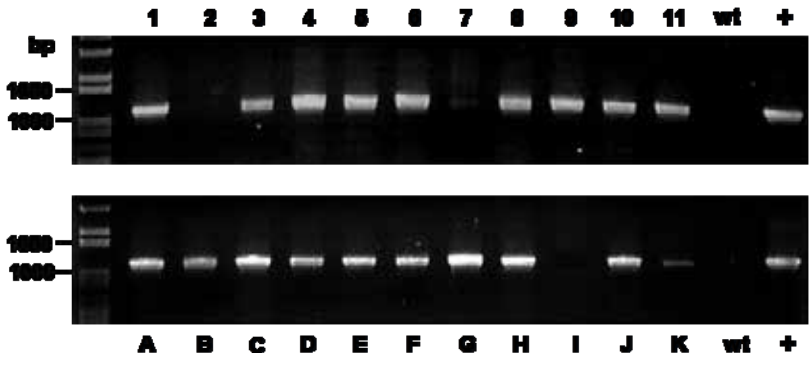

Figure 1: Confirmation of T-DNA insertion into the genome of tobacco SR-1 by PCR analysis of genomic tobacco DNA. Numbers 1-11 indicate transgenic lines that were transformed with pDU-pGIP-AD1-3, letters A-K indicate transgenic lines that were transformed with pCAMBIA-pGIP-220. Lines 2 and I do not have the T-DNA insertion. Wild type plants were used as negative controls and isolated plasmids pDU-pGIP-AD1-3 and pCAMBIA-pGIP-220 were used as positive controls (+).

RNA was extracted from all PCR positive lines and cDNA generated by reverse transcription. PCR analysis using primer pair pGIP-HAfor and HArev confirmed that all plants were expressing the transgene coding for AD1-3 **(Figure 2).**

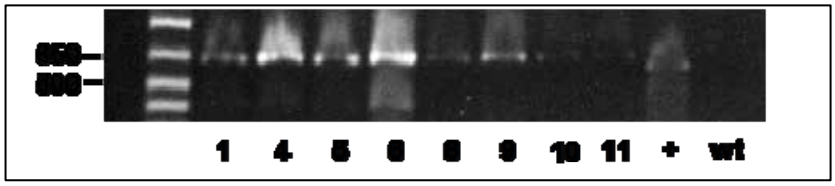

Figure 2. RT-PCR of transformed tobacco SR-1 using primer pair pGIP-HAfor and HArev. Numbers indicate transgenic lines that were transformed with pDU-pGIP-AD1-3. Wild type plants were used as negative controls and isolated plasmid pDU-pGIP-AD1-3 was used as positive control (+).

After three months, seed pods of the HA expressing T0 plants were harvested, the seeds sterilized and plated for germination of the T1-generation on MSO medium supplemented with kanamycin sulfate or hygromycin B, according to the selectable marker gene present on the T-DNA. For plants transformed with pDU-pGIP-AD1-3, 8 out of 10 plants germinated in a 1:3 segregation pattern according to Mendel on MSO supplemented with kanamycin **(Figure 3).** The germinated 75% seedlings are either homo- or heterozygous regarding the transgene. The remaining 25% seedlings are azygous. Although PCR positive, lines 3 and 7 did not germinate on the selective medium. It is possible that the transgene is located in an area of the tobacco genome where expression is silenced; these lines were not further considered. For plants transformed with pCAMBIA-pGIP-220 all 10 PCR positive lines were germinating in 1:3 segregation pattern on MSO supplemented with hygromycin B.

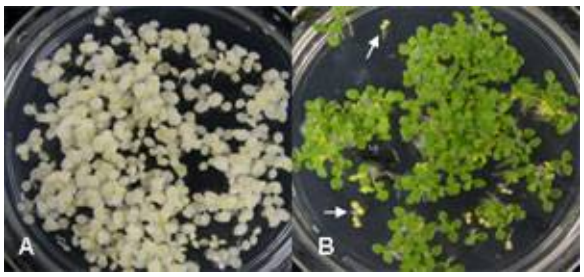

Figure 3. Tobacco seeds germinated on MSO medium supplemented with kanamycin. **A:** wild type SR-1 is not resistant. **B:** Transgenic line shows a 1:3 segregation pattern according to Mendel. Arrow indicate examples of the 25% azygous seedlings, the remaining 75% are homo- or heterozygous.

The germinated T1 generation plantlets were transferred into single pots and kept in a mist chamber (**Figure 4**). After 10 days, the plantlets were transferred to a greenhouse and grown for an additional three months until the production of T2 seeds (**Figure 5**). Plating and analysis of the germination pattern on selective medium will be repeated for the T2 seeds. T2 lines that show a germination rate of 100% are homozygous and will be used for ELISA and Western blot analysis using the anti-HA antibodies we generated in our earlier work to test for expression of HA protein. Positive plants will be challenged with Xf to determine if movement of the bacteria is inhibited or delayed in the HA-expressing tobacco plants.
Unfortunately, transformation of grapevines takes considerably more time than transformation of tobacco and we expect to obtain transformed grapevine plants in spring of 2010.

Figure 4. Germinated tobacco seedlings were transplanted from selective MSO plates to soil. Plantlets are kept in a mist chamber at the environmental horticulture facility (UC Davis, CA).

Figure 5. T_1 generation of HA-expressing tobacco. Plants are kept at the environmental horticulture facility (UC Davis, CA) until production of T_2 seeds.

CONCLUSIONS

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 (1). We showed that HA proteins are secreted and processed to a mature 220 kD protein and that they contain N-terminal hemagglutination domains (3). Taken together, this suggests that the secreted N-terminal portion of the HA proteins is responsible for cell-cell aggregation and biofilm formation. We transformed tobacco plants with the N-terminal portion of the HA proteins and could show that the genes are integrated in the tobacco genome and that HA mRNA is transcribed. We are in the process of generating T2 homozygous plants and will evaluate them for expression of HA proteins. 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 PD.

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TRANSMISSION OF *METHYLOBACTERIUM MESOPHILICUM* BY *BUCEPHALOGONIA XANTHOPHIS* FOR PARATRANSGENIC CONTROL STRATEGY OF *XYLELLA FASTIDIOSA* SUBSP. *PAUCA*

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ABSTRACT

Methylobacterium mesophilicum, originally isolated as an endophytic bacterium from citrus plants, was genetically transformed to express green fluorescent protein (GFP). The GFP-labeled strain of *M. mesophilicum* was inoculated into *Catharanthus roseus* (model plant) seedlings and further observed colonizing its xylem vessels. The transmission of this endophyte by *Bucephalogonia xanthophis*, one of the insect vectors that transmit *Xylella fastidiosa (Xf)* subsp. *pauca*, was verified by insects feeding from fluids containing the GFP bacterium followed by transmission to plants and isolating the endophyte from *C. roseus* plants. Forty-five days after inoculation, the plants exhibited endophytic colonization by *M. mesophilicum*, confirming this bacterium as a nonpathogenic, xylem-associated endophyte. Our data demonstrate that *M. mesophilicum* not only occupy the same niche of *Xf* subsp. *pauca* inside plants but also may be transmitted by *B. xanthophis*. The transmission, colonization, and genetic manipulation of *M. mesophilicum* is a prerequisite to examining the potential use of symbiotic control to interrupt the transmission of *Xf* subsp. *pauca*, the bacterial pathogen causing citrus variegated chlorosis by insect vectors.

LAYPERSON SUMMARY

We report the localization of the endophytic bacterium, *M. mesophilicum*, in *Catharanthus roseus* model plant and the transmission of this endophyte by *Bucephalogonia xanthophis.* The results indicated that *M. mesophilicum* as a good candidate for a symbiotic control strategy to reduce the spread of *Xylella fastidiosa* (*Xf*) subsp. *pauca*. The transgenic endophytic *M. mesophilicum* tested has most of the prerequisites, for a successful strategy using paratransgenesis, that is, *M. mesophilicum* that colonize citrus plants and *B. xanthophis* is amenable to isolation, culture, and transformation with foreign genes. Also, we used the green fluorescent protein gene, which does not affect the fitness of the bacteria, as a marker gene makes transfer of bacteria and the plasmid traceable, was done with success in *M. mesophilicum*. The colonization and transmission of *M. mesophilicum* in the same host tissues and insect vector of *Xf* subsp. *pauca* makes it possible to makes *M. mesophilicum* an interesting candidate for the symbiotic control of the citrus variegated chlorosis agent, e.g., through a paratransgenesis approach.

INTRODUCTION

Citrus variegated chlorosis (CVC) is a disease of sweet orange (*Citrus sinensis* (L.)) trees caused by *Xylella fastidiosa* (*Xf*) subsp. *pauca* (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). Endophytes colonize an ecological niche similar to that of phytopathogens, and this fact might favor them as candidates for biocontrol agents (Hallmann et al., 1997) because they have access to and could interact with phytopathogens (Azevedo et al., 2000). Many endophytic bacteria have been isolated from sweet orange (Araújo et al., 2002), but our research has focused on the genus *Methylobacterium*, which occupies the same ecological niche as *Xf* subsp. *pauca* in the xylem vessels of plants (Araújo et al., 2002; Lacava et al., 2006). The genus *Methylobacterium* is described as a main player in the interaction between the endophytic community and the pathogen *Xf* subsp. *pauca* (Araújo et al., 2002; Lacava et al., 2004). *Catharanthus roseus* (L.) G. Don has been shown to be an excellent experimental host for *Xf* subsp. *pauca* (Monteiro et al., 2001). *Catharanthus roseus* has also been used to study the interactions between *Xf* subsp. *pauca* and endophytic bacteria (Lacava et al., 2007). Xylem-feeding leafhoppers (Homoptera: Cicadellidae, tribes Cicadellini and Proconiini) are unique organisms in terms of their nutritional ecology; they are able to feed from xylem fluid, which is difficult to access and a nutritionally dilute food (Young, 1968). A clear association has been observed between Cicadellinae leafhoppers xylem-feeding habit and ability to transmit *Xf* (Almeida and Purcell, 2003). In Brazilian citrus groves, *Dilobopterus costalimai* Young, *Oncometopia facialis* (Signoret), and *Acrogonia citrina* Marucci & Cavichioli are the most common sharpshooters found, whereas *Bucephalogonia xanthophis* (Berg) is the most commonly trapped in citrus nurseries and young groves (Redak et al., 2004). A new genetic transformation tool, called paratransgenesis, has been used to prevent the transmission of pathogens by insect vectors to humans (Beard et al., 1998). Paratransgenesis means genetic alteration of

symbiotic microbes that are carried by insects. The overall strategy of disease prevention is called symbiotic control and is a variation on the theme of symbiotic therapy (Ahmed, 2003). The key to symbiotic control is finding a candidate microbe having an existing association with the ecosystem that includes the problem or condition at hand and that occupies the same niche as or has access to the target pathogen (Miller, 2007).

OBJECTIVES

In this work, we report the localization of the endophytic bacterium, *M. mesophilicum*, in *C. roseus* model plant and the transmission of this endophyte by *B. xanthophis*. Also, we propose *M. mesophilicum* as a candidate for a symbiotic control strategy to reduce the spread of *Xf* subsp. *pauca*.

RESULTS

When the pCM88 was introduced into the strain M. mesophilicum SR1.6/6, up to 102 transformants per μg of plasmid DNA were obtained (now called SRGFP), indicating a high efficiency of transformation. The analysis of randomly selected SRGFP transformants revealed that pCM88 was stably maintained in medium without antibiotic, expressing both the resistance to tetracycline and the gfp gene, after 20 generations in 120 h, 95%, decreasing the stability on 0.25% per generations approximately (**Table 1**). To evaluate the bacterial community of insect heads, five insects were used. After isolation, a total of 2.14×10^3 bacteria with an average of $3.56\times10^2\pm23.2$ bacteria per insect head were isolated. The original bacterial community of *B. xanthophis* was comprised of five groups: *Methylobacterium* sp., Actinomycetes, *Curtobacterium* sp., *Sphingomonas* sp., and *Bacillus* sp. (**Figure 1**). The *Methylobacterium* genus occurred naturally in *B. xanthophis*. The ecological niche occupied by the endophytic bacterium *M. mesophilicum* on *C. roseus* plants was determined by visualization with fluorescent microscope, of in vitro cultivated plants, 45 days after bacterium inoculation. A preferential colonization of plant xylem by this bacterium is clearly observed in fluorescence microscopy (**Figures 2C and D**). **Figures 2A and B** show vessels from control plants, where no fluorescent cell can be observed. The insects used in transmission experiments were monitored for the presence of the SRGFP strain 24 h after acquisition. Bacteria isolation from insect heads revealed the average population density of *M. mesophilicum* of 1.64×102 ±11.33 CFU/insect head suggesting that the bacteria are capable of colonizing the foregut of the insect as they were not washed way by the sap flux. The ability of the sharpshooter *B. xanthophis* in transmitting *M. mesophilicum* was accessed by insect acquisition of endophytic strain SRGFP and further feeding in C. roseus plants cultivated in greenhouse. Forty-five days after the insect feeding on plants, leaves on which insects were trapped, were submitted to bacterial isolation. The population density of *M. mesophilicum* found in *C. roseus* leaves 45 days after insect transmission presented an average of 2.8×103 CFU/g of fresh tissue. In analyzing inoculated plants, from 45 plants used in insect traps, six presented the SRGFP strain colonizing inner tissues endophytically. It indicates that *B. xanthophis* is able to transmit the endophytic bacteria in the same way it transmits *Xf*, with an efficiency of transmission of 13.3% (**Table 2**).

CONCLUSIONS

The results from this study suggest that the pCM88 plasmid was stably maintained in planta and sharpshooter for at least 180 generations in the transmission assay. As shown in this study, the transgenic endophytic *M. mesophilicum* has most of the prerequisites, for a successful strategy using paratransgenesis. The use of GFP, which does not affect the fitness of the bacteria, as a marker gene makes transfer of bacteria and the plasmid traceable, was done with success in *M. mesophilicum* in this work. Many aspects can influence the transmission efficiency, such as phytopathogen populations in feeding plant and the interaction between bacterial communities residing vector foregut and inoculated plant. Furthermore, bacteria community present in plants and insects could influence disease development by reducing the insect transmission efficiency due to competition with pathogens or by symbiotic control of *Xf*. The colonization and transmission of *M. mesophilicum* in the same host tissues and insect vector of *Xf* subsp. *pauca* makes it possible to study the potential interactions between these bacteria in the insect body and makes *M. mesophilicum* an interesting candidate for the symbiotic control of the CVC agent, e.g., through a paratransgenesis approach.

Table 1. Plasmidial stability of pCM88 on *Methylobacterium mesophilicum*. The percent of reaming colonies caring out the pMC88 was obtained from randomly collected samples after 24, 48, 72, and 120 h of culture cells of strain SR1.6/6 growing without antibiotic tetracycline.

Generation number	Reaming colonies caring out pCM88 (%)	SD[a]
0 (0 h)	100	0
4 (24 h)	99	1
8 (48 h)	98	3.21
12 (72 h)	95	2.08
20 (120 h)	95	1

[a] SD for four replicates

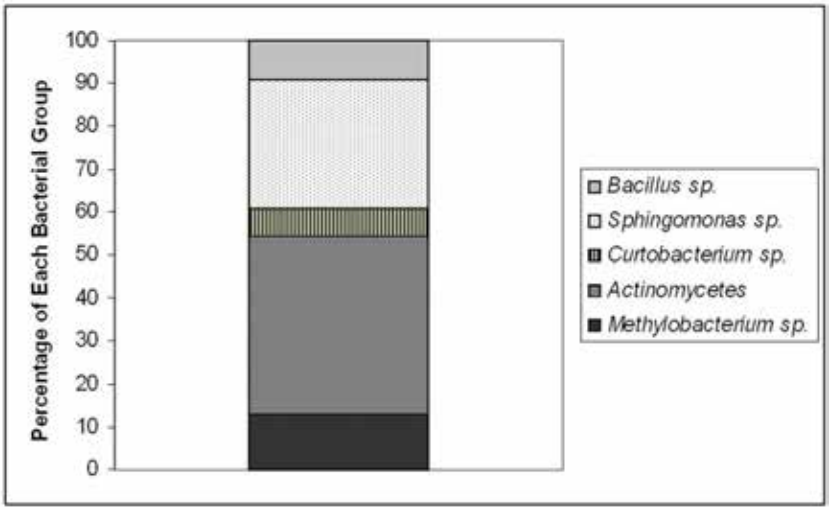

Figure 1. Most dominant group of bacteria isolated from *Bucephalogonia xanthophis.*

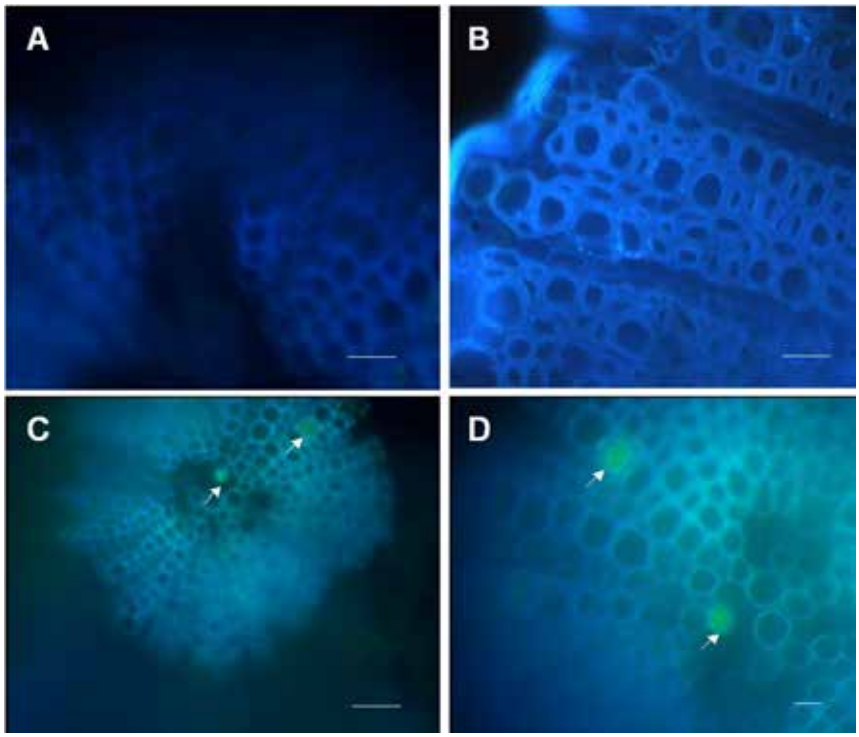

Figure 2. Fluorescent microscopy evidencing the ecological niche occupied by endophytic *Methylobacterium mesophilicum*, expressing GFP in *Catharanthus roseus* plants. Xylem vessels observed under a fluorescence microscope (Leica MZ FLIII) 45 days after inoculation. Images are the result of the overlay of images produced using filters DAPI and GFP. A and B) Xylem vessels of a control plant, scale bar=10 μm. C and D) Colonized xylem vessel, scale bar=10 μm and 5 μm.

Table 2. Evidence of the transmission of *M. mesophilicum* expressing GFP (SRGFP) to healthy plants (*C. roseus*) by insects (*B. xanthophis*). Plants were inoculated by insects, which acquired the fluorescent bacteria from membrane system. Endophytic bacteria were isolated from inoculated plants after 45 days of inoculation and fluorescent bacteria were counted. The average of SRGFP in inoculated plants was calculated as colony forming unit (CFU)/g of fresh tissue.

Number of inoculated plants	Number of plants positive to presence of SRGFP	Transmission rate	SRGFP in plants (CFU/g fresh tissue)
45	**6**	**13.3%**	**2.8×10^3**

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CONTROL OF PIERCE'S DISEASE BY METHODS INVOLVING PATHOGEN CONFUSION

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ABSTRACT
Xylella fastidiosa (Xf) produces an unsaturated fatty acid signal molecule called diffusible signal factor (DSF) that modulates gene expression in cells as they reach high numbers in plants. By increasing the expression of a variety of afimbrial adhesins while decreasing the expression of pili involved in twitching motility as well as extracellular enzyme involved in degrading pit membranes, DSF accumulation suppresses virulence of *Xf* in grape. We thus are exploring different ways to elevate DSF levels in plants to achieve "pathogen confusion." Plants expressing *rpfF* from *Xf* produce low levels of DSF and are highly resistant to Pierce's disease (PD). Chloroplast targeting of RpfF substantially increased DSF production in grape; these plants are currently being tested for disease resistance. *Xf* moved much less rapidly in *rpfF*-transformed grape, colonized many fewer xylem vessels, and achieved a much lower population size indicating that elevated DSF levels suppressed movement within the plant. Topical applications of DSF-containing bacterial extracts also reduced the severity of PD suggesting that DSF is relatively mobile within the plant and that exogenous sources of DSF might also be applied in various ways to achieve suppression of pathogen mobility and hence virulence. Some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggesting that DSF produced by rootstocks can somewhat move to scions and confer disease control' the control of disease was substantially less than that seen in transformed scions however. While certain bacteria such as *Rhizobium etli* can produce DFS when transformed with *rpfF* from *Xf*, and to colonize grape slowly after inoculation, modest levels of disease control are conferred by pre-treatment of grape suggesting that a more rapidly multiplying and internally mobile endophytic bacterium might be superior for disease control as a surrogate DSF producer. As studies of pathogen confusion will be greatly facilitated by having an improved bioassay for the DSF produced by *Xf*, we have been developing several immunological and biochemical means to assay for the presence of DSF using *Xf* itself as a bioindicator.

LAYPERSON SUMMARY:
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. Accumulation of DSF in *Xf* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in *Xf*, but the overall effect is to suppress the virulence of *Xf* in plants. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption (pathogen confusion) as a means of controlling Pierce's disease (PD). We have investigated both the role of DFS-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. Elevating DSF levels in plants should reduce movement of *Xf* in the plant. We have produced bacterial endophyte strains that can produce large amounts of DSF and, by moving within plants apparently they can alter the abundance of DSF sufficiently to reduce the virulence of *Xf*. 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. Some reduction in disease severity was observed in grape scions grafted to DSF-producing rootstocks suggesting that DSF produced by rootstocks can somewhat move to scions and confer disease control.

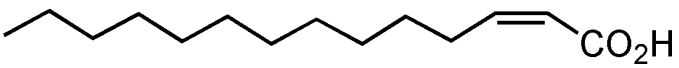


Figure 1

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 diffusible signal factor (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 DFS-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 DFS-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 Pierce's disease (PD) control.

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 PD in the scion
3. Evaluate enhanced DSF-producing endophytic bacteria for control of PD
4. Investigate DSF-overproducing strains of *Xf* as biocontrol agents for PD and whether *Xf* strains previously identified with biocontrol potential exhibit an elevated production of DSF
5. Determine if resistance to PD 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 PD is due to differences in induction of virulence factors in the pathogen by the plant

RESULTS AND DISCUSSION

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. 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.

Further tests of SR1tobacco 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

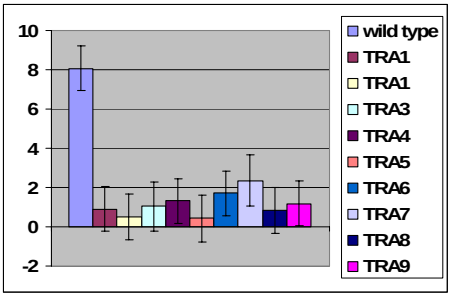


Figure 2. Disease severity (# symptomatic leaves/plant) on Freedom grape transformed with the r*pfF* gene encoding DSF production and inoculated with *Xf*.

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 PD (**Figure 2**). 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 measured the movement of Xf in these plants by measuring both the population size of *Xf* in stems and petioles at different distances from the point of inoculation, as well as to observe the fraction of vessels to which a gfp-marked strain of *Xf* had moved using fluorescence microscopy (**Figure 3**). *Xf* was greatly limited in its movement in plants producing DSF as evidenced by both a lower population size at sites distal to the point of inoculation and a lower incidence of vessel colonization at all points; both would contribute to low disease severity.

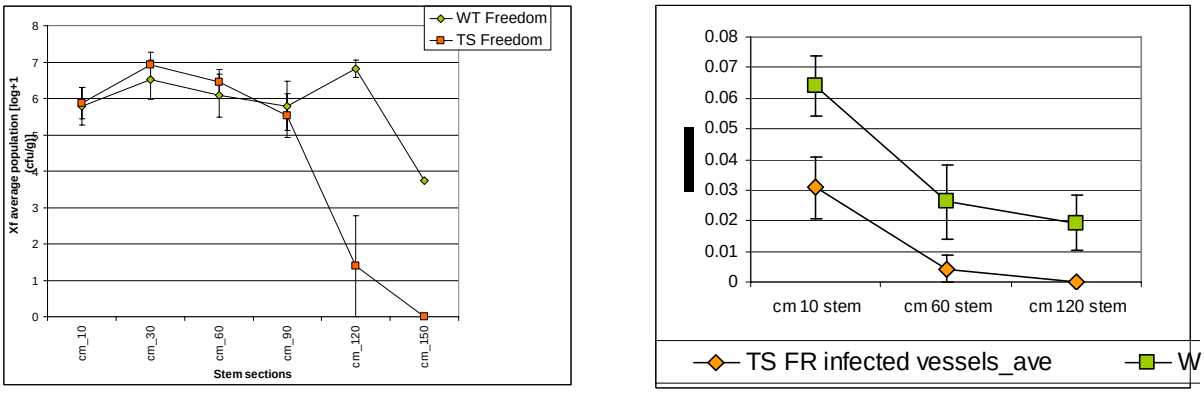


Figure 3. (Left) Population size of *Xf* in 1 cm stem segments at different distances from the point of inoculation on non-transformed Freedom grape (green) and in *rpfF*-transformed Freedom (red). The vertical bars represent the standard error of mean log population size. (Right). Proportion of xylem vessels in stems of transformed Freedom grape (green) and in *rpfF*-transformed Freedom (orange) colonized with a gfp-marked strain of *Xf* at different distances from the point of inoculation.

We have recently transformed tobacco, *Arabidopsis*, tomato, and grape 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, indicated 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. Further tests of the efficacy of chloroplast targeting of rpfF implants were preformed 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 obtained the chloroplast-targeted grape in June, 2009 and have very recently produced enough vegetative clones of these grapes and have initated pathogenicity studies; results are expected by November, 2009. We also have inoculated the chloroplast-targeted rpfF plants with a gfp-marked strain of *Xf* to assess differences in its movement within the DSF-producing and normal Thompson seedless grape using fluorescence microscopy.

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 availably 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 expressing both *rpfB* and *rpfF* transgenic plants can better complement the virulence of the non pathogenic *rpfF* mutant of *Xcc*. We thus are preparing genetic constructs to transform grape with these two genes to further enhance DSF production.

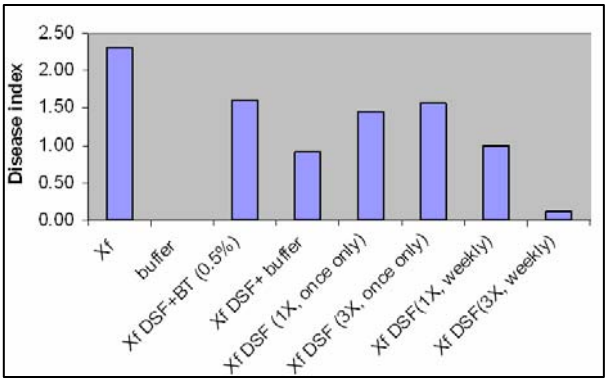
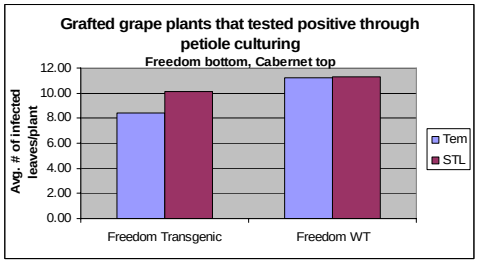


Figure 4. Disease incidence-severity relation (disease index) for grape inoculated with *Xf* and to which DSF was topically applied or introduced into the stem.

Direct application of DSF to non-transgenic grape can also confer disease control. While we have very recently tentatively determined the chemical structure and have synthesized the putative 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 4**). 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 are currently repeating these studies using DSF extracted from various surrogate hosts as well as from an rpfC mutant of *Xf* to compare the efficacy of these various sources of DSF to determine whether the amount and chemical identity of DSF from these sources are the same. This will be very helpful in our continuing efforts to

unambiguously determine the chemical structure of DSF and to justify the synthesis of large amounts of DSF for plant experiments.

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 and grape transformed with the rpfF of *Xf* are used as rootstocks to which normal SR1 tobacco or grape is grafted as a scion. These plants have been inoculated with *Xf* to test whether normal scions on DSF-producing rootstocks have a lower susceptibility to *Xf* colonization. The average disease severity rating on the normal SR1 tobacco grafted onto the rpfF-expressing rootstock was 0.97 compared to a rating of 0.84 on SR1 grafted onto normal SR1 rootstocks (control); these ratings did not differ significantly. However, the average disease rating on *rpfF*-expressing SR1 tobacco scion grafted onto an *rpfF*-expressing rootstock was only 0.24, which was significantly lower than that of a normal scion grafted onto an *rpfF*-expressing rootstock or onto a normal rootstock. These preliminary results suggest that putative DSF production in the scion is much more effective on reducing the movement and growth of *Xf* in the scion than that of the rootstock. This work is being repeated.



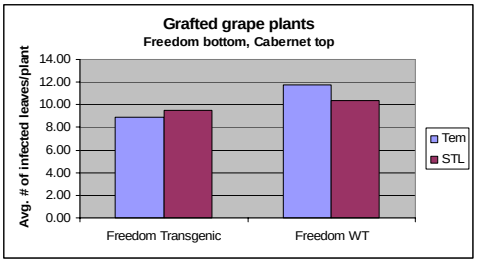


Figure 5. Severity of PD (left box) or number of leaves per vine infested with *Xf* (right box) of Freedom grape having a Cabernet sauvignon grape scion grafted onto the rootstock noted on the abscissa. Plants were inoculated with either *Xf* strain Temecula (blue) or strain STL.

Non-chloroplast targeted RpfF-expressing transgenic Freedom grape were also sued as a rootstock for green-grafted Cabernet Sauvignon scions. Initial estimates of disease severity indicate that there were about 30% less symptomatic leaves of the normal Cabernet scion when grafted onto a *rpfF*-expressing rootstock compared with plants on a normal Freedom rootstock (**Figure 5**). Thus, like in the studies of the *rpf*F-expressing tobacco, it appears that DSF production in the scion is more efficacious for disease control than is the expression of *rpfF* in the rootstock. We are repeating these experiments and will be inoculating the plants in a variety of ways to determine the efficiency of disease control from rootstock modification.

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*). We recently were able to transform a putative efficient endophyte of plants, *Rizobium etili* 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. 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 3 cm away within one week, and population sizes of this strain increased 100-fold within three weeks after inoculation. Studies are continuing to determine the maximum population size that this strain can achieve in grape leaves. The evidence, however, suggests that the bacteria move relatively slowly in grape, and thus such strains would have to be inoculated into grape substantially in advance of the pathogen in order to achieve high levels of disease control. We thus are exploring the possibilities of introducing these bacteria throughout the plant by various means as a way to rapidly increase their population size in the plant, and thus their ability to elevate the DSF levels within the plant. As the use of surfactants to introduce the bacteria into the plant sometimes resulted in some phytotoxicity to leaves, we are exploring an experimental strategy of forcing the bacteria physically into leaves using either pressure or a vacuum in order to achieve leaves that have high levels of bacteria but without any leaf damage that will complicate the interpretation of disease control by such bacteria.

Various DSF-producing bacteria were tested for their ability to control PD 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 6**) 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 and 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 co-inoculated with the pathogen (**Figure 7**). 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.

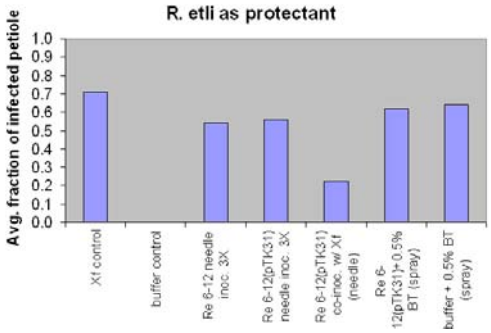


Figure 6 Incidence of colonization of petiols of grape by *Xf* when plants were treated with DSF-producing *R. etli* in various ways.

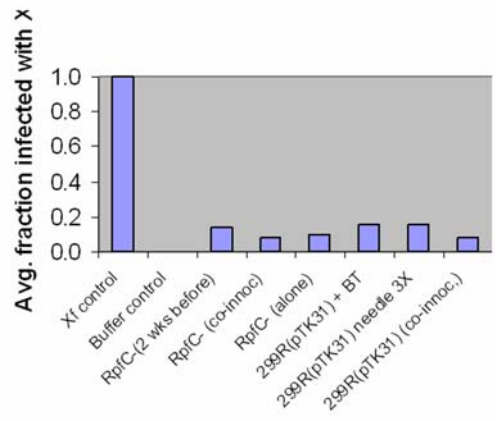


Figure 7 Incidence of colonization of petioles of grape by *Xf* when plants were treated with RpfC mutants of *Xf* in various ways.

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 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 much lower efficiency then the *Xanthomonas* DSF since the two molecules apparently differ slightly. Elsewhere have described another project in which we are developing 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* but which will be most responsive to DSF from *Xf.* We have made much effort in this reporting period to developing methods to use *Xf* itself to detect DSF**.** Among the several genes that we know to be most strongly regulated by DSF 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 and WT strain 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. For example, cells of the *rpfF*- mutant which are not adherent, and thus which do not form cell-cell aggregations became much more adherent to each other when DSF was added to shaken broth cultures. The increased adherence is readily visualized as an enhanced ring of cell-cell aggregates that forms at the liquid-air interface of shaken cultures (**Figure 8**). Thus it appears that we may be able to assess the concentration of DSF in samples using a cell adhesion assay using *Xf* cells, although both assays are time consuming and somewhat qualitative.

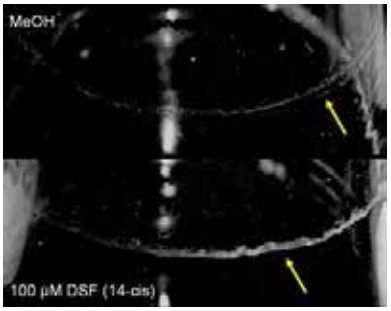


Figure 8. Cellular aggregations that formed at the air-liquid interface in broth cultures of a *rpfF*- mutant of *Xf* grown in XFM minimal medium without added DSF (top) or with 100 uM of added C14-cis enoic acid, the presumptive DSF produced by grape strains of *Xf*. The yellow arrows note the presence of the ring of adhered cells.

Figure 9. Staining of colony lifts of streaks of WT Xf (left), a GGDEF mutant (center) and a GGDEF mutant in which gene PD 0279 has been over-expressed in trans (right) with Alcian Blue.

We are also exploring several other methods of assessing the presence of DSF using *Xf* itself as a bioindicator. We are taking 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 are fusing this gene to a *gfp* reporter gene that has been optimized for expression in E. coli (and thus hopefully will also have higher levels of expression in *Xf* – see progress report for project 08-0170 for more details) and will 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. Likewise, we have cloned the gene encoding alkaline phosphatase from *Xf* and are determining if it can be used in in vitro bioassays when fused to DSF-responsive genes in an alkaline phosphatase-deficient background in *Xf*. 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.

In addition to estimating the transcriptional expression of genes known to be regulated in response to accumulation of DSF, we are also exploring ways of measuring the amounts of gene products (proteins) or EPS that are made in response to the presence of DSF. For example, we are exploring whether 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. Our initial results suggest that DSF-deficient RpfF- mutants of *Xf* 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.

We are also exploring the use of a simple staining procedure to estimate the abundance of EPS produced by *Xf*. For example, our work on the mechanism by which DSF mediates changes in gene expression in *Xf* has uncovered the important role of cyclic di-GMP as a so-called second messenger within cells. We have made mutants in gene PD 0279 which encodes a GGDEF domain protein the apparently functions in the synthesis of cyclic di-GMP. The GGDEF mutant produces much more EPS ion culture as apparent from observing colonies, and this difference in EPS can be readily visualized by staining of colony lifts on nitrocellulose filters by staining with Alcian Blue; the GGDEF mutant stains a much darker blue than the wild type, which the over-expression of this GGDEF proteins stains much less intensely (**Figure 9**). This Alcian blue staining method thus is being pursued as a means to estimate DSF-mediated changes in EPS production in an *rpfF* mutant of *Xf* exposed to different amounts of DSF.

We have also explored the use of immunofluorescence to detect other DSF-regulated proteins in *Xf*. This work is very promising. Antibodies to XadA were provided by Dr. Alessandra Souza who had developed this tool to detect a homologous protein in CVC strains of *Xf*. The antibodies cross-reacted strongly to the XadA from grape strains of *Xf*. In preliminary experiments we find that cells of an *rpfF* mutant of *Xf* harbor very little XadA when grown on XFM minimal medium without added DSF, but that significant amounts of XadA is detected with the antibody when DFS-containing extracts from an rpfC mutant of *Xf* were added (**Figure 10**). These are very exciting results in that it suggests that such a biosensor would be very useful within plants to monitor the temporal and spatial patterns of DSF production within plants, as well as allow us to monitor the dispersal and stability of DSF that has been applied to plants, or of DSF which has been produced by

transgenic plants themselves. We also observed a very curious effect of DSF on XadA; while some of this protein is cell-associated, a much lower proportion of this protein is released to the outside of the cell when cells are exposed to DSF (**Figure 11**). This phenomenon also should provide a useful means to estimate DSF abundance in vitro. We are exploring the use of other antibodies such as those directed against PilC as well for such studies.

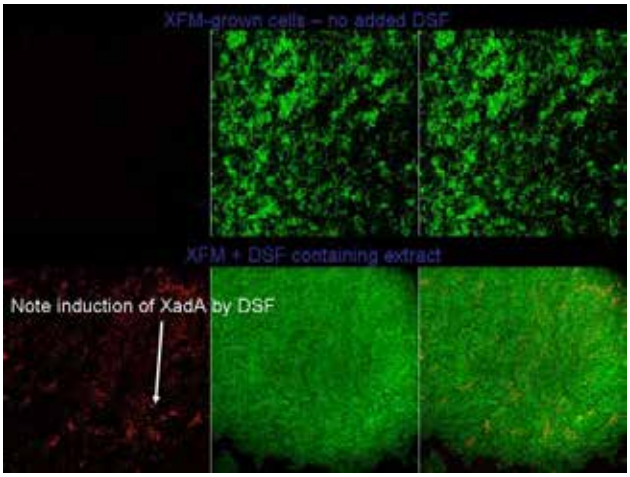


Figure 10 Cells of rpfF- mutant of *Xf* grown on a minimal medium and then stained with Syto-9 (green) and probed with rhodamine-labeled anti-XadA antibody (red). Cells were grown in XFM minimal medium without added DSF extract (top) or with added DSF-containing cell culture extract (Bottom).

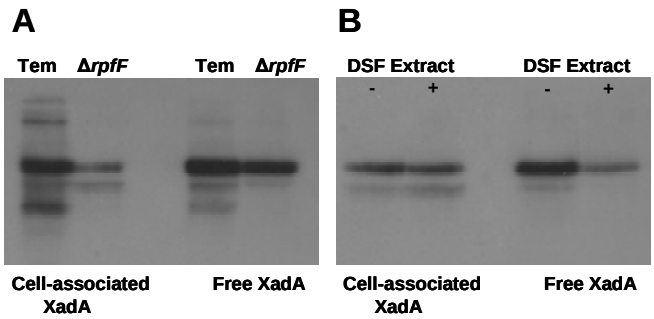


Figure 11. (**A**) Western blot analysis of cell-associated and cell-free XadA in *Xf* Tem and Δ*rpf*F after 14 days of growth on minimal media (XFM) supplemented with pectin. (B) Effect of *Xf* Δ*rpfC* DSF extract on XadA level in Δ*rpf*F.

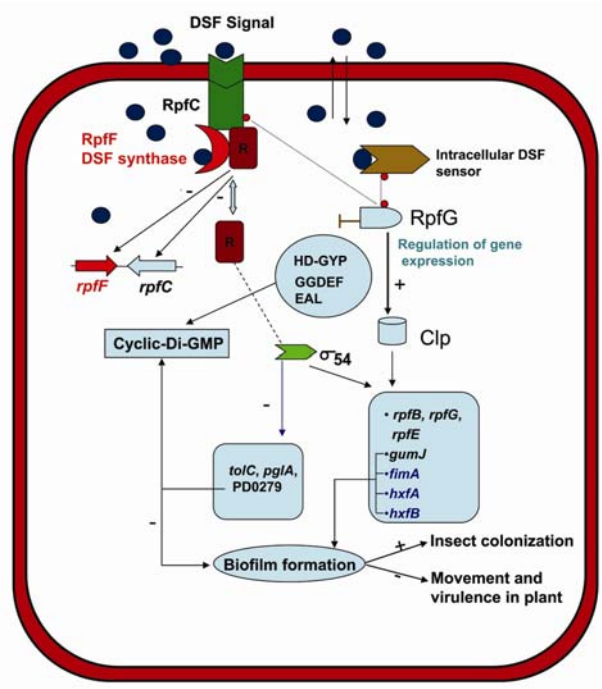


Figure 12. A proposed model for DSF-mediated cell-cell signaling regulation in *Xylella fastidiosa*.

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 a *rpfF* and *rpfC* mutant background as well as a double mutant. The results have enabled the production of a more complete model of DSF-dependent gene expression in *Xf* (**Figure 12**). A central role for modulation of cyclic d-GMP in altering expression of cell surface features central to virulence of *Xf* has been noted (**see Figure 9**). The several genes identified here will be examined by RT-PCR in cultures of *Xf* to which plant extracts have been applied as proposed.

CONCLUSIONS

Since we have shown that DSF accumulation within plants is a major signal used by *Xf* to change its gene expression patterns and since DFS-mediated changes all lead to a reduction in virulence in this pathogen we have shown proof of principle that disease control can be achieved by a process of "pathogen confusion". Several methods of altering DSF levels in plants, including direct introduction of DSF producing bacteria into plants, topical application of such bacteria to plants with surfactants, and direct application of DSF itself to plants appear promising as means to reduce PD. Transgenic DSF-producing plants appear particularly promising and studies indicates that such plants provide at least partial protection when serving as a rootstock instead of a scion. Based on work done on other plant species in which a chloroplast-targeted DFS synthase has provided much higher levels of DSF production, we are hopeful that such a construct in grape will provide even higher levels of PD control in our current studies. While the principle of disease control by altering DSF levels has been demonstrated, this work addresses the feasibility of how achieve this goal, and what are the most practical means to achieve disease control by pathogen confusion. Our continuing work will address which method is both most practical and efficacious.

FUNDING AGENCIES

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EXPLOITING PATHOGEN SIGNAL MOLECULES FOR CONTROL OF PIERCE'S DISEASE

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ABSTRACT
The movement of *Xylella fastidiosa (Xf)* in plants as well as insect transmission is controlled by a small diffusible signal factor (DSF) that accumulates when cells are at high cell densities. Pathogen behavior can be 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 most abundant DSF produced by grape strains of *Xf* under the control of the *rpfF* gene as 2-Z-tetradecenoic acid (hereafter called C14-cis). Additional related chemicals forms of DSF with biological activity may exist, and we are currently determining their relative activity and if such molecules cooperate in regulating gene expression in *Xf*. The DSF of *Xf* 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 uM of DSF produced by *Xcc*, more than about 100 uM 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 EPS production and production of various cell adhesins are induced in the presence of DSF in *Xf*, we are developing *Xf*-based 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 uM exogenous C14 cis while cell-cell adhesiveness and cell-surface adhesiveness was enhanced. 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. Initial results suggest that the responsiveness of *Xf* to C14-cis is dependent on the physiological state of cells; young, actively-growing cells appear to respond less than older cells. 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; modest decreases in disease control with these treatments revealed a need to better assess the efficiency with which DSF was introduced into plants. Substantial decreases in disease severity and incidence of vessel colonization with cells of *Xf* were obtained after application of DSF-containing bacterial extracts, revealing the importance of efficient methods of introduction of this signal molecule for disease control. We have also designed and synthesized some DSF-analogs and will soon test them for their ability to alter pathogen gene expression and behavior in culture as well as control disease.

LAYPERSON SUMMARY
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. Accumulation of DSF in *Xf* cells, which presumably normally occurs as cells become numerous within xylem vessels, causes a change in many genes in *Xf*, but the overall effect is to suppress the virulence of *Xf* in plants. The DSF produced by grape strains of *Xf* has tentatively been characterized as a 14 carbon, unsaturated molecule we will refer to as C14-cis. Both its relatively lower biological activity as assessed in a bioassay in the bacterium *Xanthomonas campestris* pv. *campestris* (*Xcc*) than that of the DSF from *Xcc* itself and lesser activity in an *Xcc* bioassay indicates that there is considerable specificity in the structure-function relationships between different bacterial DSF signal molecules. We have focused on developing a bioassay for the DSF made by *Xf* in *Xf* itself so that we can monitor the fate of DSF applied to grapes for the purpose of pathogen confusion. Preliminary studies conducted in the greenhouse in which synthetic DSF and DSF extracted from bacterial cultures was applied as a topical spray to plants, and by direct injection into the stems of plants before inoculation with *Xf* have shown that some reduction of disease symptoms and extent of colonization of grape vessels is achieved by these treatments.

INTRODUCTION
Research in the Lindow lab has provided considerable evidence for a diffusible signal factor (DSF) encoded by *rpfF*, which we have now shown to be a fatty acid derivative, 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 coordinate the plant lifestyle and convert cells into the insect acquisition phase.

Our earlier work demonstrated that the severity of Pierce's disease (PD) is reduced when the levels of DSF are increased in the plant in various ways. For example, the severity of PD 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 PD. In very exciting results, the DSF-expressing grape are MUCH less susceptible to PD. 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 PD 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 and that optimum production is on solidified media after growth for 10 days or more. We also expressed *rpfF* from *Xf* in *E. coli* and *Erwinia herbicola* strain 299R under strong promoters. The yield of DSF as detected in *Xcc* 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 compared to *E. coli*.

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 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 Ethyla 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. The spectral data for the acid isolated from *E. herbicola* match those obtained for the synthetic 2-*Z*-tetradecenoic acid.

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 tentatively conclude that DSF from *Xf* is C14-cis (**Figure 2**). The putative DSF from *Xf* differs somewhat from the DSF made by *Xcc* in that it has a longer, but unbranched acyl chain.

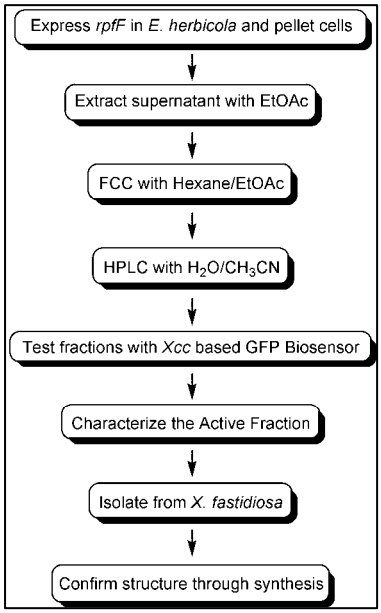

Figure 1. Process by which *Xf* DSF as detected in *Xcc* was isolated and characterized.

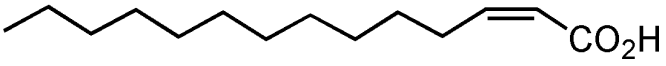

Figure 2. Putative structure of C14-cis, the DSF made by *Xf* that can be detected in *Xcc*.

We are continuing to investigate whether other, related enoic acids are made by *Xf* in addition to the numerically dominant C14-cis. The possibility exists that some of these related enoic acids may be biologically active and perhaps act in synergy with C14-cis to regulate gene expression in *Xf*.

The biological activity of C14 cis was initially 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 uM of DSF produced by *Xcc*, more than about 100 uM of C14-cis is required for detection. (**Figure 3**). It is important to note that the biological activity of C14-cis was much less than that of 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 in *Xcc* (**Figure 3**).

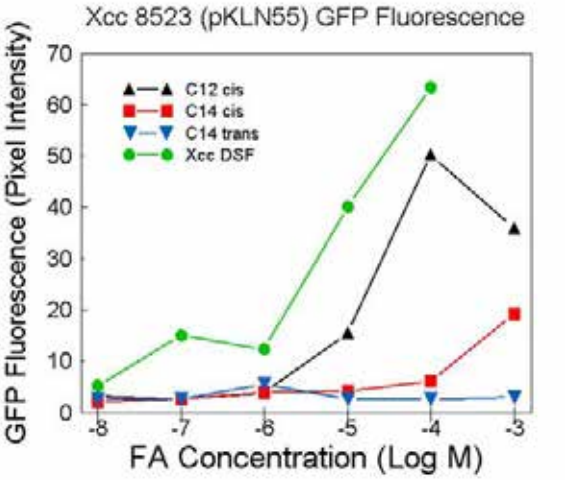

Figure 3. Dose response relationship for DSF from *Xcc* and that from *Xf* as well as other related enoic acids.

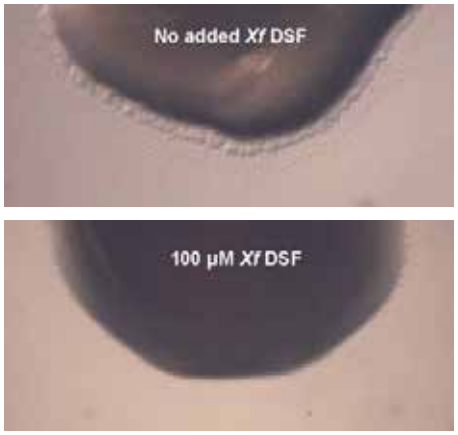

Figure 4. Twitching motility of *Xf* evident as a fringe around the coloniy of an rpfF mutant (top) on PWG medium but not around the colony when grown on medium containing C14-cis.

Biological assays for the activity of C14-cis are also being developed in *Xf* to ensure that the C14-cis molecule detected in *Xcc* is also biologically active in Xf. This is a critical step in also monitoring the translocation and stability of DSF in treated plants (Objective 3). As the expression of genes conferring type IV pili and thus twitiching 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 should respond to exogenous C14-cis. Twitching motility of the *rpfF* mutant was suppressed in the presence of as little as 1 uM exogenous

C14 cis while cell-cell adhesiveness and cell-surface adhesiveness was enhanced (**Figure 4**). The twitching assay tends to be highly variable from one assay to another, presumably due to small differences in the physiological state of the *Xf* indicator bacteria or of the agar surface on which twitching is being assayed. More quantitative assays based on expression of the genes involved in twitching motility and in adhesion to surfaces are being developed using quantitative RT-PCR to assess expression of genes such as *hxfA*, *fimA*, and *pilA*. Since it is possible that a functional *rpfF* gene may be needed to properly respond to DSF, the responsiveness of these genes to exogenous DSF is being assessed in both a WT strain as well as an *rpfF* mutant of *Xf*. Initial results suggest that the responsiveness of *Xf* to C14-cis is dependent on the physiological state of cells; young, actively-growing cells appear to respond much less than older cells. Since RT-PCR assays are time consuming and expensive, we are also exploring the use of cell "dot blots" to directly test for expression of EPS and afimbrial adhesins using antibodies obtained from the Kirkpatrick lab and from Dr. de Souza from Brazil.

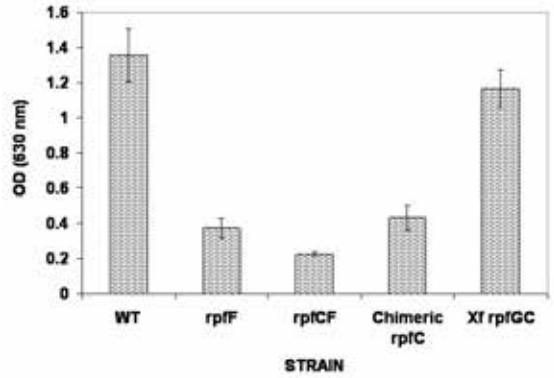

Figure 5. EPS production in various *Xcc* strains as estimated by total sugar content of extracted cells as measured by an anthrone assay

The current biodetector 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 have done considerable work on developing an improved DSF biosensor in *Xcc*. Much of our work has focused on producing a chimeric *rpfC* that will recognize DSF from *Xf* but will function in signal transduction in *Xcc*. Our analysis of RpfC from *Xf* indicates that it has a similar cytoplasmic domain as that from RpfC from *Xcc*. In fact, models that predict the 3-dimentional structure of proteins predict that the cytoplasmic domain of these two proteins will have very similar structure.In contrast to the cytoplasmic domain which is predicted to function in signal transduction by phosphorelay, the transmembrane domain of the RpfC of *Xf* is somewhat shorter than that of RpfC from *Xcc*. Given that the transmembrane domain is thought to serve as the DSF binding domain, we hypothesized that an improved DSF biosensor could be made in *Xcc* by replacing its native RpfC with a chimeric RpfC which had the N-terminal transmembrane domain of *Xf* with the cytoplasmic phospho-transfer domain of *Xcc*. This has now been accomplished by forming the hybrid protein with an appropriate fusion point.

The production of EPS in *Xcc* normally increases in response to accumulation of DSF. We therefore assessed the regulation of EPS production wild type and *rpfF* mutants of *Xcc*. There is a large reduction of EPS production as measured by total sugar concentration in extracted cells of an *rpfF* mutant compared to wild type, while EPS content of an rpfC mutant is even lower (**Figure 5**). Introduction of a chimeric *rpfC* into the *rpfFC* double mutant *in trans* restored production of EPS to levels similar to that in an rpfF mutant, suggesting that it functioned in a manner similar to the native *Xcc rpfC*. In contrast, the *rpfC* from *Xf* conferred high levels of EPS production in this mutant background, suggesting that it was inappropriately de-repressed in *Xcc*. Thus the chimeric RpfC appears to be functioning properly in *Xcc*. We are currently determining the levels of EPS production in an *rpfFC* double mutant of *Xcc* harboring the chimeric rpfC in the presence and absence of added DSF; we how to see elevated EPS levels with added DSF.

We have done extensive work to develop alternative reporter genes for use in *Xf*. In our past work we found that gfp and ice nucleation reporter genes were not efficiently expressed, and significant expression of these reporter genes could be detected only when transcription was driven by strong promoters such as the 16S rRNA promoter. We thus have explored the use of two other reporter genes. In one example, we have cloned the gene encoding alkaline phosphatase (*phoA*) from *Xf* and introduced it into a stable plasmid vector suitable for introduction into *Xf* (**Figure 6**). We also have knocked out the expression of the indigenous alkaline phosphatase gene in *Xf* since such a background would be required to measure expression of this gene in response to an environmentally-responsive gene such as those encoding EPS production, adhesins or other genes that are regulated upon increases in DSF concentration. We are in the process of determining if alkaline phosphatase activity can be detected in strains harboring various fusions to this *phoA* reporter gene. We also have obtained variants of a gfp reporter gene that confer much higher levels of expression in *E. coli* than the native gfp reporter gene. We are determining whether fusions to these gfp variants yield sufficient green fluorescence for detection in *Xf*.

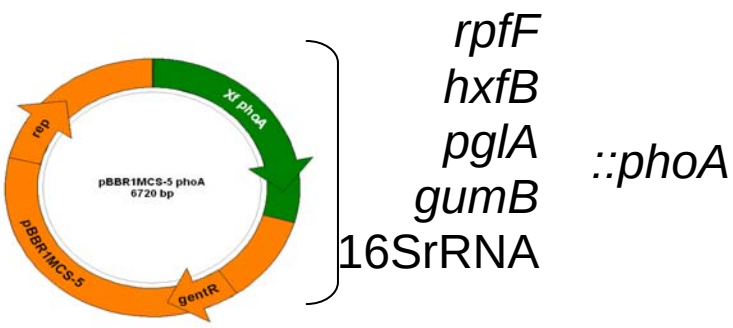

Figure 6 Expression vector harboring *phoA* from Xf being tested for expression in a *phoA* mutant of Xf.

Objective 2. Design and synthesize DSF analogs. We have made several synthetic analogs of C14-cis for testing for biological activity in *Xf* (**Figure 7**). As these materials have only recently been synthesized the biological activity of most have not yet been assayed. We have been waiting to assay them in *Xf* until we have produced a better bioassay for DSF in this pathogen. 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 *Xcc*. In addition to the DSF analogs noted in **Figure 7**, various halogenated variants will also be synthesized.

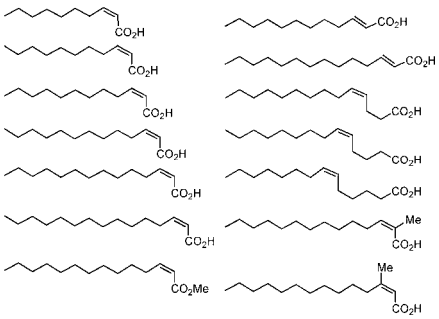

Figure 7 Analogs of the DSF produced by *Xf* that have been synthesized.

Objective 3. Testing of DSF in *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. To understand how best to test these synthetic materials we have used bonafide DSF-containing extracts from both a RpfC mutant of *Xf* as well as from an *E. herbicola* strain harboring the *rpfF* gene from *Xf* and applied them to grape in different ways before inoculating with *Xf.* These materials were injected into stems in initial studies to determine their efficacy for disease control.

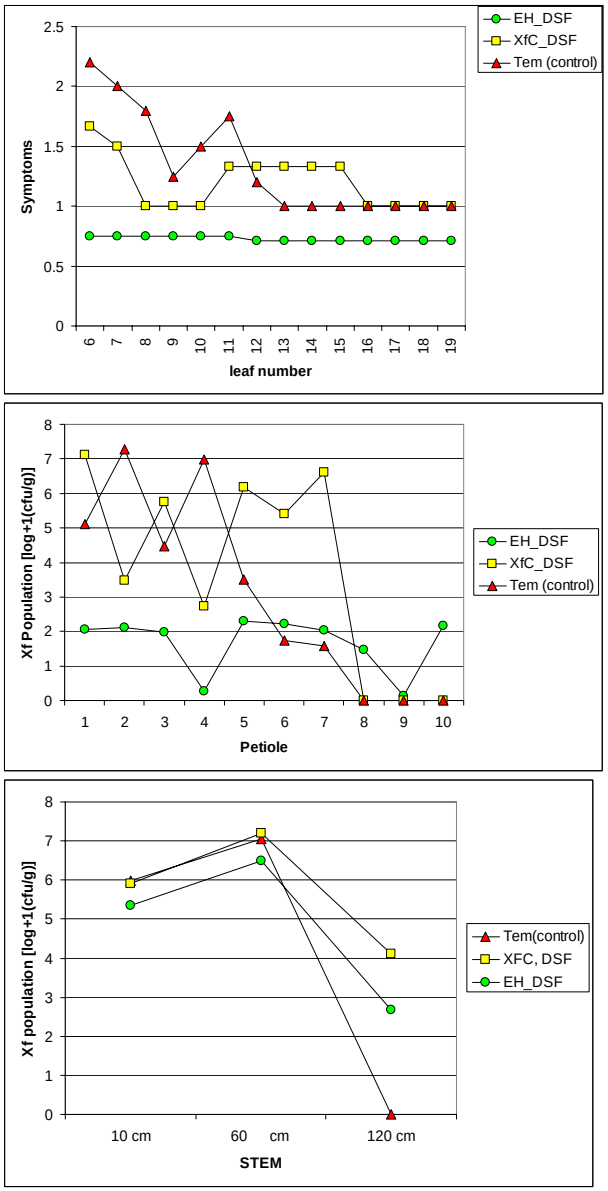

Figure 8. (Top) Severity of disease symptoms on grape injected with a DSF-containing extract from an *E. herbicola* strain harboring *rpfF* from *Xf* (green), from an RpfC mutant of *Xf* (yellow) or with methanol alone (red) before being inoculated with *Xf*. Disease severity of leaves (numbered) at various distances above the point of inoculation is shown. Disease severity was rated as 0= healthy, 1= minor marginal necrosis, 2 = moderate marginal necrosis, and 3= leaves dead. (Middle) Population size of *Xf* in petioles at different distance from the point of inoculation after pre-treatment with DSF as noted above. (Bottom) Population size of *Xf* in 1 cm stem segments collected at different distances from the point of inoculation after pre-treatment of plants with DSF solutions as noted above.

The DSF-containing extracts, particularly those from the *E. herbicola* surrogate harboring *rpfF* from *Xf* substantially reduced both disease severity when injected into stems of grape before *Xf*, as well as reduced the multiplication of *Xf* both in petioles and stems of treated plants (**Figure 8**). The reduction in population size of *Xf* increased with increasing distance from the point of inoculation, suggesting that the DSF reduced the movement of *Xf* within the plant. These results are very promising and have enabled us to initiate further tests to compare different means of introducing synthetic and extracted DSF for disease control.

CONCLUSIONS

Since we have shown that DSF accumulation within plants is a major signal used by *Xf* to change its gene expression patterns and since DFS-mediated changes all lead to a reduction in virulence in this pathogen we have shown proof of principle that disease control can be achieved by a process of "pathogen confusion." This study addresses an obvious means of achieving pathogen confusion since direct introduction of DSF via topical application to plants should enable us to alter the abundance of this signal molecule. While the principle of disease control by altering DSF levels has been demonstrated, this work addresses the feasibility of how achieve this goal using synthetic DSF. Our results show promising effects of topical application of DSF in disease control. We have made considerable progress in developing a biological sensor for the DSF produced by *Xf* so that we can better assess methods by which DSF can be introduced into plants and monitor its fate after introduction. Our continuing work will address whether this is a practical means to achieve disease control by pathogen confusion.

FUNDING AGENCIES

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

RESPONSES OF ADDITIONAL 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 November 2008 to October 2009.

ABSTRACT

Nine additional plant species and one other cultivar were evaluated in 2009 for reactions to *Xylella fastidiosa (Xf)* mechanical inoculations. We investigated relative safety for species use in and near Texas vineyards at risk for Pierce's disease. Seedlings started in the cool season were grown in containers in greenhouse and screenhouse until caulescent and flowering. Mechanical inoculation used Texas *Xf* isolates from grape, weeds, shrub, and tree (*Vitis vinifera, Ambrosia trifida* var. *texana*, *Helianthus annuus, Nerium oleander, Platanus occidentalis*). SCP buffer was the control. Each plant was twice needle-inoculated on different days into xylem of two adjacent lower internodes with two 10-µm drops of ca. 10^8 cfu/ml SCP-suspended cells. No obvious symptoms developed. Evaluations used ELISA on stem tissue that we recovered after several weeks, from the inoculation site and above the inoculation site. Experiments were repeated but repetitions of yarrow and blanketflower experiments were not yet complete at this writing because late planted seedlings were acaulescent through the summer. Safety as estimated by mechanical inoculation reflected the number of isolates that colonized (OD≥0.300), mean OD, and whether grape isolates colonized the species. All SCP controls were negative. All species were colonized to some extent by at least one isolate. Tissue location for ELISA (inoculation zone vs. above the inoculation zone) was significant ($P<0.05$) only for the four species with safer reactions. Those safe species were *Coreopsis tinctoria* (plains coreopsis 'Dwarf red'), *Coriandrum sativum* (cilantro), *Fagopyrum sagittatum* (buckwheat), and *Silene armeria* (catchfly). Species rated as unsafe under conditions of these studies were *Achillea millefolium* (yarrow), *Gaillardia aristata* (blanketflower), *Linum rubrum* (scarlet flax), *Machaeranthera tanacetifolia* (Tahoka daisy), *Oenothera speciosa* (showy primrose), and *Petunia* x *violaceae* (petunia 'Laura Bush'). *Coreopsis tinctoria* (plains coreopsis), *Verbena rigida* (tuber vervain), and *Lolium multiflorum* (annual ryegrass) met safe plant criteria in previous greenhouse and screenhouse work. Future vineyard evaluations of plant safety may differ from these results due to interactions of plant species and vector species phenologies.

LAYPERSON SUMMARY

An aggressive inoculation technique was used to screen selected groundcover plant species for potential safe use in and near Texas vineyards at risk for Pierce's disease. Relatively safe reactions were detected in 2009 in *Coreopsis tinctoria* (plains coreopsis 'Dwarf Red'), *Coriandrum sativum* (cilantro), *Fagopyrum sagittatum* (buckwheat), and *Silene armeria* (catchfly).

FUNDING AGENCIES

Funding for this project was provided by a cooperative agreement between USDA Animal and Plant Health Inspection Service, and Texas A&M University.

Section 5:
Crop Biology and Disease Epidemiology

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 October 2008 to October 2009.

ABSTRACT
In response to a recommendation by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Research Scientific Advisory Panel to express plant genes for particularly effective inhibitors of *Xylella fastidiosa* (*Xf*) polygalacturonase (PG) in transgenic grape, optimal plant polygalacturonase inhibiting proteins (PGIPs) are being selected and expressed in grape rootstocks to enhance grapevine Pierce's disease (PD) resistance. This project includes integrated approaches aimed at the eventual deployment of that strategy in commercial lines. To ease the path to commercialization, PIPRA investigators are examining the relevant Intellectual Property and regulatory issues associated with the use of selected PGIPs in transgenic grape rootstocks in combination with elite scion lines. The PGIPs that most effectively inhibit *Xf*PG are predicted to be the best candidates for providing significant PD resistance. Recombinant *Xf*PG is being developed to screen diverse PGIPs selected from a wide variety of plant sources for their ability to effectively inhibit the *Xf*PG enzyme. We are cloning the selected PGIPs so they can be expressed in plants for the tests of their efficacy in inhibiting *Xf*PG. Grape rootstock lines will be transformed with the most effective PGIPs and signal and target sequences will be used as needed to maximize PGIP expression in the rootstock and its export to the non-transgenic scions. At the conclusion of the project, the capacity of non-transgenic scions to resist PD and produce high quality grapes when grafted on transgenic rootstocks will be tested.

LAYPERSON SUMMARY
Plant proteins have been selected to inhibit a key enzyme called polygalacturonase (PG) that *Xylella fastidiosa (Xf)* uses to spread from the point of inoculation throughout the grapevine and cause Pierce's disease (PD). Proteins called PG-inhibiting proteins (PGIPs) are produced by many plants. PGIPs are selective in their ability to inhibit the PG enzymes of plant pathogens. We know that the pear fruit PGIP can inhibit *Xf*PG and that expression of the pear PGIP in transgenic grapevines slow PD development. We also know that pear PGIP produced in a rootstock can move into scions by crossing the graft union in the water-conducting tissues. The PGIPs from different plants are being tested for their ability to inhibit *Xf*PG and structural modeling is being used to characterize what parts of the PGIP are important for inhibition. The best inhibiting PGIPs will be expressed in grape and their ability to reduce PD development in grafted scions will be determined. Regulations regarding the release and use of transgenic rootstocks and intellectual property considerations associated with this approach are being addressed to maximize the commercial potential of this PD management strategy, an approach that has been advocated by the CDFA PD/GWSS Advisory Panel.

INTRODUCTION
Xylella fastidiosa (Xf), the causative agent of Pierce's disease (PD) in grapevines, has been detected in infected portions of vines. Several lines of evidence support the hypothesis that *Xf* uses cell wall-degrading enzymes to digest the polysaccharides of plant pit membranes separating the elements of the water-conducting vessel system, the xylem, of the vines (Thorne et al., 2006). *Xf* cell wall degrading enzymes break down these primary cell wall barriers between cells in the xylem, facilitating the systemic spread of the pathogen. The genome of *Xf* contains genes predicted to encode a polygalacturonase (PG) and several β-1,4-endo-glucanases (EGase), cell wall degrading enzymes that are known to digest cell wall pectin and xyloglucan polymers, respectively. To demonstrate that these wall degrading proteins facilitate *Xf* systemic movement and PD development, 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, the *Xf*PG is a virulence factor of the bacteria that contributes to the development and spread of PD. Labavitch et al. (2006) reported that introduction of PG and EGase enzymes into explanted stems of uninfected grapevines caused breakage of the pit membranes and demonstrated that substrates for these enzymes, pectins and xyloglucans, are present in grapevine pit membranes (Labavitch, 2007).

PG-inhibiting proteins (PGIPs) produced by plants limit damage caused by fungal pathogens (*B. cinerea*, or gray mold) as well as by insects (*Lygus hesperus*, the western tarnished plant bug) (Powell et al., 2000; Shackel et al., 2005) because PGIPs

are selective inhibitors of the PGs produced by fungal pathogens and insects (Cervone et al., 1990). Agüero et al. (2005) demonstrated that by introducing a pear fruit PGIP gene (Stotz et al., 1993; Powell et al., 2000) into transformed grapevines, the susceptibility to both fungal (*B. cinerea)* and bacterial (*Xf)* pathogens decreased. This result implied that the pear PGIP provided protection against PD by inhibiting the *Xf* PG, reducing its efficiency as a virulence factor. Using *in vitro* assays with *Xf*PG expressed in *E. coli*, Roper (2006) demonstrated that the recombinantly expressed *Xf*PG can be inhibited by the pear PGIP (Labavitch, 2006). In a key preliminary observation for the PD control approach investigated in this project, Agüero et al. (2005) demonstrated that transgenic pear PGIP protein 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 reduce the virulence of *Xf*.. The project is designed to identify specific PGIPs that optimally inhibit the virulence factor, *Xf*PG, and to express efficiently the optimal PGIPs in grape rootstocks to provide PD protection in scions. The optimization of the expression of PGIPs includes the use of transformation components with defined intellectual property (IP) and regulatory characteristics, as well as expression regulating sequences that result in the maximal production of the PGIPs in rootstocks and efficient transport of the proteins through the graft junctions to the aerial portions of the vines so that *Xf*PG produced by the pathogen in scions is inhibited.

OBJECTIVES

1. Define a path to 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

Objective 1: Regulatory issues associated with commercialization of transgenic rootstocks

A visit by PIPRA staff to federal agencies regulating the environmental release into the environment of genetically modified (GM) plants gave us insights on the regulatory issues related to *PGIP* expressing grape rootstocks. From a USDA/APHIS perspective, it will be recommended that field trials start with GM rootstocks and wild type scions will be grafted on later for deregulation of future commercial products. From an EPA standpoint, one potential issue for this project to address will be gene-flow from transgenic pollen. For regulatory approval only, it will be necessary to allow rootstocks to flower in commercial settings even though under normal practices, the rootstocks will not be allowed to flower. The EPA will have the final word on defining if grapes harvested from non-GM scions grafted on the *PGIP*-expressing rootstocks will be considered transgenic. This will determine if the FDA needs to be consulted before commercialization.

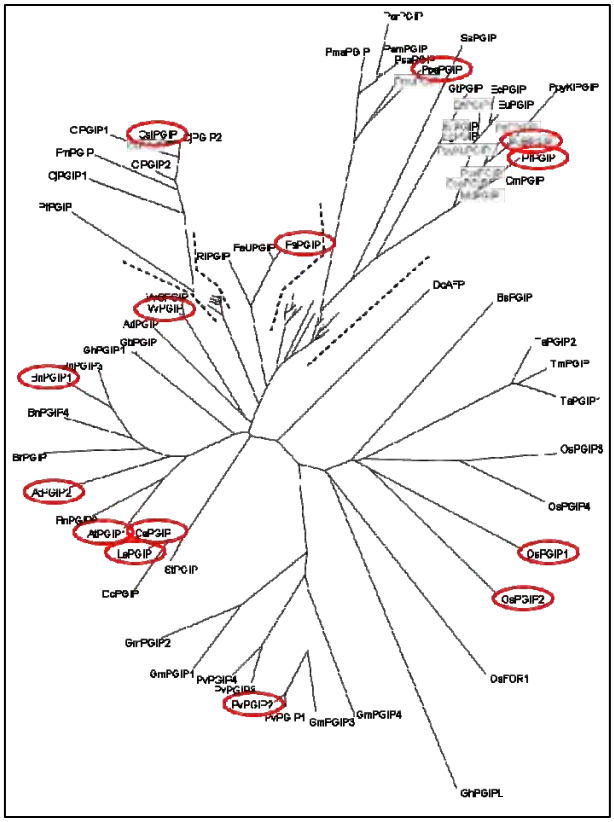

Figure 1. Unrooted phylogenetic tree of PGIP proteins. The 14 candidate PGIPs for *Xf*PG inhibition are circled in red. The protein names and organisms are given in Labavitch, 2008.

Objective 2: Propagation and grafting of existing grape lines expressing and exporting pear PGIP

Agüero et al. (2005) described the use of transgenic grapevine cultivars 'Thompson Seedless' and 'Chardonnay' expressing the 'Bartlett' pear fruit PGIP (*PcBPGIP*). These plants have been maintained in our greenhouse facilities with the intent to use them in grafting and *Xf* inoculation experiments. Vegetative propagation efforts to increase the total number of plants for these experiments yielded 66% efficiency last winter. PCR analysis has been used to verify the transgene identity in both grape cultivars containing either the *PcBPGIP* transgene or the empty vector.

Further work related to this objective has been delayed substantially due to quarantine measures implemented in response to a Panicle Rice Mite infestation in the greenhouse facilities. For more information regarding these matters, please refer to the August 2009 progress report for CDFA contract number 08-0171. The CDFA and UC Davis issued directives for treating the affected grape vines including drastic pruning and isolation, resulting in rootstocks with only one or two viable buds remaining prior to intensive chemical disinfestations treatments. Some of the

grape lines did not survive the mandated action and all remaining lines have shown slower than normal regeneration. Due to the considerable amount of time and effort to follow these directives and regenerate the plants, the grafting experiments to be conducted in years 1 and 2 have been delayed until the affected grapevines can be moved back to the appropriate facilities. As of October 2009, the grapevines remain in isolation.

Objective 2: Selection of PGIPs

The previously reported phylogenetic tree of PGIP sequences (Labavitch 2008) has been expanded upon to include a total of 68 PGIP-like amino acid sequences. These PGIPs represent a diverse array of plant families and expression patterns. The PG inhibition activities of some of them are known. The full-length protein sequences from GenBank were aligned using ClustalX 2.0.9. An unrooted, neighbor-joining tree (**Figure 1**) was constructed in ClustalX and visualized with TreeView 1.6. PGIPs are typically characterized by 10 leucine rich repeats (LRR) in the region thought to influence inhibition of PGs. The PGIP sequence diversity in this phylogenetic tree mirrors the diversity among plant families, crediting the use of PGIP sequence data in plant family classification studies.

Fourteen candidate PGIPs (**Figure 1, Table 1**) were selected from the phylogeny, representing the major clades of the tree and the inherent sequence variation dividing them. The candidates were also chosen by their predicted total protein charge at a given pH. The predicted charges were calculated for all 68 PGIP sequences but the lower total charges predicted for the candidates should prevent interference or repulsion between each PGIP and the highly charged *Xf*PG. The large positive charge on AtPGIP2 and the minimal charge on OsPGIP2 will be particularly informative as we correlate *Xf*PG inhibition with total PGIP charge.

Table 1. Predicted total protein charge analysis for the 14 candidate PGIPs and *Xf*PG in different pH environments.

Common name	Organism	Protein	Charge of Protein (at certain pH)					
			3.5	*4.0*	***4.5***	*5.0*	*5.5*	*6.0*
Thale cress	*Arabidopsis thaliana* (Col.)	AtPGIP1	27.5	20.9	**14.2**	10.0	7.4	5.2
Thale cress	*Arabidopsis thaliana* (Col.)	AtPGIP2	35.4	28.5	**21.6**	17.0	14.2	11.8
Rapeseed	*Brassica napus* cv. DH12075	BnPGIP1	30.5	22.2	**14.2**	9.4	6.8	4.8
Pepper	*Capsicum annum* cv. arka abhir	CaPGIP	20.7	15.2	**9.5**	5.9	3.8	2.2
Sweet orange	*Citrus sinensis* cv. Hamlin	CsiPGIP	28.0	21.7	**15.2**	11.1	8.7	6.7
Strawberry	*Fragaria x ananassa*	FaPGIP	25.4	18.7	**12.1**	8.0	5.6	3.7
Rice	*Oryza sativa* cv. Roma	OsPGIP1	18.4	12.9	**7.6**	4.3	2.2	0.2
Rice	*Oryza sativa* cv. Roma	OsPGIP2	17.5	9.3	**1.6**	-3.1	-6.1	-8.8
Common bean	*Phaseolus vulgaris* cv. Pinto	PvPGIP2	22.7	17.6	**12.9**	10.2	8.5	7.1
Peach	*Prunus persica*	PpePGIP	28.7	21.9	**14.9**	10.3	7.5	5.3
Chinese Firethorn	*Pyracantha fortuneana*	PfPGIP	16.9	11.7	**6.6**	3.4	1.4	-0.3
Bartlett pear	*Pyrus communis* cv. Bartlett	PcBPGIP	23.1	16.1	**9.3**	5.0	2.6	0.7
Tomato	*Solanum lycopersicum* cv. VFNT Cherry	LePGIP	29.8	23.4	**17.0**	12.8	10.1	7.7
Grape	*Vitis vinifera* cv. Pinotage	VvPGIP	30.5	24.0	**17.7**	13.6	11.1	8.7
		*Xf*PG	41.0	31.3	**22.2**	16.4	11.9	6.8

Objective 2: Express PGIPs and test for optimal inhibition of XfPG

The 14 candidate PGIPs will be tested for their ability to inhibit *Xf*PG. The previously reported plant transformation strategy (Labavitch 2008) is being used to generate plant transformation vectors containing a PGIP sequence under control of the CaMV 35S constitutive promoter and linked to a C-terminal poly-His tag for protein purification. These plant proteins are highly glycosylated (**Figure 3B**) and therefore require expression in a plant-based system. Arabidopsis lines transformed to express each PGIP will be used to obtain the proteins necessary for *in vitro* radial diffusion assays, testing the inhibitory capacity of each PGIP. Cloning each of the candidate PGIPs from its source species into the proper plant transformation vector is in progress (**Table 2**). The stably expressing Arabidopsis lines will provide PGIPs for inhibition assays against the PD causing *Xf*PG, as well as against PGs from other pathogens and pests linked to many plant diseases and resulting crop losses.

Table 2. Research progress for cloning the 14 candidate PGIPs. "X" = completed checkpoint, "O" = work in progress, "-"= checkpoint to be completed.

Protein	Cloning Progress Checkpoints				
	Plant tissue acquired	PGIP cDNA isolated	Transformed into *E. coli*	Transformed into *A. tumefaciens*	PGIP ready for plant transformation
AtPGIP1	X	X	X	O	-
AtPGIP2	X	X	X	O	-
BnPGIP1	O	-	-	-	-
CaPGIP	X	O	-	-	-
CsiPGIP	X	O	-	-	-
FaPGIP	X	X	-	-	-
OsPGIP1	X	O	-	-	-
OsPGIP2	X	O	-	-	-
PvPGIP2	X	O	-	-	-
PpePGIP	O	-	-	-	-
PfPGIP	X	O	-	-	-
PcBPGIP	X	X	X	X	O
LePGIP	X	X	X	X	O
VvPGIP	O	-	-	-	-

The *in vitro* assays require optimal expression and activity of *Xf*PG, a topic covered below. We are developing another assay to test each candidate PGIP's ability to inhibit *Xf*PG *in planta*. This assay will provide an environment more similar to the potential PG-PGIP interaction taking place in the plant apoplastic space. Separate plant transformation vectors carrying a candidate PGIP and the *Xf*PG coding sequence will be used to transiently co-express both proteins in tobacco leaves by *Agrobacterium tumefaciens* pressure infiltration. An analogous assay was used to test the effectiveness of a grape PGIP (VvPGIP) in inhibiting a PG from *B. cinerea* (Joubert et al., 2007). Both the PG and PGIP were transiently expressed in tobacco leaves by co-infiltration of *A. tumefaciens* clones carrying the genes of interest. Expression of the PG alone resulted in PG-dependent lesions which were visible and could be measured. Co-infiltration of PG and PGIP-expressing clones resulted in inhibition of PG-mediated lesion development (**Figure 2**). *Xf* is known to cause local lesions in tobacco leaves after infection. Two plant transformation constructs have been developed containing *Xf*PG for this experiment: one with the native coding sequence and one with an apoplastic targeting sequence attached upstream of the coding region to ensure *Xf*PG secretion by the plant cells. We expect to see results similar to those from the earlier work: local lesions induced by the expression of *Xf*PG will be lessened when the *Xf*PG is co-expressed with an inhibiting PGIP. This will support rapid comparisons of the effectiveness of each PGIP in inhibiting *Xf*PG *in planta*.

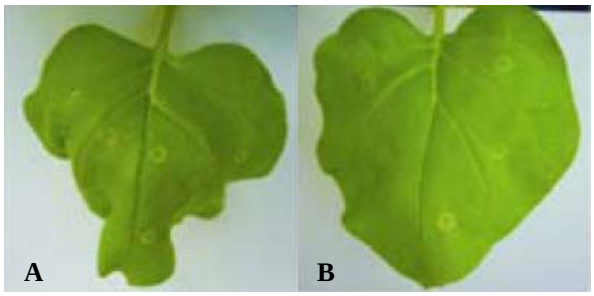

Figure 2. Co-expression of *Bcpg2* with either (A) empty vector control or (B) *Vvpgip* in *N. benthamiana* leaves at 24 h postinfiltration. Leaves were infiltrated with the two *A. tumefaciens* strains in a 1:1 ratio. Figure from Joubert et al., 2007.

Objective 2: XfPG cloning and expression

The *Xf*PG gene was subcloned from the pET29b vector into pMT/BiP/V5-HisA, a vector compatible with the Drosophila protein expression system used by R. Booth. The construct was confirmed by performing digestions with EcoRI, XhoI, EcoRV, and sequencing by the UC Davis DNA Sequencing facility. Transfections were performed with the confirmed *Xf*PG construct or an expression vector containing a GFP marker as the positive control; non-transfected cells were tested as the negative control. Cellular components (pellet) and supernatant (SN) from the transfected lines were collected. Protein expression was validated by SDS-PAGE and Western Blot analyses (**Figure 3**). The *Xf*PG protein has an apparent molecular weight of 70 kD, slightly greater than expected, possibly due to the effects of glycosylation. Crude extracts will be assayed for PG activity while further steps to purify the protein using the attached His-tag will provide *Xf*PG for future *in vitro* PGIP inhibition assays.

Figure 3. Lanes 1-4 are protein collected from the SN of the transfection reaction. Lanes 5-8 are protein collected from the pellet. Lanes 1, 5: negative control; lanes 2, 3, 6, 7: *Xf*PG transfection; lanes 4, 8: positive control of GFP marker-expressing cells.

Objective 2: Model PGIP and XfPG interactions to aid in optimal PGIP prediction for PD defense

The interaction between PG and PGIP proteins influences whether the plant PGIP is able to successfully inhibit the pathogen virulence factor, *XfPG*. The crystal structure of PvPGIP2 (Di Matteo et al., 2003) has facilitated structural inquiries into what regions of the PGIP are responsible for PG inhibition. One study found that a single amino acid, Q224, is responsible for *Fusarium moniliforme* PG (*Fm*PG) inhibition by PvPGIP2 by comparison to PvPGIP1, which is unable to inhibit *Fm*PG (Leckie et al., 1999). While sequence variation can account for some of the specificity, the ability of PvPGIP2 to have competitive, non-competitive, and mixed modes of inhibition for *Fm*PG, *A. niger* PGII, and *B. cinerea* PG1, respectively, suggests that additional recognition and specificity sequences or motifs occur (Federici et al., 2001; King et al., 2002; Sicilia et al., 2005). PGIPs are heavily glycosylated proteins with 7 potential N-linked glycosylation sites on PcBPGIP (Lim et al., 2009) thereby adding 14.5 kD to the molecular weight (Powell et al., 2000). It has been hypothesized that differing glycosylation patterns also affect PGIP specificity.

Homology modeling efforts by D. King created *in silico* interactions between the predicted structures of *Xf*PG and each of the 14 candidate PGIPs to visualize the possible interactions and predict the likelihood of a successful inhibition. Each structural model was created by threading the PGIP amino acid sequence onto the PvPGIP2 crystal structure. Models were then optimized with molecular mechanics, MM3, using the Swiss PDB Viewer DeepView 3.7 and the modeling suite BioMedCACche 6.1 (**Figure 4A**). Glycosylated versions of the models were created by attaching three $Man_3XylGlcNAc_2$ and four $Man_3XylGlcNAc_2Fuc$ groups to the appropriate sites (NxS/T) on the optimized protein structures as previously determined for PcBPGIP (**Figure 4B**; Lim et al., 2009). The putative *Xf*PG model was visualized and optimized with the same techniques used for each PGIP.

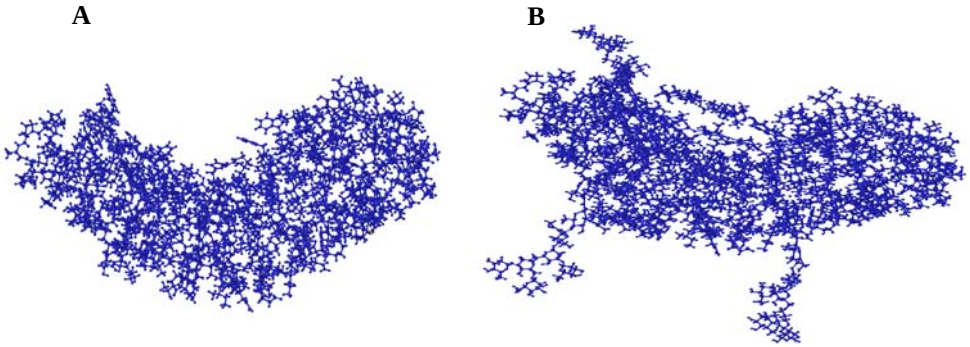

Figure 4. Homology models of (A) the PcBPGIP protein and (B) the protein with N-linked glycosylations. The concave face of the PGIP is thought to interact with PGs.

The inhibitory capability of each PGIP was determined through a series of dynamic reaction simulations where the effectiveness of the potential inhibition was measured by the ability of amino acids or glycosylations on the PGIPs to block key amino acids on the surface of the *Xf*PG that are responsible for cleaving the modeled polygalacturonan (PGA) substrate. The *Xf*PG model was put through a series of simulations with the PGA in its active cleft and keeping various groups of amino acid residues on the outer β sheet locked in place until immobilizing a particular group of residues inhibited the enzyme's *in silico* cleavage of the substrate (**Figure 5A**). It was determined that two clusters of amino acids, 63-74 & 223-226, control the ability of *Xf*PG to cleave its substrate. Dynamic reaction simulations were carried out with the PG, PGIP, and PGA substrate to determine if the PGA was cleaved and therefore, to what extent the PGIP inhibited the PG (**Figure 5B**). The dynamic reaction simulations were supplemented by preliminary surface chemistry mapping in BioMedCACche to determine

if compatible acid/base regions were present on the LRR face of the PGIP and the previously demonstrated controlling region of *Xf*PG. Both techniques identified PcBPGIP, CsiPGIP, and OsPGIP1 as the potentially most effective inhibitors of *Xf*PG.

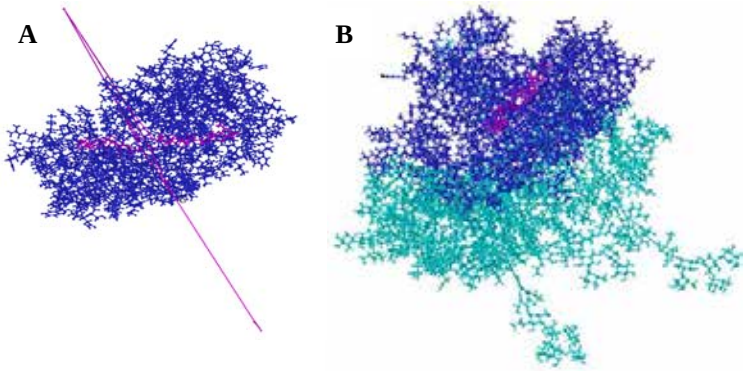

Figure 5. *Xf*PG homology models. (A) *Xf*PG (blue) with PGA (pink) being cleaved in its active site. (B) Dynamic reaction simulation of PcBPGIP (green) inhibiting substrate cleavage by blocking certain residues away from the cleft of *Xf*P.

Objectives 3 and 4
No activity planned for this reporting period.

CONCLUSIONS

1. Fourteen selected PGIPs have been identified that are likely candidates to effectively inhibit *Xf*PG.
2. Cloning has progressed to obtain each of the selected PGIPs in a format so their *in planta* and *in vitro Xf*PG inhibiting activities can be tested.
3. Molecular modeling has progressed so that differences in ability to inhibit *Xf*PG can be related to unique conformational properties of the selected PGIPs.
4. *Xf*PG has been expressed in Drosophila cells to obtain material for *in vitro* analysis of the inhibition activity of the selected PGIPs.
5. Relevant federal agencies have been consulted for regulatory issues related to commercial product development.

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DO CELL WALL STRUCTURES LIMIT *XYLELLA FASTIDIOSA* DISTRIBUTION IN INOCULATED, PIERCE'S DISEASE SUSCEPTIBLE AND RESISTANT GRAPEVINES?

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ABSTRACT
The development and progression of Pierce's disease (PD) symptoms depends largely on the ability of the pathogen to spread via xylem, more specifically, its vessel system, in the infected grapevine. We believe that to the *Xylella fastidiosa* (*Xf)* entering vessels, pit membranes (PM) separating neighboring vessels should become barriers which the pathogen must digest to enhance its systemic spread. Production of occlusions (tyloses and pectin-rich gels) in vessels in response to the presence of *Xf* may also be related to disease symptom development or the host plant's resistance. The research included in this report focuses on these two factors of the host plant which should affect *Xf*'s systemic spread in the host plant. Our data revealed that grape varieties with different PD resistance were different in some cell wall polysaccharides of PMs, that intervessel PMs may be modified in infected PD susceptible grapes and that development of many vessel-obstructing tyloses in response to the presence of *Xf* should contribute to the PD symptom development of the host plant. These observations provide information for understanding of the possible roles of these factors in grape's resistance to PD and are also likely to contribute to identification of an efficient approach for control of the disease.

LAYPERSON SUMMARY
Several of the approaches currently being investigated as strategies for management of Pierce's disease (PD) in vineyards are based on studies that identified the way the disease becomes established in a grapevine. The relative resistance/susceptibility of range of grape genotypes has been studied in the past decade. The work described here asks whether the pathway used by *Xylella fastidiosa* (*Xf)* to spread through a grapevine, the so-called pit membranes (PMs), differ between susceptible and tolerant grape lines. It also asks whether the development of vascular system obstructions, barriers that could either prevent *Xf* spread or shut down vine water transport, or both, differ in susceptible and resistant vines. The data suggest that the polysaccharide compositions of the PMs are different (in terms of kinds or amounts of polymers present) in susceptible and resistant vines. Whether these differences are important in determining whether a given grape germplasm will be PD resistant or tolerant is not yet known.

INTRODUCTION
Pierce's disease (PD) is a devastating grapevine disease caused by the xylem-limited bacterium, *Xylella fastidiosa* (*Xf).* It is clear that vine death is caused by the systemic spread of the locally introduced *Xf* throughout the vine (Krivanek and Walker, 2005; Labavitch, 2007; Lin, 2005; Lindow, 2006a, b, 2007a, b; Rost and Matthews, 2007). The initial introduction of *Xf* by the glassy-winged sharpshooter (GWSS) involves only in few vessels. To spread throughout the grapevine, *Xf* cells must move successively from one vessel to another. The neighboring vessels are separated from one another by the so-called pit membranes (PMs), primary cell wall "filters." Since the meshwork of PMs is too small to permit *Xf* passage, an increase in PM porosity is a prerequisite for spread of the *Xf* population in a host plant (Labavitch et al., 2004).

Xf's genome contains genes encoding cell wall-degrading enzymes (CWDEs), including polygalacturonase (PG) and a few ß-1,4-endo-glucanases (EGase). We believe that *Xf* cells use the CWDEs to digest the polysaccharides of the PMs, opening the primary cell wall barrier and allowing *Xf* passage. 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.

Research in the laboratories of the PIs on the present proposal has shown that the substrates for *Xf*'s CDWEs, pectins and xyloglucans, are present in grapevine PMs (Labavitch, 2007; Labavitch and Sun, 2008) and 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 (Lindow, 2007a, b). Cooperator Andy Walker and his colleagues have identified a grapevine QTL that contains the Pierce's disease 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 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 that are expressed as differences in porosity, polysaccharide composition or susceptibility to *the pathogen'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.

Grape genotypes show differential PD resistance. Most *vinifera* varieties are susceptible to PD, while wild *Vitis* species and some of their hybrids with *vinifera* varieties have been demonstrated to have PD tolerance or resistance in greenhouse and field evaluations. Quantitative analyses of the concentration and distribution of the pathogen have clarified that *Xf*'s spread from the inoculation site in resistant genotypes is limited relative the its spread in susceptible *vinifera* varieties (Lindow, 2007a), suggesting differences in PM polysaccharide composition among the genotypes with differential PD resistance. Therefore, the clarification of any possible cell wall compositional differences in PMs of those grape varieties/genotypes is essential to the better understanding of the natural PD resistance mechanisms of grapes.

While the production of gels and tyloses in response to infection have been examined in several programs (e.g., Lin, 2005; Stevenson et al., 2004), detailed information about the spatial and temporal distributions and of vascular occlusions in susceptible and resistant germplasm is still lacking. This information is crucial to clarify the role of the vascular occlusions in PD symptom development or disease resistance of host plant. An efficient system to evaluate the development of vascular occlusions in grapevines quantitatively and qualitatively has been developed by Co-PI Sun (Sun et al., 2006, 2007 and 2008) and was used in this study. The utility of immunohistochemical techniques in identifying the polysaccharides of grapevine PMs and vascular occlusions 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 grape germplasms where differential resistance to PD has 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. These results may provide the information useful for finding an effective approach for control of grape PD.

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?

   (**Note**: The original proposal had four Objectives, but only Objectives 1 and 2 were approved for funding.)

RESULTS AND DISCUSSION

Differences in cell wall polysaccharide compositions of pit membranes of grapevines with differential PD resistance

In this research, we have used the following grape genotypes/varieties with different PD susceptibility: *Vitis vinifera var.* Chardonnay (susceptible), *Muscadinia rotundifolia* (highly tolerant) and 89-0908 (resistant, a hybrid of *V. arizonica* x *rupestris*). The immunohistochemical techniques and confocal laser scanning microscopy we established previously were used to identify and compare polysaccharide compositions of the vessel PMs in these genotypes/varieties. The research covered both intervessel PMs and vessel-parenchyma PMs, which exist in vessel lateral walls. The former are the barriers to *Xf*'s systemic spread, while the latter are related to the development of vascular occlusions (tyloses and gels) and may contribute to disease resistance or symptom development.

Our experiments focused on two major groups of cell wall polysaccharides: homogalacturonans (the predominant components of pectin) which polygalacturonases may attack, and xyloglucans (XyGs, a major group of hemicellulosic polysaccharides), the substrates of endo-glucanases. We have used three different kinds of monoclonal cell wall antibodies to identify the polysaccharide composition of PMs: JIM5, JIM7 and CCRC-M1. JIM5, JIM7 and CCRC-M1 can recognize weakly methyl-esterified homogalacturonans (low Me- HGs), heavily Me-esterified HGs (high Me-HGs), and fucosylated XyGs, respectively. Our aim is to determine whether there are any differences in the presence or distributions of these two groups of polysaccharides in the PMs of the four genotypes/varieties studied.

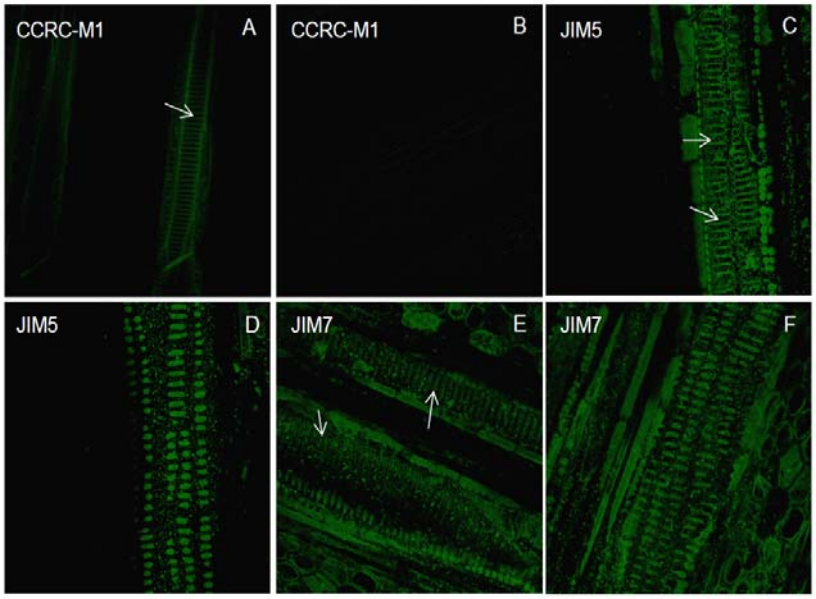

Figure 1. Cell wall compositions in intervessel pit membranes (A, C, E) and vessel-parenchyma PMs (B, D, F) in 89-0908, a PD-resistant *Vitis* genotype. A-B, No green fluorescence from intervessel PMs (A) and vessel-parenchyma PMs (B) in xylem tissue treated with CCRC-M1, indicating that fucosylated XyGs in both types of PMs are below the detectable level. C-D. PM composition revealed by JIM 5. Low Me-HGs are detected in vessel-parenchyma PMs (arrowed, D) but not in intervessel PMs (arrows, C). E-F. PM wall composition revealed by JIM7. Very weak fluorescence and relatively strong fluorescence are detected from intervessel PMs and vessel-parenchyma PMs, respectively, indicating that high Me-HGs are at a low concentration in intervessel PMs but in larger amount in vessel-parenchyma PMs.

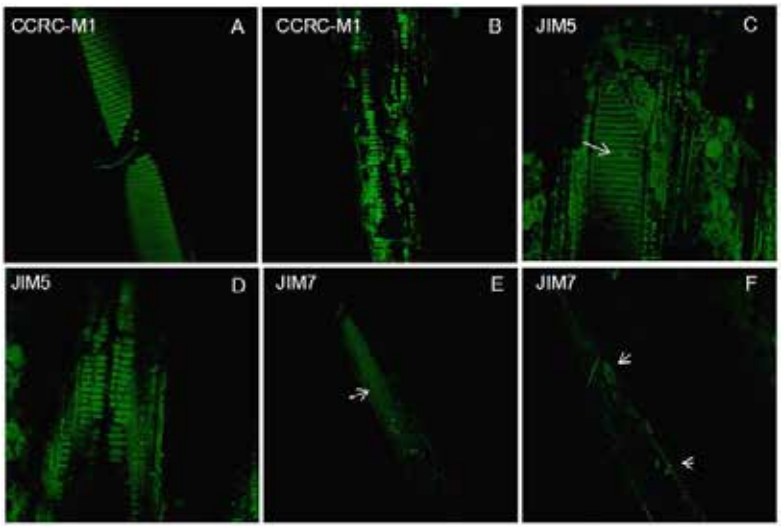

Figure 2. Cell wall compositions in intervessel PMs (A,C,E) and vessel-parenchyma PMs (B,D,F) in *Muscadinia rotundifolia*, a highly PD-tolerant grape genotype. A-B, Cell wall composition revealed by CCRC-M1, showing the presence of fucosylated XyGs in both intervessel PMs (A) and vessel-parenchyma PMs (B). C-D. Cell wall composition revealed by JIM5. Low Me-HGs are not obvious in intervessel PMs (C) but are present abundantly in vessel-parenchyma PMs (D). E-F. Cell wall composition revealed by JIM7. Fluorescence signal is detected from both intervessel PMs and vessel-parenchyma PMs, but is relatively weak, indicating a limited amount of high Me-HGs in both types of PMs.

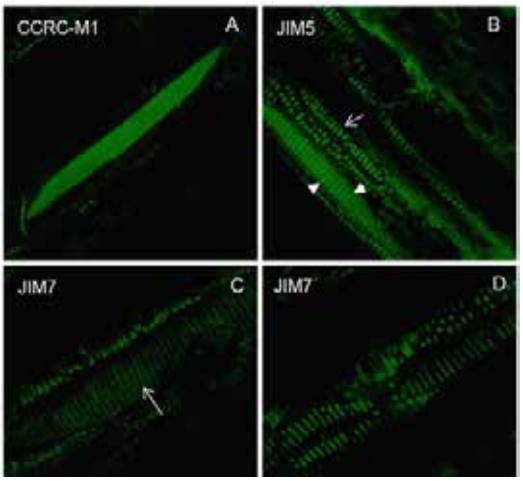

Figure 3. Cell wall compositions of intervessel PMs (A,C,E) and vessel-parenchyma PMs (B,D,F) in *Vitis vinifera* cv. Chardonnay, a PD-susceptible genotype. A. Intervessel PMs have strong fluorescence when incubated with CCRC-M1, indicating the abundant presence of fucosylated XyGs. B. Xylem tissue incubated with JIM5, showing that low Me-HGs are common components of both intervessel PMs (arrow head) and vessel-parenchyma PMs (arrow). C-D. Xylem tissue incubated with JIM7. Fluorescence is below the detectable level in intervessel PMs (arrow, C) and is strong from vessel-parenchyma PMs (D), indicating high Me-HGs is weakly present in intervessel PMs (C) but is abundantly present in vessel-parenchyma PMs (D).

Our results have indicated that the four genotypes with different PD susceptibility all have intervessel PMs and vessel-parenchyma PMs in their vessel lateral walls. Individual intervessel PMs are transversely elongated across the whole surface of the shared (i.e., common) wall of neighboring vessels and are arranged in a tight scalariform pattern along the vessel long axis (Fig. 2A). Vessel parenchyma PMs are round, oval or slightly transversely elongated (**Figure 2D**).

The genotypes also showed differences in the polysaccharide compositions of intervessel and vessel-parenchyma PMs. In 89-0908, both intervessel PMs (**Figure 1A**) and vessel-parenchyma PMs (**Figure 1B**) lack fucosylated XyGs In addition, their intervessel PMs do not have a detectable amount of low Me-HGs (**Figure 1C**) or high Me-HGs (**Figure 1E**). However the vessel-parenchyma PMs contain both low Me-esterified (**Figure 1D**) and high Me- HGs (**Figure 1F**). In *Muscadinia rotundifolia*, strong fluorescence signals were detected from both intervessel PMs (**Figure 2A**) and vessel-parenchyma PMs (**Figure 2B**) when incubated with CCRC-M1 (showing fucosylated XyGs) in both types of PMs. Some high Me-HGs are also present in both types of PMs (**Figures 2E and 2F**). Low Me-HGs occur in vessel-parenchyma PMs (**Figure 2D**) but are not detected in intervessel PMs (**Figure 2C**). In *V. vinifera var.* Chardonnay, fucosylated XyGs (**Figure 3A**) and low Me-HGs (**Figure 3B**) are abundantly present in both intervessel PMs and vessel-parenchyma PMs. High Me-HGs occur in a large quantity in vessel-parenchyma PMs (**Figure 3D**), but are undetectable in intervessel PMs (**Figure 3C**).

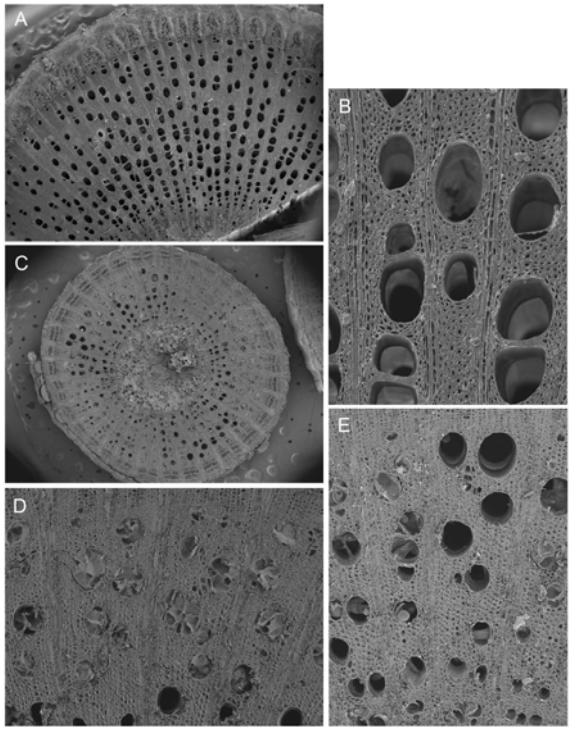

Figure 4. Xylem structure of control (A and B) and inoculated (C-E) vines. A-B. No vascular occlusions occurred in secondary xylem vessels (A); a closer image shows that vessel lumens are empty (B). C. Vascular occlusions developed in secondary xylem of inoculated branches and showed uneven distribution. D. A xylem region with extensive vascular occlusions, showing most vessels blocked by tyloses. E. Xylem region with fewer vascular occlusions and some empty vessels.

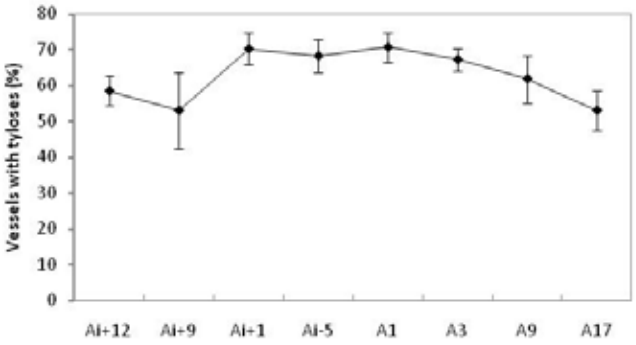

Figure 5. Comparison of vascular occlusion occurrence among different internodes of the two shoots of a same vine. "Ai" and "A" are the shoots with *Xf* inoculation and without inoculation, respectively. The number following "Ai" indicates a specific internode with the positive or negative number showing that the counting of internode started from the inoculated internode and moved upward (positive) or downward (negative), respectively. The number following "A" shows the internode in the non-inoculated shoot, counted from its base.

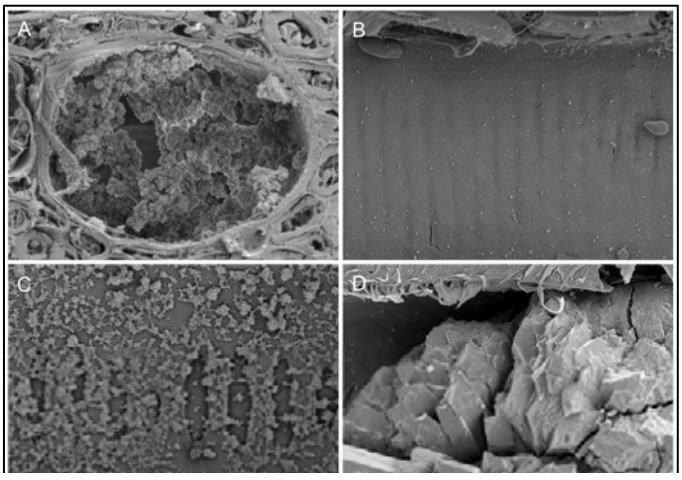

Figure 6. Other types of vascular occlusions in infected grapevines. A. Gels in a vessel lumen. B. Gels covering the lateral wall of a vessel. C. Gels sparsely attached to the vessel lateral walls. D. Crystals filling a vessel lumen.

Comparison of vascular occlusion formation between control vines and sick vines

PD susceptible Chardonnay vines were used in our experiment. Each chardonnay vine on rootstock was pruned back with only two buds left at the base. The two buds thus develop into two branches. When the branches are six weeks-old, one branch of each treatment vine was needle-inoculated with *Xf* at the 12th internode from the base. Vines for controls were inoculated at the corresponding internode with phosphate buffer also on one of the two branches for each vine. Both

branches of each vine (control and treatment) were kept about 25 nodes in height by pruning the top off. Samples were collected from both branches of each vine for both control and treatment vines at different times after the inoculation. Included here are only the data from the vines at Week 12 after inoculation when severe external PD symptoms of the treatment vines have developed.

The vines inoculated with *Xf* and those inoculated with buffer showed obvious differences in secondary xylem structure (**Figure 4**). In control vines, no vascular occlusions were observed in secondary xylem, even in the internode with the inoculation of buffer (**Figures 4A and B**). In vines treated with *Xf*, extensive formation of vascular occlusions occurred in secondary xylem vessels (**Figure 4C**). Vascular occlusions in infected vines were not even in vessels across the transverse section. Instead, in some regions of xylem, they were present in most of the vessels (**Figure 4D**), while in other regions, some vessels were free of vascular occlusions (**Figure 4E**). The cause for patchy occurrence of vascular occlusions in secondary xylem is not known.

Investigation of the spatial distribution of vascular occlusion indicated that it occurred to the internodes of both branches of each infected vine, no matter how far away the internodes were from the inoculation site. Quantitative analysis of vascular occlusions revealed that the percentage of the vessels with one or more vascular occlusions was usually around 60% in all the examined internodes and that no big difference can be distinguished between the two branches of each vine as well as among different internodes of each branch (**Figure 5**).

When tracking through vessels in the longitudinal direction, we found that tyloses did not always continuously block a whole vessel; a given vessel may have some gaps where no occlusions developed. With this in consideration, the actual percentage of vessels affected by vascular occlusions should be higher than the value measured at any transverse section. The effect of vascular occlusion on hydraulic conductivity of xylem is to be evaluated.

Our investigation also clarified that three types of vascular occlusions excluding *Xf* formed in secondary xylem. Tyloses are the predominant type and accounted for over 95% of the occlusions in vessels (**Figures 4C and D**). Pectin-rich gels were another type of occlusion observed; these formed usually in less than 3% of the total vessels (**Figures 6A-C**). Occasionally, crystals were found in the vessels of infected vines and may partially or completely block the affected vessels (**Figure 6 D**).

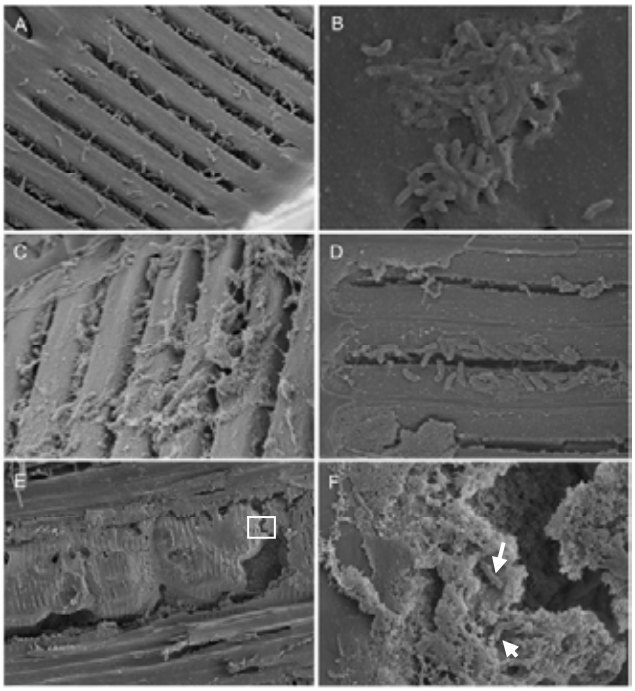

Figure 7. Distribution of *Xf* in infected vines. A. Bacteria are mostly present freely in the internode just above the internode with the inoculation site. B. Many bacteria in an aggregate in the 9th internode (the counting started from the inoculated internode with it as zero). C. Some free bacteria and some bacteria in an aggregate in the 9th internode of the non-inoculated shoot (the counting started from the shoot base with the lowest internode as one). D. Free bacteria in the 17th internode of the non-inoculated shoot (the counting started from the shoot base with the lowest internode as one). E. A vessel filled with tyloses in the lowest internode of the non-inoculated shoot. Gels were present between tyloses. F. Enlargement of the rectangle region in E, showing bacteria embedded in the gels.

Distribution of Xf after inoculation

In the vines with severe external PD symptoms, *Xf* cells were observed in all the examined internodes of the two branches (**Figure 7**). This indicated that the bacteria could move not only upward from the inoculation site in the shoot, but also travelled downward, from the inoculated shoot to the trunk shared by the two branches, and then moved into the non-inoculated branch and travelled up towards its top internodes.

Our observations also indicated that bacteria in the vines with severe external PD symptoms were present in very few vessels. Vessels with *Xf* were usually less than 10% and 3% of all vessels in the inoculated and non-inoculated shoots, respectively. The number of bacteria in the affected vessel was also larger in the internodes of an inoculated shoot than in those of a non-inoculated shoot. However, no vessels with enough bacteria to completely block vessels were observed, as suggested by some earlier studies . Since *Xf* are only present in few vessels in limited amount, a direct influence of bacterial inhabitation on the water transport through the vessel system should be very limited.

Xf were present in vessel lumens in several different forms. Most commonly, they occurred as free individuals (**Figures 7A and D**). Bacteria in this form were observed in the internodes of both inoculated and non-inoculated shoots. Aggregates of 2-6 cells were also common, in which bacteria are loosely bound together through a filamentous network (**Figure 7C**). Occasionally, aggregates formed by tens or hundreds of bacteria were observed in some vessel lumens (**Figure 7B**). Bacteria were also observed between loosely or compactly arranged tyloses (**Figures 7E and F**). In this case, bacteria were always embedded in gels whose origin (tylose or bacterium) is not clear.

CONCLUSIONS

1. Grape varieties/genotypes with differential PD resistance show differences in the cell wall polysaccharide composition of intervessel PMs. The intervessel PMs of resistant genotypes lack fucosylated xyloglucans and weakly Me-esterified HGs, and contain only a little amount of heavily Me-esterified HGs, while the PMs of the more susceptible genotypes/varieties all have fucosylated xyloglucans, and contain substantial amounts of either heavily Me-esterified HGs or weakly Me-esterified HGs. The absence of polysaccharide substrates for *Xf*'s CWDEs in intervessel PMs of resistant genotypes may limit the ability of the pathogen to move away from the inoculation point and, thus, may contribute to the localized distribution of *Xf* in host plant and its PD resistance.
2. Multiple types of vascular occlusions (tyloses, gels and crystals) may develop in infected vines, but tyloses are the principal occlusion type which blocks the majority of vessels, contributing the symptom development.
3. *Xf* may occur in diverse forms (singly, or in groups) and in different parts of the vines with severe PD symptom, but the *Xf* cells are present in only few vessels where they are too low in number to block the vessels.

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SEASONAL TRANSMISSION OF *XYLELLA FASTIDIOSA* BY GLASSY-WINGED SHARPSHOOTER FROM GRAPEVINES: SHARPSHOOTER PREFERENCE FOR INFECTED GRAPEVINE TISSUE

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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)*. Over the course of this two year project we have determined that GWSS males and females choose to feed on young leaf, petiole, and stem tissue compared to the same tissues on older parts of the grapevine cane, regardless of the time of year. However, they will feed on old stem tissue, which logically should result in more rapid chronic infection than feeding on young tissue. GWSS adults frequently change position between various tissues through the day, which may contribute to the apparent effectiveness in spreading *Xf*. We have determined that GWSS adults do not feed on cordon tissue, regardless of the time of year. In winter studies, we found that GWSS prefer to feed on grapevine tissue that is infected with *Xf* over tissue that is not infected. This has tremendous implication for bacterial acquisition during the dormant periods of the year, and since GWSS adults retain *Xf* for life, this represents another interesting feature of this invasive vector that may contribute to Pierce's disease (PD) spread. In fall studies when vines were in full flush, the preference for infected tissue was not present. Both GWSS and the closely related smoketree sharpshooter (STSS) fed equally on infected and non-infected grapevine tissue. This work shows yet another aspect of GWSS and STSS biology that is important to the spread of *Xf*.

LAYPERSON SUMMARY
The detailed experiments that have been conducted in this project have tremendous implication for the movement of *Xylella fastidiosa* (*Xf)* by the glassy-winged sharpshooter (GWSS). We have learned that GWSS showed a strong preference for grapevine canes from infected vines in the winter months. This aspect of GWSS biology is interesting and contributes to its status as a vector of *Xf* in grapevines. If we can determine the cause of this preference, we may be able to design methods to reduce it. Studies in the fall months did not reveal a preference for infected or non-infected grapevine tissue. We found that GWSS and smoketree sharpshooter (STSS) move readily between infected and non-infected tissue, again a behavior that would contribute to *Xf* in the field. Studying these detailed behaviors contributes to our understanding of the epidemiology of Pierce's disease vectored by GWSS and STSS.

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, GWSS, 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 glassy-winged sharpshooter (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). 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) (Perring et al. 2008), 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 observed GWSS feeding in this tissue (Almeida et al. 2005b), 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 these past studies with present and future research 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.

OBJECTIVES:

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 two, three, or four 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 two, three, or four 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.

We were forced to modify the original objectives due to the fact that suspected infections of our grapevines were not present. At the time we started in July 2007, selected vines in our field cages had been needle-inoculated in May 2003, May 2004, and May 2005 by cooperator Groves. An evaluation of all the vines on August 28, 2007 showed almost no infection with *Xf*. It is unclear why the infections did not become systemic, but the fact that we had no multi-year infections dictated a revision of our original plans. We re-inoculated the set of vines that had been inoculated in 2003 by scraping the bark on the cordons to expose green tissue for needle inoculation. This procedure was done on November 5, 2007 and September 8, 2008 and has yielded severe infections for us to use. While waiting for infections, we proceeded with experiments to document GWSS feeding biology through the season in choice and no-choice studies. Below we summarize these studies, the data of which are presented in Perring et al. (2008).

RESULTS AND DISCUSSION

Choice and No-choice Studies

Choice studies were conducted in the fall 2007 (August 29, and September 11, 2007), winter 2008 (January 16, and February 6, 2008) and summer 2008 (July 1, 2008). For this research, we placed GWSS adults individually in observation cages fabricated from acetate cylinders (25cm x 17cm diameter) with organdy 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. We made hourly observations during daylight hours over three consecutive days to determine the location of each GWSS. 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.

No-choice studies were conducted in the winter 2008 (February 26, March 4), summer 2008 (July 15), and fall 2008 (September 19). Individual GWSS were caged on selected grapevine tissue in 50 ml polypropylene centrifuge tubes 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. 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^2. 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. The sharpshooters were allowed to feed for four days.

In these 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.

GWSS preference for infected/non-infected grapevine tissue

We selected canes from putative infected and non-infected Cabernet Sauvignon and Chardonnay grapevines to study GWSS choice for infected or non-infected tissue. Trials were conducted with GWSS on February 19-21 and February 25-27 2009. Because of the time of year, there were no leaves or petioles on the canes. All tissue had a brown hardened outward appearance, but we confirmed that the internal tissue was green, so GWSS would be able to feed. We placed GWSS adults individually in observation cages, which were placed over a section of cane from an infected vine and a section of cane from a non-infected vine (**Figure 1**). The infected cane was marked with a small wire label. All sharpshooters were placed on the cage, so they were forced to make a choice to find a feeding host. The ends of the observation cage were sealed giving a single GWSS in each cage access to infected or non-infected cane tissue. Twenty cages were used for each trial. We made hourly observations from 8am to 5pm over three consecutive days to document the cane (infected or non-infected) on which the GWSS fed.

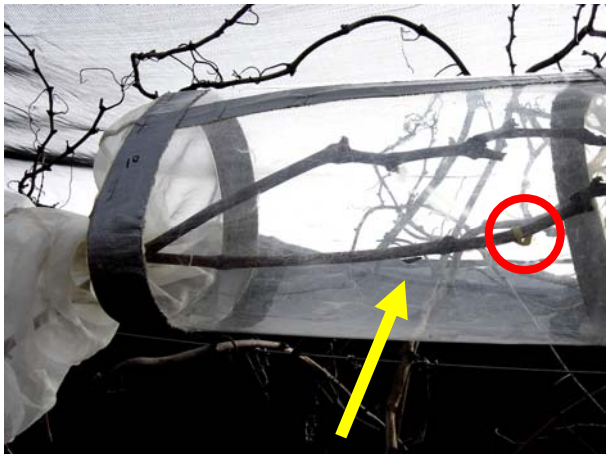

Figure 1. Acetate cage uses to evaluate GWSS feeding preference for infected (marked with yellow wire label (in circle) and non-infected grapevine tissue. Notice GWSS feeding in center of infected cane (arrow).

At the conclusion of the studies, we conducted a variety of procedures to verify the infection status of the cane tissue to which the GWSS were exposed. First each section of both canes that were inside the acetate cages was removed from the vine and a small section (0.5 in) was macerated and subjected to ELISA immediately after the trial was concluded. Second, the cane sections were marked and planted into pots. Following growth of these cuttings, we conducted ELISA and culturing to determine the infection status of the section of cane to which GWSS was exposed. Third, when we pruned the vines, we selected six canes and planted an approximately 14 inch section from each cane into pots to grow in the greenhouse. After they pushed leaves, we assayed these plants by ELISA. Finally, each vine was visually assessed in the fall for symptoms of *Xf* infection. Symptomatic canes were sampled and subjected to ELISA.

From the various tests, we determined the infection status of all the canes used in the experiments and discarded the cages in which we were unable to make a confident determination. We also discarded cages in which the GWSS died, because this indicated the inability of the insect to successfully feed on either cane. This filtering resulted in nine total cages for the February 19-21 trial (four Cabernet Sauvignon, five Chardonnay, four females and five males) and a total of 11 cages for the February 25-27 trial (four Cabernet Sauvignon, seven Chardonnay, six females, and five males). Because of the small numbers present in each variety and gender, the data are presented as totals for each trial.

Results from both trials showed that GWSS was found more often on the infected vines (**Figure 2**). For the February 19-21 test, GWSS were present on the infected tissue 71% of observed times, while they were on non-infected tissue just 22% of the time. They were found on the cage only 7% of the time. In the second trial (February 25-27), they again were found more often on the infected cane (71%) compared to the non-infected cane (22%) or the cage (7%). We were surprised that the proportions for each of these trials were the same, and have no explanation for this similarity. This is particularly remarkable, given that there was a total of 215 observation times in the first trial and 303 observation times in the second trial (**Table 1**) and the two trials were conducted with different insects on different canes, often from different vines, and at two distinct times.

Also interesting were the movements that sharpshooters made throughout the studies. More GWSS moved to infected canes and stayed for three or more hours than to non-infected canes (**Table 1**). Additionally, there were more sharpshooters that fed on infected canes, left these canes and returned to the infected canes, than those on non-infected canes. Clearly there was something unique about the infected canes that the sharpshooters preferred. It also is apparent that sharpshooters in this study moved about the cages often (17 of a possible 215 observations in trial 1 (8%) and 36 of a possible 303 observations (12%) in trial 2).

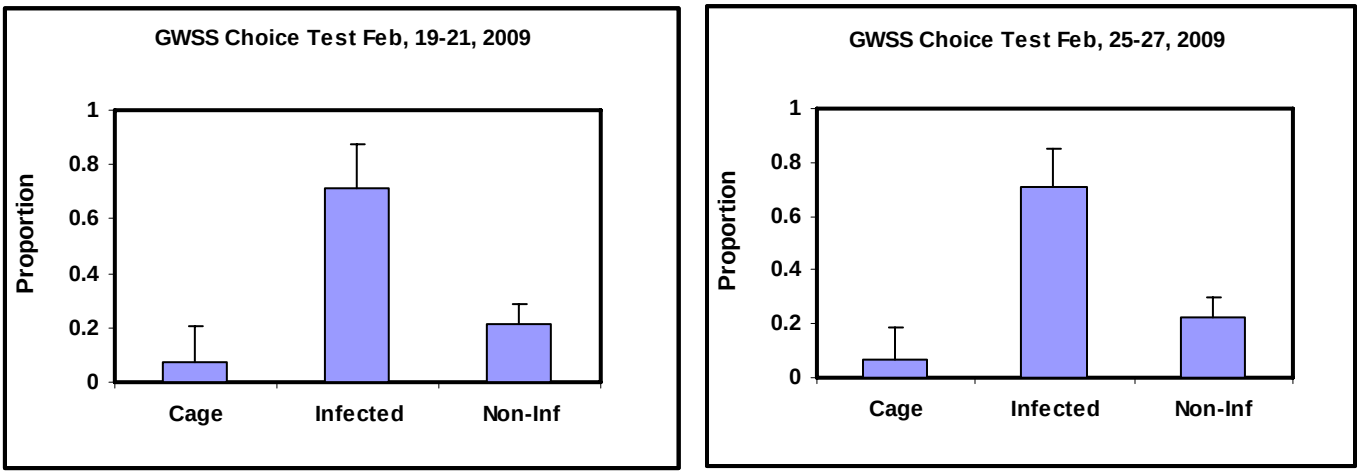

Figure 2. GWSS preference on field-grown Cabernet Sauvignon and Chardonnay grapevines in choice experiments initiated on 19 February (left) and 25 February (right), 2009. Bars represent average proportions of GWSS (± SE) observed on the cage, on the infected canes, and on the non-infected canes.

Table 1. Actions taken by GWSS in two trials (February 19-21 and February 25-27, 2009). Sharpshooters were given a choice between infected and non-infected cane tissue over the 3 day period and observations were made hourly during the daylight hours.

Parameter	Trial 1 (Feb 19-21)	Trial 2 (Feb 25-27)
Chose Infected and stayed 3h or more	12	13
Chose Non-Infected and stayed 3h or more	3	3
Chose Inf. for 3h, left, returned for 3h or more	3	4
Chose Non-I for 3h, left, returned for 3h or more	0	0
Moved from Cage to Inf.	7	12
Moved from Cage to Non-I	1	7
Moved from Inf. to Cage	5	5
Moved from Non-I to Cage	1	4
Moved from Inf. to Non-I	1	4
Moved from Non-I to Inf.	2	4
Total number of Times insect moved	17	36
Total number of Observed Times	215	303

A second set of choice experiments was conducted in September, 2009. These studies, which had the same design as those conducted in February, utilized infected canes that were severely diseased. A healthy, asymptomatic cane was paired with each diseased cane and the canes were stripped of all but 1 leaf within the experimental cage. Twenty cages were established on Chardonnay vines on September 17, and into each cage we introduced a single GWSS female. Observations were made hourly from 8am to 6pm for three days. Utilizing the same 20 cages on the same canes, a second trial was initiated on September 20 with 20 female smoketree sharpshooters (STSS). Observations again were made each hour from 8am to 6pm for a period of three days.

Sharpshooter responses from these trials were distinctly different from the studies conducted in February. In the September 17-19 trial, a slightly higher proportion of GWSS were observed on the non-infected cane (56%) than on the infected canes (40%), with just 4% of the observations on the cage (**Figure 3**). Interestingly, similar results were found for the STSS. This species showed a slight preference for the non-infected canes (51%) rather than the infected canes (40%), with 9% of the observations on the cage (**Figure 3**). We will be collecting the canes from this study in an effort to analyze the xylem sap to see if any particular chemical constituents were present in the canes on which sharpshooters predominantly fed.

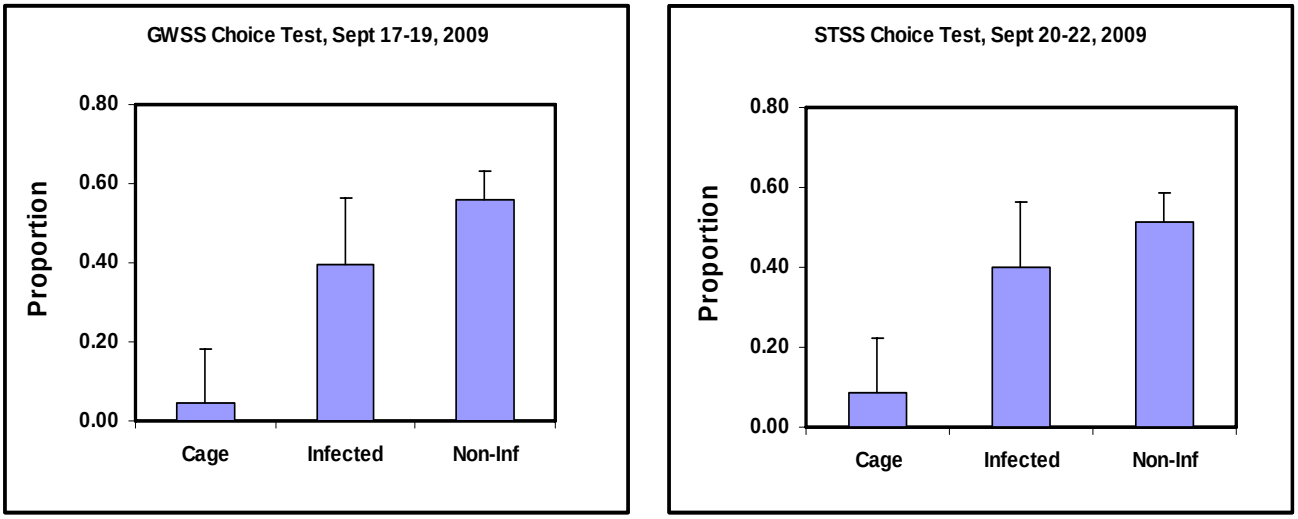

Figure 3. Female GWSS preference (left graph) and STSS preference (right graph) on field-grown Chardonnay grapevines in choice experiments initiated on September 17 (STSS) and September 20 (STSS). Bars represent average proportions of sharpshooters (± SE) observed on the cage, on the infected canes, and on the non-infected canes.

Table 2. Actions chosen by GWSS (September 17-19) and STSS (September 25-27) in choice studies between infected and non-infected cane tissue over the three day period . Observations were made between 8am and 6pm.

Parameter	GWSS (Sept. 17-19)	STSS (Sept. 25-27)
Chose Infected and stayed 3h or more	11	21
Chose Non-Infected and stayed 3h or more	19	25
Chose Inf. for 3h, left, returned for 3h or more	3	9
Chose Non-I for 3h, left, returned for 3h or more	10	5
Moved from Cage to Inf.	15	23
Moved from Cage to Non-I	14	26
Moved from Inf. to Cage	3	17
Moved from Non-I to Cage	10	14
Moved from Inf. to Non-I	12	10
Moved from Non-I to Inf.	8	12
Total number of Time insect moved	62	102
Total number of Observed Times	485	633

Sharpshooters moved slightly more often in this set of experiments than in the February study. In the GWSS trial, insects moved 62 out of a possible 485 observations (13%) and 102 out of 633 observations (16%) (**Table 2**). There were more GWSS that settled and had prolonged feeding (at least 3 hr) on non-infected canes than on infected canes. There was only a slightly higher number of STSS that had prolonged feeding on the non-infected canes than the infected canes.

CONCLUSIONS

In winter choice studies in which GWSS were given access to infected and non-infected grapevine tissue, GWSS were found more often on grapevine tissue that was infected with *Xf* over tissue that was not infected. The reason why this choice was made is unknown, but likely is related to the biochemical components in the various cane tissues (Anderson et al. 1992). Regardless, the fact that GWSS prefers infected tissue has important epidemiological ramifications. Specifically, feeding on infected tissue increases the likelihood of sharpshooters acquiring *Xf*. Movement by these sharpshooters to non-infected tissue, which occurred 1/17 (6%) and 4/36 (11%) times in the two winter trials, could rapidly move the bacteria causing new infections. It is important to remember that the cane tissue was woody (although green inside) and sharpshooters easily fed on this tissue.

In fall experiments, on vines containing green leaves, sharpshooter preference for infected tissue was not apparent. Both GWSS and STSS fed equally on infected and non-infected grapevine tissue. These data suggest that there was nothing in either infected or non-infected tissue that caused sharpshooters to feed preferentially. Both insect species moved readily from infected to non-infected tissue (12/62 = 19% and 10/102 = 10% for GWSS and STSS, respectively). They also moved from non-infected tissue to infected tissue with similar frequency. These results suggest that transmission between infected and healthy vines may be greater at this time of year.

The work reported here is valuable to our understanding of GWSS and STSS feeding behavior that can influence transmission of *Xf*. These studies fill an important data gap in our knowledge of GWSS- and STSS-vectored epidemiology at various times of the year. We plan to continue studies through next year, to confirm the preference of sharpshooters for infected tissue. During this work, we will conduct biochemical assays similar to Andersen et al. (1992) to determine what components are correlated with GWSS and STSS feeding.

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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 2009.

ABSTRACT
This project is designed 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 (GWSS) and smoketree (STSS) sharpshooters can acquire and transmit *Xf* into grapevines. In our studies *Xf* was successfully isolated from needle-inoculated alfalfa, basil, lima bean, tomato, annual bluegrass, cheeseweed, wild-type sunflower, goosefoot, London rocket, Spanish broom, tree tobacco, annual ryegrass, black mustard, Blando brome, New Zealand White clover, Hykon Rose clover, cowpea, fava bean, Miranda field pea, meadow barley, California Red oats, and White sweetclover. We were unable to recover *Xf* from bell pepper, cotton, black nightshade, common groundsel, Evening Sun sunflower, horseweed, Zorro annual fescue, birdsfoot trefoil, or sudangrass plants. We have confirmed successful transmission of *Xf* by GWSS for alfalfa-to-alfalfa, alfalfa-to-grapevine, basil-to-basil, basil-to-grapevine, tomato-to-tomato, Blando brome-to-Blando brome, Blando brome-to-grapevine and cowpea-to-cowpea. GWSS transmission of *Xf* from tomato-to-grapevine, cowpea-to-grapevine, fava bean-to-fava bean, and fava bean-to-grapevine could not be confirmed with culturing. We have determined that STSS can transmit *Xf* between alfalfa plants, from alfalfa to grapevines, between Blando brome plants, from Blando brome to grapevines, and between fava bean plants. We were unable to confirm successful transmission by STSS from tomato-to-tomato, tomato-to-grapevine, cowpea-to-cowpea, or cowpea-to-grapevine, or from fava bean to grapevines. Goosefoot appears to be a poor host for GWSS, STSS, and *Xf*, as nearly all the vectors died before the end of the 48-hr acquisition access period. *Xf* isolates obtained from goosefoot were few and slow-growing.

LAYPERSON SUMMARY
Evaluating the potential of various common plant species found in and near vineyards to serve as reservoirs of Pierce's disease (PD), and the ability of glassy-winged (GWSS) and smoketree (STSS) sharpshooters to acquire and transmit PD from these alternative plant hosts, is fundamental to managing the primary spread of PD in California vineyards. Identifying the plants that contribute to primary spread enables growers to target these plants around their vineyards as a mechanism to reduce spread. Understanding how these two vectors contribute to primary and secondary spread can assist in the development of alternatives to the area-wide management program. To reduce primary spread, efforts must focus on reducing bacteria-carrying vectors from entering healthy vineyards through continued area-wide or local treatment programs outside the vineyard, barriers, trap crops, and/or removal of pathogen sources outside the vineyard.

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 the glassy-winged sharpshooter (GWSS), an insect with a broad host range, theoretically increases the probability of disease spread from the 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 a 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

Needle Inoculated Plants

Thirty-four plant species have been needle-inoculated with *Xf* (**Table 1**). *Xf* does not appear to be able to survive in bell pepper, cotton, black nightshade, common groundsel, Evening Sun sunflower, horseweed, Zorro annual fescue, birdsfoot trefoil, or sudangrass plants. A few positives were detected at two-weeks post-inoculation with ELISA for bell pepper, cotton, common groundsel, and horseweed, but no plants tested positive by ELISA at four weeks, nor were they positive by culturing. These results suggest a transient infection or detection of dead *Xf* cells by the early ELISA. Evening Sun Sunflower tested positive by ELISA for all 20 plants, but the cultures were clean and negative. However, the Evening Sun sunflower also died very quickly, which may explain why it was not detected by culture. Final results are pending for filaree, Shepherd's purse, and stinging nettle.

Xf was successfully isolated from needle-inoculated alfalfa, basil, lima bean, tomato, annual bluegrass, cheeseweed, wild-type sunflower, London rocket, goosefoot, Spanish broom, tree tobacco, annual ryegrass, black mustard, Blando brome, New Zealand White clover, Hykon Rose clover, cowpea, fava bean, Miranda field pea, meadow barley, California Red oats, and White sweetclover. We recovered one isolate of *Xf* for lima bean in the first needle-inoculation set, so we repeated this test. No isolates were recovered from a second needle-inoculated set, suggesting that lima bean is a poor host for *Xf*. We did not isolate *Xf* from basil until 16 weeks post-inoculation. All ELISA tests for Basil were positive, including those for the negative controls, indicating that the commercial kit for *Xf* from Agdia, Inc. is not reliable for testing this plant species. The cultures for the negative controls were always negative, including at 16-weeks post-inoculation when the positive cultures from other plants were obtained. We only recovered one isolate from annual bluegrass. Cultures from annual bluegrass and fava bean have been routinely heavily contaminated, regardless of plant age or inoculation status. Other microbes present in the plants may be obscuring the presence of *Xf* in those species. Healthy grapevines also were needle inoculated with every inoculation group as positive controls for each set.

Table 1. ELISA and culture results for plant species needle-inoculated with *Xf*.

Type	Common Name	Scientific Name	ELISA +	Culture +	*Xf* Recovered?
Agriculture Crops	Alfalfa	*Medicago sativa*	20/20	14/20	Yes
	Basil, Italian Large Leaf	*Ocimum basilicum*	20/20*	10/20	Yes
	Bell Pepper, Taurus	*Capsicum annuum*	5/20**	0/20	No
	Cotton, Upland	*Gossypium hirsutum*	2/15**	0/15	No
	Lima Bean, Fordhook 242	*Phaseolus lunatus*	2/38	1/38	Yes
	Tomato, Rutgers	*Solanum lycopersicum*	15/39	8/38	Yes
Weeds	Annual Bluegrass	*Poa annua*	8/20	1/20****	Yes
	Black Nightshade	*Solanum nigrum*	0/20	0/20	No
	Cheeseweed	*Malva parviflora*	7/20	16/20	Yes
	Common Groundsel	*Senecio vulgaris*	3/20**	0/20	No
	Common Sunflower, Evening Sun	*Helianthus annuus*	20/20*	0/20	No
	Common Sunflower, wild-type	*Helianthus annus*	19/20	7/20	Yes
	Filaree	*Erodium species*	Tests in Progress		
	Goosefoot	*Chenopodium species*	7/40***	5/33	Yes
	Horseweed	*Conyza Canadensis*	2/20**	0/20	No
	London Rocket	*Sisymbrium irio*	5/20	13/20	Yes
	Shepherd's Purse	*Capsell bursa-pastoris*	Tests in Progress		
	Spanish Broom	*Spartium junceum*	17/20	17/20	Yes
	Stinging Nettle	*Urtica species*	Tests in Progress		
	Tree Tobacco	*Nicotiana species*	12/20**	2/20	Yes

Table 1. ELISA and culture results for plant species needle-inoculated with *Xf* (continued).

Type	Common Name	Scientific Name	ELISA +	Culture +	*Xf* Recovered?
Cover Crops	Annual Ryegrass	*Festuca species*	6/20	6/20	Yes
	Annual Fescue, Zorro	*Lolium multiflorum*	0/20	0/20	No
	Black Mustard	*Brassica nigra*	17/20	13/20	Yes
	Blando Brome	*Bromus hordeaceus*	16/20	13/20	Yes
	Birdsfoot Trefoil	*Lotus species*	10/20	0/20	No
	Clover, New Zealand White	*Trifolium repens*	15/20	2/20	Yes
	Clover, Hykon Rose	*Trifolium hirtum*	16/20	10/20	Yes
	Cowpea, California Blackeye	*Vigna unguiculata*	22/40	16/35	Yes
	Fava Bean, Windsor	*Vicia faba*	30/40	7/20****	Yes
	Field Pea, Miranda	*Pisum sativum*	14/39	3/11	Yes
	Meadow Barley	*Hordeum brachyantherum*	9/20	4/20	Yes
	Oat, California Red	*Avena sativa*	12/20	2/20	Yes
	Sudangrass	*Sorghum bicolor var. sudanense*	0/20	0/20	No
	Sweetclover, White	*Melilotus alba*	20/20	16/20	Yes

* 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 Transmission

Last year we lost our clean, captive-reared GWSS and STSS colonies to infestations of the parasitoid wasp, *Gonatocerus ashmeadi.* Several of the needle-inoculated plant species died before we were able to rebuild our colonies and perform transmission with them. Therefore, we re-grew and needle-inoculated new sets of those alternative host plants to use for transmission after the colonies sufficiently recovered. To date, transmission using both vector species has been completed for alfalfa, basil, tomato, annual bluegrass, cheeseweed, wild-type sunflower, goosefoot, London rocket, tree tobacco, annual ryegrass, Blando brome, cowpea, fava bean, California Red oats, and White sweetclover. Although transmission has been completed, we are still evaluating the test plants for basil, annual bluegrass, cheeseweed, wild-type sunflower, London rocket, annual ryegrass, California red oats, and White sweetclover, and final data are still pending. At the time of preparing this report, transmission was underway for Spanish broom, black mustard, New Zealand White clover, Hykon Rose clover, Miranda field pea, and meadow barley, with the final data expected to be available in four-eight weeks.

We have confirmed (by culture) successful transmission of *Xf* by GWSS for alfalfa-to-alfalfa, alfalfa-to-grapevine, basil-to-basil, basil-to-grapevine, tomato-to-tomato, Blando brome-to-Blando brome, Blando brome-to-grapevine and cowpea-to-cowpea (**Table 2**). GWSS transmission of *Xf* from tomato-to-grapevine, cowpea-to-grapevine, fava bean-to-fava bean, and fava bean-to-grapevine tested negative by culturing. 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.

We confirmed successful transmission of *Xf* by STSS for alfalfa-to-alfalfa, alfalfa-to-grapevine, Blando brome-to-Blando brome, Blando brome-to-grapevine and fava bean-to-fava bean (**Table 2**). We were unable to confirm successful transmission by STSS from 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.

Table 2. Results for transmission of *Xylella fastidiosa* by GWSS and STSS to date.

			GWSS		STSS	
Host Plant Type	PD Acquisition Host	PD Inoculation Host	ELISA +	Culture +	ELISA +	Culture +
Agriculture Crop	Alfalfa	Alfalfa	4/5	4/5	5/5	3/5
	Alfalfa	Grapevine	4/5	4/5	4/5	4/5
	Basil	Basil	9/9	9/9	Tests in Progress	
	Basil	Grapevine	8/9	8/9	Tests in Progress	
	Tomato, Rutgers	Tomato, Rutgers	3/5	1/5	1/5	0/5
	Tomato, Rutgers	Grapevine	2/5	0/5	3/5	0/5

Table 2. Results for transmission of *Xylella fastidiosa* by GWSS and STSS to date (continued).

			GWSS		STSS	
Host Plant Type	PD Acquisition Host	PD Inoculation Host	ELISA +	Culture +	ELISA +	Culture +
Weed	Annual Bluegrass	Annual Bluegrass	Tests in Progress		Tests in Progress	
	Annual Bluegrass	Grapevine	Tests in Progress		Tests in Progress	
	Cheeseweed	Cheeseweed	Tests in Progress		Tests in Progress	
	Cheeseweed	Grapevine	Tests in Progress		Tests in Progress	
	Common Sunflower, wild-type	Common Sunflower, wild-type	Tests in Progress		Tests in Progress	
	Common Sunflower, wild-type	Grapevine	Tests in Progress		Tests in Progress	
	Goosefoot	Goosefoot	0/0	0/0	0/0	0/0
	Goosefoot	Grapevine	0/1	0/1	0/0	0/0
	London Rocket	London Rocket	Tests in Progress		Tests in Progress	
	London Rocket	Grapevine	Tests in Progress		Tests in Progress	
	Spanish Broom	Spanish Broom	Tests in Progress		Tests in Progress	
	Spanish Broom	Grapevine	Tests in Progress		Tests in Progress	
	Tree Tobacco	Tree Tobacco	Tests in Progress		Tests in Progress	
	Tree Tobacco	Grapevine	Tests in Progress		Tests in Progress	
Cover Crop	Annual Ryegrass	Annual Ryegrass	Tests in Progress		Tests in Progress	
	Annual Ryegrass	Grapevine	Tests in Progress		Tests in Progress	
	Black Mustard	Black Mustard	Tests in Progress		Tests in Progress	
	Black Mustard	Grapevine	Tests in Progress		Tests in Progress	
	Blando Brome	Blando Brome	1/4	1/4	4/4	3/4
	Blando Brome	Grapevine	2/4	1/4	0/4	1/4
	Clover, New Zealand White	Clover, New Zealand White	Tests in Progress		Tests in Progress	
	Clover, New Zealand White	Grapevine	Tests in Progress		Tests in Progress	
	Clover, Hykon Rose	Clover, Hykon Rose	Tests in Progress		Tests in Progress	
	Clover, Hykon Rose	Grapevine	Tests in Progress		Tests in Progress	
	Cowpea, California Blackeye	Cowpea, California Blackeye	4/5	2/5	5/5	0/5
	Cowpea, California Blackeye	Grapevine	3/5	0/5	2/5	0/5
	Fava Bean, Windsor	Fava Bean, Windsor	2/5	0/5	1/5	1/5
	Fava Bean, Windsor	Grapevine	1/5	0/5	4/5	0/5
	Field Pea, Miranda	Field Pea, Miranda	Tests in Progress		Tests in Progress	
	Field Pea, Miranda	Grapevine	Tests in Progress		Tests in Progress	
	Meadow Barley	Meadow Barley	Tests in Progress		Tests in Progress	
	Meadow Barley	Grapevine	Tests in Progress		Tests in Progress	
	Oat, California Red	Oat, California Red	Tests in Progress		Tests in Progress	
	Oat, California Red	Grapevine	Tests in Progress		Tests in Progress	
	Sweetclover, White	Sweetclover, White	Tests in Progress		Tests in Progress	
	Sweetclover, White	Grapevine	Tests in Progress		Tests in Progress	

CONCLUSIONS

Bell pepper, cotton, black nightshade, common groundsel, Evening Sun sunflower, horseweed, Zorro annual fescue, birdsfoot trefoil, 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 particularly good news for horseweed since it is an extremely common weed in vineyards and is reported to be resistant to herbicides. In their PD management program, growers can choose to target weeds other than those identified here, knowing that these species do not sustain infection with *Xf*. In addition, growers can safely select

Zorro annual fescue, birdsfoot trefoil, and sudangrass as cover crops with confidence that their choice will not contribute to PD spread in their vineyards.

We recovered *Xf* from at least 50% of test plants for alfalfa, basil, cheeseweed, London rocket, Spanish broom, black mustard, Blando brome, Hykon Rose clover, and White sweetclover, indicating that these can serve as hosts for *Xf* in the field. We obtained isolates from three-46% of needle-inoculated plants for lima bean, tomato, wild-type sunflower, goosefoot, tree tobacco, annual ryegrass, New Zealand White clover, cowpea, fava bean, Miranda field pea, and meadow barley. The results from the transmission studies using these plants (pending) should provide 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 be sustained. 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.

Alfalfa and Blando brome are good hosts for *Xf*, GWSS, and STSS, indicating that they can serve as a reservoir of *Xf* and source of infection in the field for these vectors. Both GWSS and STSS successfully transmitted *Xf* between alfalfa plants, between Blando brome plants, from alfalfa into grapevines, and from Blando brome into grapevines. These two plant species (one a crop plant and the other a cover crop) should not be around or in vineyards where *Xf* or sharpshooters are present.

GWSS successfully transmitted *Xf* between basil plants, from basil to grapevines, between cowpea plants, between tomato plants, and from fava bean to grapevines, but not between fava bean plants, or from cowpea to grapevines. STSS also successfully transmitted between fava bean plants, but not from fava bean into grapevines, between cowpeas, or from cowpea into grapevines. It is possible that *Xf* isolates were obscured by other microbes present in the plants and on the media plates (cowpea and fava bean contain numerous other microbes that grow on media plates for PD), and positive transmission occurred, but was not detected. It also is possible that these plant species would not naturally serve as acquisition sources by these vectors, but because they were unnaturally needle-inoculated, some transmission did occur. Pending further results, careful consideration should be applied when using cowpea or fava bean as cover crops in vineyard areas with known *Xf* infection, or sharpshooter populations, since they can serve as sources. In the unlikely event that basil is grown near vineyards, it could may be a major contributor to the spread of PD, since both GWSS and STSS favor this host, and it could sustain high populations of vectors and harbor *Xf*. The final transmission results (pending) will provide a better understanding of which plant hosts, in combination with GWSS or STSS vectors, are more important in the epidemiology of this plant pathogen.

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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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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 1,841 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. In November, seedlings from 12 crosses made in 2008 were tested with molecular markers and 159 resistant plants identified from 319 individuals. Greenhouse screening was completed on 150 selections and 63 of the 64 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 maker 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 125 individuals was completed with 25 being resistant.

LAYPERSON SUMMARY
Although Pierce's disease (PD) has existed in California since the late 1800s, the introduction of the glassy-winged sharpshooter to California in the late 1990's significantly increased the spread and damage caused by PD. A collaborative breeding program was started in 2000 to develop PD resistant table and raisin grapes with high fruit quality comparable to that existing in markets today. The first crosses to make the BC4 generation of table and raisin grapes with *V. arizonica* source of PD resistance were made this year. These families will have high fruit quality as they consist of 97% *V. vinifera*. An example of increased fruit quality would be this year's selection of five raisin grapes made from BC3 *V. arizonica* families which will be propagated for production trials. An additional fifteen BC2 *V. arizonica* raisin selections were made and will be propagated for production trials. The use of molecular markers has allowed the selection of PD resistant seedlings while they are still in test tubes. Three hundred twenty-seven resistant seedlings were selected from 885 seedlings this year, thereby making the program more efficient. Advanced selections are screened in the greenhouse to verify PD resistance. Powdery mildew (PM) resistance is being combined with PD resistance and this year 54 of 97 PD resistant seedlings showed PM resistance after greenhouse screening. A family from BD5-117 source of resistance that is different from *V. arizonica* has been made to develop molecular markers for this source of resistance. To date, 154 seedlings have been tested with 70 fluorescent labeled SSR markers. The PD resistance of 125 of these seedlings has been tested in the greenhouse. This collaborative research between USDA/ARS, Parlier and University of California, Davis has the unique opportunity to develop high quality PD resistant table and raisin grape cultivars for the California grape industry where PD might restrict the use of conventional table and raisin grape cultivars.

INTRODUCTION
Pierce's disease (PD) has existed in California since the late 1800s 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 AND DISCUSSION

Objective 1

Fifty-one crosses using *V. arizonica* and SEUS (BD5-117 and Zehnder) sources of resistance were made in 2009 and produced 5,918 berries, 6,661 ovules, and 1,719 embryos (25% embryos/ovules) (**Table 1**). The first BC4 crosses (97% *Vitis vinifera*) was made this year and consisted of 31 crosses of which 19 produced 3,931 berries, 5,000 ovules and 1,372 embryos. These crosses were made before observing fruit on the 18 month old seedlings and the size of the seed/aborted seed was unknown at bloom. The seed traces were too small to culture from six of these crosses. The seedlings obtained from these crosses should have high fruit quality as they now have 97% *V. vinifera* in their background. In additional to the BC4 crosses, 17 BC3 crosses were made and consisted of 1,313 berries, 987 ovules and 312 embryos. Five and three crosses combined *V. arizonica* and SEUS PD resistance respectively with powdery mildew resistance. No seeded crosses were made in 2009.

Leaves were taken from seedlings in test tubes in November from 2008 crosses and tested for resistance with molecular markers for the PdR1 locus on chromosome 14. Results for three BC1 and ten BC3 seedless x seedless families (89-0908 *V. arizonica* source of resistance) is shown in **Table 2**. A total of 885 individuals were tested with SSR markers and 812 showed markers on both sides of the PdR1 region as expected. A total of 327 individuals (43% of those showing markers) were resistant and planted to soil in cups for growth in the greenhouse before planting to the field in April, 2009. Thirty-nine percent of the plants showing markers were susceptible. This is very similar to the percent resistant and susceptible plants obtained for over 1,600 F1, BC1, BC2 table and raisin seedlings reported by Riaz et al. 2009. The susceptible and recombinant individuals were discarded making more efficient use of greenhouse and field space. A total of 172 seeded by seedless BC3 seedlings were also screened with molecular markers and 34% and 44% were resistant and susceptible respectively. Only the resistant plants were planted in the field. Four BC3 crosses made in 2008 combined PD resistance from *V. arizonica* with powdery mildew (PM) resistance from *V. romanetii*. Usually resistance from this source of PM resistance segregates in a 1:1 resistant:susceptible ratio. The seedlings that had PD resistant markers were screened in the greenhouse for PM resistance. Of the 97 PD resistant seedlings screened to date, 54 were resistant, which is as expected. Inoculation of plants with *Xylella* in the Greenhouse (method by Krivanek et al. 2005, Krivanek and Walker 2005) was done to determine resistance of 105 selected individuals from BC2 *V. arizonica* and F1, BC1 SEUS (Table 3). Sixty-three resistant individuals were from *V. arizonica* and only one was from SEUS source of resistance. This shows that a high level of resistance is being passed on by *V. arizonica*. Greenhouse testing is absolutely necessary to make the final decision about resistance of individual selections. The highest level of resistance is being obtained from *V. arizonica* and BD5-117 and their use as parents will be emphasized.

Fifty percent of the 400 resistant BC2 and BC3 *V. arizonica* seedlings planted in 2008 produced fruit. From these seedlings, 3 BC2 and 3 BC3 tray dried raisin, and 2 natural DOV raisin selections were made that are good enough for propagation into advanced production trials. An additional 15 tray dried raisin, 4 natural DOV raisin, 10 table grape and 2 wine selections were kept for use as parents and for additional evaluations. Raisin samples were dried from 41 PD resistant seedlings from 2 BC2 raisin families. Nine seedlings were selected for propagation in production trials and as parents. One selection had a rating of 52 which was higher than Selma Pete (50), Fiesta (48) and DOVine (47) and equal to Diamond Muscat (52). Nine table grape selections from BC2 *V. arizonica* have been selected for further observation and as parents. Four have been tested in the greenhouse for *Xylella* infection and were resistant. Five table grapes and nine raisin grapes were also selected from 227 BC2 *V. arizonica* seedlings that were planted in 2007 and fruited for the first time in 2009. Three of the raisin selections will be propagated for yield trials. Twelve advanced selections, with a range of PD resistant in greenhouse tests, have been planted in a replicated plot at the USDA ARS research station, Weslaco, Texas. Samples were taken in September, 2009 to determine if any plants have become infected after one year's growth

Objective 2

The PD resistant grape selection BD5-117 from Florida was hybridized with the seedless table grape selection C33-30 and 300 individuals are fruiting. Fruit samples were taken from all seedlings for cluster weight, berry weight and seed/seed trace weight as an indication of fruit quality. Greenhouse testing for PD resistance is complete on 125 individuals, with 25 being resistant (**Table 3**). Three hundred additional plants were planted this year to increase the family size to over 500 individuals. Of the 105 SSR polymorphic primers identified last year, 70 have been labeled with fluorescent dyes and run on all 154 individuals plus the parents. The additional 35 labeled polymorphic primers are being tested on the 154 seedlings.

Table 1. Successful 2009 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 *V. rupestris* x *V. arizonica*						
B71-60	07-5054-12	Table BC4	2,823	229	408	234
Scarlet Royal	07-5054-12	Table BC4	3,115	11	20	6
07-5061-04	A63-85	Raisin BC4	2 bags[a]	252	285	68
07-5061-04	A50-33	Raisin BC4	3 bags[a]	391	411	148
07-5061-14	Y143-39	Raisin BC4	3 bags[a]	256	460	69
07-5061-14	B82-43	Raisin BC4	4 bags[a]	643	1,168	97
07-5061-14	Y144-157	Raisin BC4	2 bags[a]	298	441	239
A49-82	07-5061-34	Raisin BC4	3,000	74	50	24
A50-39	07-5061-34	Raisin BC4	3,315	402	394	100
A50-91	07-5051-28	Raisin BC4	1,500	108	117	21
A50-91	07-5061-146	Raisin BC4	1,830	160	178	44
A61-79	07-5058-16	Raisin BC4	2,800	142	144	37
B82-43	07-5052-43	Raisin BC4	2,606	223	250	64
Y142-76	07-5061-34	Raisin BC4	496	42	45	14
Y142-76	07-5061-72	Raisin BC4	1,492	88	84	13
Y142-76	07-5061-106	Raisin BC4	1,156	38	31	7
Y143-161	07-5053-33	Raisin BC4	3,946	207	229	28
Y144-132	07-5061-34	Raisin BC4	2,390	32	50	10
05-5501-27	Y129-176	Table BC3	5 bags[a]	36	3	1
05-5501-27	05-5501-28	Table BC3	4 bags[a]	170	16	2
05-5501-28	Y133-191	Table BC3	1,564	21	23	4
05-5502-25	Y129-176	Table BC3	2,631	163	162	30
05-5501-68	C57-60	Table BC3	5 bags[a]	240	287	99
05-5501-68	C45-64	Table BC3	5 bags[a]	48	13	2
05-5501-40	Y129-161	Table BC3	5 bags[a]	51	52	7
05-5502-15	04-5514-2443	Table BC3	2,708	382	290	39
05-5551-19	A63-58	Raisin BC3	650	77	94	18
04-5514-28	C61-123	Table BC2	1,375	75	93	23
07-5061-14	Y308-344	PM Raisin BC4	3 bags[a]	210	235	149
Total			**39,397**	**44,256**	**6,033**	**1,597(26%)**
SEUS source of resistance (BD5-117 or Zehnder)						
03-5003-10	C45-64	Table BC1	5 bags[a]	2	2	1
03-5003-10	Y308-314	PM Table BC1	1,554	69	88	20
Y305-58	C61-123	PM Table BC1	3,097	308	436	101
Total			**5,285**	**3,291**	**4,836**	**1,179(23%)**

[a]Parents with female flowers were not emasculated, only bagged and pollinated.

Table 2. Determination of seedling resistance based on molecular markers for 89-0908 BC2 families made in 2008.

Family	Type Cross	No. Resistant[a]	No. Susceptible[b]	No. Recombinant[c]	No data[d]	Off Types	Total
08-5001	Table BC3	46	17	13	1	19	96
08-5002	Table BC1	40	35	29	9	14	127
08-5003	Table BC3	6	6	2	1	0	15
08-5054	Raisin BC1	38	37	13	5	0	93
08-5055	Raisin BC1	29	33	18	8	1	89
08-5056	Raisin BC3	27	45	14	16	0	102
08-5057	Raisin BC3	29	28	6	2	2	67
08-5058	Raisin BC3	6	2	0	3	4	15
08-6002	Table BC3 PM	35	28	18	9	3	93
08-6003	Table BC3 PM	2	2	0	2	0	6
08-6052	Raisin BC3 PM	5	3	1	6	2	17
08-6053	Raisin BC3 PM	62	59	18	11	10	160
08-6054	Raisin BC3 PM	2	2	1	0	0	5
Total		**327 (43%[e])**	**297 (39% [e])**	**133 (18% [e])**	**73**	**55**	**885**
	Seeded x Sdlss						
08-5504	Table BC3	7	8	3	4	0	22
08-5505	Table BC3	1	0	1	0	0	2
08-5552	Raisin BC3	47	66	16	6	0	135
08-6501	Table BC3 PM	3	2	5	2	0	12
08-6502	Table BC3 PM	1	0	0	0	0	1
Total		**59 (37% [e])**	**76 (48% [e])**	**25 (16% [e])**	**12**	**0**	**172**

[a]Resistant = marker on both sides of *PdR1* region.
[b]Susceptible = no *PdR1* markers.
[c]Recombinant= genotypes that amplified with one *PdR1* marker.
[d]No data = genotypes that failed to amplify properly.
[e]%= Number of seedlings in each category / total number of seedlings showing markers properly.

Table 3. Results of greenhouse test for determination of PD reaction.

Population	Resistance Source	Total sent	Testing Compete	
			No. tested	No. resistant
BD5-117 map	BD5-117	154	125	25
Arizonica	PdR1	113	105	63
Other PD	SEUS	65	45	1
Total		**332**	**275**	**109**

CONCLUSIONS

Families for the development of PD resistant seedless table and raisin grape cultivars continue to be produced. Emphasis was placed on *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 frame work 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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BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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ABSTRACT
The use of marker-assisted selection (MAS) using DNA markers tightly linked with Pierce's disease (PD) resistance (see our companion report) and the acceleration of the seed-to-seed breeding cycle to three years have allowed very rapid progress towards the creation of PD resistant winegrapes. Seedlings from the 2008 crosses were screened for PD resistance with MAS and only those seedlings with the markers were planted in the field. The goals of the 2009 crosses were to: 1) create 97% *V. vinifera* seedlings with PD resistance using *PdR1* from *V. arizonica* hybrid– F8909-08; 2) create 75% *V. vinifera* seedlings with PD resistance from *V. arizonica* b40-14; and 3) enlarge the *V. arizonica/girdiana* b42-26 PD resistance mapping population by remaking the *V. vinifera* F2-35 x b42-26 cross. Numerous greenhouse-based PD resistance screens were performed on breeding lines, mapping populations and new PD resistant rootstocks. Selections with *PdR1* at the 87.5% and 75% *vinifera* level at our Beringer, Napa County trial were inoculated and a similar trail at the 93.75% *vinifera* level was planted. Finally, small-scale wine lots were made from three 93.75% *vinifera* and five 87.5% *vinifera PdR1* selections. Fruit evaluation and juice analysis were performed on numerous other promising progeny at the 93.75% *vinifera PdR1* level.

LAYPERSON SUMMARY
Rapid progress breeding Pierce's disease (PD) resistant winegrapes continues to be made by combining the use of MAS with *PdR1* and aggressive vine training to produce clusters in a seedling's second season to produce the next generation crosses of PD resistant populations. Wines were made this Fall from PD resistant selections that contain 94% *vinifera*. These selections resulted from the original cross of *vinifera* x F8909-08 (the *V. arizonica/candicans* resistance source from Monterrey, Mexico), followed by crossing back to a *vinifera* parent over three more generations (modified Back Cross 3 – mBC3). This Fall, wine was made from three of these 94% *vinifera*, PD resistant selections. Two years of wines have also been made from the previous generation 88% *vinifera* (mBC2) PD resistant selections with very favorable results. This year also saw the creation of the next generation back cross to *vinifera* (mBC4), which will result in 97% *vinifera* seedlings with *PdR1* resistance for planting in Spring 2010. PD resistant selections are also being tested at the Beringer ranch in Napa Valley, and wines will be made from these vines. Major advances have also been made in the production and greenhouse testing of seedling populations that will allow the characterization of PD resistance genes from multiple backgrounds (see companion project on the genetics of resistance to PD).

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 having 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 made wine from vines that are 93.75% *V. vinifera*, and possess resistance from the b43-17 *V. arizonica*/*candicans* resistance source. There are two sources of *PdR1*, 8909-08 and 8909-17 – sibling progeny 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 yet 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 MAS with the b43-17 resistance sources and their progeny. The breeding goal at this point is to introgress PD and *PdR1* resistance sources into a large number of *V. vinifera* winegrape backgrounds. Now that the backcross four (BC4) (96.9% *V. vinifera*) level has been produced (seedlings will be planted in 2010) larger numbers of progeny within populations will be produced to increase chances of selecting the best winegrape quality in a PD resistant background. **Table 1** shows the crosses made in 2009. The goals of the 2009 crosses were to: 1) use the *PdR1* allele from F8909-08 to advance the *vinifera*

winegrape populations to the 96.9% *vinifera* level; 2) create populations of 93.75% *vinifera* with the *PdR1* F8909-08 resistance allele; 3) select *V. vinifera* x *V. arizonica* b40-14 progeny and produce 75% *vinifera* populations with an alternative PD resistance source; and 4) increase the *V. arizonica/girdiana* b42-26 mapping population by remaking the *V. vinifera* F2-35 x b42-26 cross.

During this period, eight groups of plants were tested in the greenhouse for *Xf* resistance (**Table 2**). Group A tests confirmed the resistance of the parents used in the 2008 crosses, previously selected on the basis of their DNA markers; initiated the exploration of the *PdR1* alleles among resistant genotypes and *vinifera* parents; and tested the progeny from one initially promising 87.5% *vinifera* VR (*vinifera* x *rotundifolia*) hybrid from Olmo's breeding program. Unfortunately, all of these VR progeny were ELISA tested as PD susceptible at over 1,000,000 cfu/ml (data not shown), confirming the complex nature of PD resistance derived from *rotundifolia*. Groups B, D and E evaluated the greenhouse-based PD resistance of the 50% *vinifera*, 25% b40-14 *V. arizonica* resistance source 07744 and 07386 populations. **Table 3** shows that for the 07744 population, 24% were classed as resistant, approximately 26% were in either of two intermediate classes and 50% were in the susceptible class. The clearly PD resistant genotypes identified in groups B & E allowed us to make 75% *vinifera* crosses in 2009 and advance this promising new PD resistance line by another generation (**Table 1c**). Group D was tested to evaluate the impact of between pot spacing on mean ELISA cfu/ml values in an 87.5% *vinifera PdR1* background. Consistent with Baumgartel (2009), tighter spacing increased the mean ELISA values relative to the standard spacing in both susceptible and resistant selections. Group F consisted of additional 9621 population recombinants that were tested to aid the fine scale mapping of *PdR1*. Concurrently, additional 2007 crosses were tested to continue the exploration of *PdR1* resistance initiated in Group A. Groups G & H focus on the F8909-17 allele of *PdR1* to elaborate differences in resistance behavior of this allele compared to the F8909-08 allele. The new PD resistant rootstocks were tested in Group G. Chardonnay was used as a susceptible scion to determine if high *Xf* levels in the scion increase *Xf* levels in the rootstock downward across the graft union. A81-17, a rigorously tested 75% *vinifera PdR1* genotype, was used as the resistant scion to determine whether grafting on *PdR1* rootstocks impacts the titer of *X. fastidiosa* found in the scion.

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/girdiana 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 this population focused on table grape breeding, and found that the 0023 population (F1, 1/4 b42-26) had about 30% resistant progeny. The 0023 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. In 2007, we tested the 05347 (*vinifera* F2-35 x b42-26) population to examine the b42-26 resistance source in a background without the confounding effect of *V. rupestris*. That same year, crosses using elite *V. vinifera* wine type pollen were made to a number of females in this population and 140 genotypes were planted in 2008, which flowered for the first time in Spring 2009. We planted an additional 100 05347 genotypes to the field in May 2009 and again repeated this cross, producing approximately 200 seeds, to further expand this F1 mapping population.

V. shuttleworthii Haines City – Based on encouraging greenhouse screen results for this resistance source, in 2008 the BC1 (75% *vinifera*), and BC2 (87.5% *vinifera*) using a BC1 from earlier table grape work were made. This BC1 is very resistant and has reasonable winegrape characteristics. If reliable genetic markers for *Xf* resistance from this resistance source can be developed, it will be relatively easy to incorporate this form of resistance with sources carrying *PdR1* at later stages of the program, with the goal of broadening resistance.

V. arizonica b40-14 – Over the last seven years, 45 F1 progeny of PD susceptible *V. rupestris* Wichita Refuge crossed with PD resistant *V. arizonica* b40-14 (the R89 series) have been tested. Forty-two were highly resistant and three had intermediate reactions (data not shown). In 2006, the 06339 population (*V. vinifera* F2-35 x b40-14) was made and contains 198 seedlings for testing. In 2007, *V. vinifera* cv. Airen was crossed onto two of the PD resistant R89 series genotypes and a total of 163 progeny were planted in Spring 2008. One of these is the 07744 population (F1 50% *vinifera*, 25% b40-14 – see **Tables 1c, 2 and 3**). Preliminary mapping of this population places PD resistance from b40-14 on LG14 but in a different location than *PdR1*. To date greenhouse testing has been completed for seven 06339 genotypes (F1 50% *vinifera*, 25% b40-14); they all lack PD symptoms and have low ELISA values. The progeny of the 06339 x *V. vinifera* crosses made in 2008 will be used for further mapping efforts to better characterize this very strong, and morphologically and genetically different source of PD resistance.

Given that low levels of *Xf* exist in resistant plants it will be important to have PD resistant rootstocks to graft with resistant scions, thus preventing failure if *Xf* moved into the rootstock. The rooting and grafting ability (with two scion varieties) of eight selections with PD resistance from *PdR1* have been tested, and they will soon be greenhouse tested for resistance and

examination of *Xf* movement across the graft union. The best selections will be tested for nematode and phylloxera resistance followed by field testing.

Field and Wine Evaluations – Field testing of advanced PD resistant selections continues at the Beringer vineyard in Yountville, CA. Natural sharpshooter vectoring is not depended on, rather each plant is needle inoculated with *Xf* each Spring. Selections from the BC3, 94% *vinifera* crosses from the 07355 (U0505-01 x Petite Sirah) and 07370 (*vinifera* F2-35 x U0502-38) populations were grafted onto Dog Ridge (currently the only certified virus-free PD resistant rootstock) in February 2009 and planted at Beringer in June 2009. These genotypes have been marker tested and their PD resistance status will be confirmed by greenhouse testing in the coming months. This Spring, selections from the 045554 (BC2, 87.5% *vinifera*) population were needle inoculated for the second time and selections from the A81 population (BC1, 75% *vinifera*) both with the *PdR1b* (F8909-08) allele were inoculated for the third time. This year the seven most promising 87.5% *vinifera PdR1* wine types (06325-42, 06325-43, U0502-01, U0502-10, U0502-35, U0502-38, U0502-41 – 2 white and 5 red; 6 reps each) grafted onto Dog Ridge and planted at the Beringer site for small-scale winemaking trials.

In 2006, at least six vines of eight 87.5% *vinifera PdR1* selections (50% Syrah or Chardonnay from the last cross) were planted for small-scale wine making tests. Wine lots of these selections made in 2007 and 2008 have shown significant promise. Sensory evaluation as well as fruit evaluation and must analysis from numerous other genotypes from crosses involving elite wine cultivars were reported in our last two progress reports. These wines were also evaluated at the UCD Viticulture and Enology alumni gathering on May 15, 2009 and at the North American Grape Breeders Conference in Tallahassee, Florida on August 7, 2009 with similar results. In 2008, at least six vines of four other particularly promising 87.5% *vinifera PdR1* selections, siblings of the 2006 plantings, were planted. Concurrently we planted at least six vines of eight 93.75% *vinifera PdR1* selections (50% Petite Syrah, Chardonnay or F2-35 from the last cross). This fall, 12 fermentations were made: three (two red, one white) at the 94% *vinifera* level; five (four red, one white) at the 87.5% *vinifera* level; and four (two red, two white) *vinifera* and PD controls. Vine, fruit and juice analyses are presented in **Tables 4a-c** and images of the vines, leaves and fruit are in **Figures 1 and 2**. Numerous other genotypes from crosses involving elite *vinifera* wine cultivars were examined for fruit evaluation and must analysis. ETS Laboratories (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.

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 to allow 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, then again in 2008 and they scored remarkably well. Small-scale wine lots from advanced selections with 93.75% *vinifera* were made in Fall 2009 and appear even more promising.

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Table 1. 2009 Wine and mapping crosses with estimated number of seeds produced.

Resistant Type	*Vinifera* Parent of Resistant Type	*Vinifera* Parents used in 2009 Crosses	Est. No. Seeds
1a. Monterrey *V. arizonica/candicans* resistance source (F8909-08) to produce progeny with 96.875% *V. vinifera* parentage.			
07354-50	Merlot	Cab. Sauv., Chard.	125
07355-020	Petite Syrah	Cab. Sauv., Chard., Chenin blanc, Zinfandel	1750
07370-039	F2-35 (Cab. Sauv. x Carignane)	Cab. Sauv., Chenin blanc, Riesling, Sylvaner, Zinfandel	1450
07370-097	F2-35	Cab. Sauv., Chard., Chenin blanc, Pinot noir	650
07370-28	F2-35	Cab. Sauv., Chenin blanc, Pinot noir, Zinfandel	950
07371-19	F2-35	Cab. Sauv., Chard., Chenin blanc, Sylvaner	375
07371-20	F2-35	Cab. Sauv., Chenin blanc, Pinot noir, Sylvaner	925
07371-36	F2-35	Cab. Sauv., Chard., Chenin blanc, Mourvedre, Riesling, Zinfandel	800
1b. *Vitis arizonica/candicans* resistance source (F8909-08) to produce progeny with 93.75% *V. vinifera* parentage.			
07307-10	Zinfandel	Cab. Sauv., Chenin blanc	115
1c. Crosses to the b40-14 *V. arizonica* resistance source to produce progeny that are 75% vinifera and 12.5% the resistance source.			
07744-038,-120	Airen	Cab. Sauv., F2-35, Malaga Rosada	790
1d. Cross to increase the b42-26 *V. arizonica* x *vinifera* mapping population.			
b42-26	F2-35 (Cab. Sauv. x Carig.)	F2-35	200

Table 2. PD resistant winegrape progeny completed or currently in greenhouse screening for PD resistance.

Group	Genotypes	# Genotypes	Inoculation Date	ELISA Date	Resistance Source(s)
A	2008 *PdR1* Parents, mini-mapping, e6-23 VR series	47	11/25/2008	2/26/2009	b43-17 (both alleles), VR
B	07744 mapping population	37	12/16/2008	4/2/2009	b40-14
C	Greenhouse spacing trial	NR	1/15/2009	5/21/2009	F8909-08
D	07386 Mapping population	45	2/3/2009	5/21/2009	b40-14
E	07744 mapping populations	70	2/12/2009	5/21/2009	b40-14
F	9621 recombinants, 2007 crosses of interest	122	4/21/2009	8/13/2009	F8909-08
G	04191 mapping population, PD Rootstocks	82	6/23/2009	9/22/2009	F8909-08
H	04191 mapping pop (MPP)	130	10/8/2009	1/7/2010	F8909-08

Table 3. Greenhouse screen results for the 07744 cross (R8918-05 x Airen).

Resistance Class	Resistance Class Parameters	No. in Class	% Total
R	mean cfu/ml ~<100k	26	24%
R?	mean cfu/ml >~100k and max cfu/ml <1M cfu/ml	9	8%
S?	mean cfu/ml >~300k and <~3M cfu/ml	18	17%
S	mean cfu/ml >~1M and max cfu/ml >3.5M	54	50%
Total		107	100%

Table 4a. Phenotypic observations of reference varieties and select progeny with the *PdR1* resistance source used for small lot winemaking in 2009.

Genotype	Parentage	Percent *vinifera*	2009 Bloom Date	Berry Color	Berry Size (g)	Ave Cluster Wt. (g)	Ripening Season	Prod 1=v low, 9=v high
Barbara	Historic	100%	05/09/09	B	2.4	290	late	6
Chardonnay	Gouais blanc x Pinot noir	Historic	05/14/09	W	1.0	190	early	5
07355-12	U0505-01 x Petite Sirah	93.75%	05/10/09	B	1.0	137	early-mid	6
07355-75	U0505-01 x Petite Sirah	93.75%	05/07/09	B	1.3	234	early	8
07713-51	F2-35 x U0502-48	93.75%	05/07/09	W	1.4	210	early	8
U0501-12	A81-138 x Syrah	87.50%	05/18/09	B	1.1	194	late	4
U0502-10	A81-138 x Chardonnay	87.50%	05/07/09	B	1.4	198	early	7
U0502-20	A81-138 x Chardonnay	87.50%	05/14/09	W	1.7	313	late	8
U0502-26	A81-138 x Chardonnay	87.50%	05/10/09	B	1.6	375	mid	7
U0505-35	A81-138 x Cab. Sauvignon	87.50%	05/10/09	B	1.1	158	early	6
Blanc du Bois	Fla D6-148 x Cardinal	~66%	05/14/09	W	1.2	125	mid-late	7
Lenoir	*V. aestivalis* hybrid	<50%	05/20/09	B	0.8	201	late	6

Table 4b. Analytical evaluation of advanced selections with the *PdR1* resistance source used for small lot winemaking in 2009. 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)
07355-12	2.79	26.8	2050	3.42	0.78	275	127	585	2178
07355-75	2.88	28.2	2180	3.49	0.74	217	5	680	1941
07713-51	1.31	23.4	1700	3.56	0.49	146	-	-	-
U0501-12	2.11	21.8	1610	3.46	0.58	263	49	555	1026
U0502-10	3.97	24.9	2170	3.60	0.73	362	48	1006	1162
U0502-20	4.18	23.3	2230	3.51	0.76	383	-	-	-
U0502-26	2.24	24.0	1900	3.40	0.73	237	67	411	947
U0505-35	4.03	28.7	2450	3.66	0.81	476	47	886	1446

Table 4c. Sensory evaluation of reference varieties and advanced selections with the *PdR1* resistance source used for small scale winemaking in 2009.

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
Chardonnay	green-gold	medium	apple, pear	sl fruity	1	4	nutty	4
07355-12	red	med-dark	red fruit	plum, berry	3	3.5	woody, spicy	1
07355-75	red	medium	plum, fig	jam,prune	2	3	hot, woody	2
07713-51	green-gold	medium	apple, pear	neutral	2	3.5	woody, spicy	3
U0501-12	red	med-dark	fruity	fruit jam	2	4	neutral	2
U0502-10	pk-red-orng	med-dark	slight vegetal	sl fruity	1	4	nutty, spicy	1
U0502-20	green	medium	neutral, fruity	grass	1	4	spicy, bitter	1
U0502-26	pink	medium	bright, spicy	fruity	2	4	nutty	3
U0505-35	red	medium	CS-veg, berry	sl CS-veg	2	4	spicy	2
Blanc du Bois	gold	med-dark	floral, vegetal	sl vegetal	1	4	spicy, bitter	4
Lenoir	red	dark	mildly fruity	fruity	1	4	nutty	4

Figure 1. Images of the two red 94% *vinifera* PD resistant winegrape selections (U0505-01 x Petite Sirah) used for small-scale winemaking at UCD in Fall 2009. 07355-12 is above and 07355-75 is below.

Figure 2. Images of the white 94% *vinifera* PD resistant wine grape selection (F2-35 (Cabernet Sauvignon x Carignane) x U0502-48) used for small-scale winemaking at UCD in Fall 2009.

MAP-BASED IDENTIFICATION AND POSITIONAL CLONING OF *XYLELLA FASTIDIOSA* RESISTANCE GENES FROM KNOWN SOURCES OF PIERCE'S DISEASE RESISTANCE IN GRAPES

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Reporting Period: The results reported here are from work conducted August 2009 to October 2009

ABSTRACT
This report presents updated results on the refined mapping of the Pierce's disease (PD) resistance locus, *PdR1*, in the 04190 (397 plants) and 9621 population (900 plants). In both populations, the resistance locus is within a 1cM distance. The flanking markers VVCh14-78 and VVCh14-81 were added to key recombinant plants from both populations and greenhouse screening was repeated to avoid any error. Genetic mapping was initiated in the 07744 (resistance from *V. arizonica* b40-14), 04191 (resistance from F8909-17 *PdR1a*) and 05347 (resistance from *V. arizonica/girdiana* b42-26) populations. A total of 152 markers were completed for 07744 to develop the framework map and greenhouse screening of the 07744 population is complete and underway for the 04191 population. In 07744, preliminary analysis indicated that PD resistance (*PdR1c*) resides on chromosome 14, in the same region where *PdR1a* (resistance from F8909-17) and *PdR1b* (resistance from F8909-08) mapped from the b43-17 background. However, the SSR alleles for resistance are very different between b43-17 and b40-14. Between October 2007 and March 2009 two BAC libraries, each with one restriction enzyme (*Hind* III and *Mbo* I), were completed and the screening of the *Hind* III BAC library with flanking markers was initiated. The Pinot noir genome sequence was used to develop markers to screen the BAC library, and these SSR markers were used to reduce the physical distance to *PdR1*. Two screenings of the libraries identified 24 (with markers VVCh14-56 and VVCh14-10) and 17 positive BAC clones (with marker VVCh14-58). Complete sequencing of two clones (H23P13 and H64M16), representing the two haplotypes of b43-17 was completed. Five clones were positive with VVCh14-56 and VVCh14-58. Clone 'H69J14' (which is bigger than 200Kb) was selected for 454-based sequencing. This clone spans scaffold 21 and nine of the Pinot noir genome sequence. A total of 42,000 sequences were assembled with the help of two different assembly programs. The DNASTAR program was used to obtain assembly at 99% stringency, and yielded more than 79 contigs larger than 5Kb in size. Primers will be designed from the ends to improve the assembly with BAC walking by filling the gaps and verifying the sequences on the ends of contigs. Assembled sequence will be used for the identification of resistance gene(s).

LAYPERSON SUMMARY
Genetic mapping efforts have identified a Pierce's disease (PD) resistance region on chromosome 14 termed *PdR1*, which originated from *Vitis arizonica/candicans* b43-17. This resistance acts as a single dominant gene and we have mapped the two forms from the homozygous parent – *PdR1a* from F8909-17 and *PdR1b* from F8909-08. We have also mapped another form of *PdR1* from *V. arizonica* b40-14, and are examining how the multi-gene PD resistance from *V. arizonica/girdiana* b42-26 maps and relates to *PdR1*. In the future these multiple resistance forms will be combined in our PD breeding program to ensure the strongest resistance possible. The combination of these forms of PD resistance can only be done with the tightly linked genetic markers discovered in these mapping efforts so that the combination of the various forms of resistance can be confirmed in the interbred progeny. These mapping efforts are also essential to physically locating and characterizing PD resistance genes. At present, the chromosome region that *PdR1* exists on has been sequenced and these pieces of sequence are being arranged and compared to the Pinot noir genome sequence and that of other plants to characterize their function and determine which are likely to be involved in PD resistance.

INTRODUCTION
This project continues to genetically map Pierce's disease (PD) resistance in forms of *V. arizonica* (Riaz et al. 2007). These efforts are closely linked to a breeding program focused on developing PD resistant winegrapes (see companion report). The breeding program produces and greenhouse screens the seedling populations upon which this genetic mapping program depends. While the tightly linked genetic markers generated in these mapping efforts are used to optimize and greatly accelerate the PD breeding program (Riaz et al. 2009). These markers are also essential to the successful introgression of resistance from multiple sources, and thus for the production of broader and more durably resistant grapevines (Riaz et al. 2008a). Genetic maps associate DNA markers with phenotypic traits, and allow the linking of these traits with markers positioned relative to each other on linkage groups, which since the sequencing of the Pinot noir genome, are now known to be chromosomes. Fine scale mapping of given regions and careful screening of recombinant progeny (those with a given genetic marker but without resistance, or vice versa, because of a recombination event) is critical to the identification of relatively short genetic regions that can then be sequenced so the genes responsible for PD resistance can be characterized and their function studied (Riaz et al. 2008b).

OBJECTIVES

1. Completely characterize and refine the PD resistance locus on chromosome 14 by genetically mapping in four populations that derive resistance from *V. arizonica/candicans* b43-17 and its *V. rupestris* x b43-17 progeny F89090-08 (*PdR1b*) and F8909-17 (*PdR1a*): 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/candicans* b43-17).
2. Genetically map PD resistance from other forms of *V. arizonica*: b42-26 (*V. arizonica/girdiana*) and b40-14 (*V. arizonica*).
3. Develop a BAC (bacterial artificial chromosome) 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. We previously reported that the genetic position of the *PdR1a* resistance locus was between marker VVCh14-56 and VVCh14-70. In the past three months, we have developed three additional SSR markers derived from Pinot noir genome sequence that allowed us to narrow down the physical distance from 300Kb to 200 Kb. These markers (VVCh14-77, VVCh14-78 and VVCh14-81) were added to the composite set of recombinants from the 9621 population as well as to the resistant and susceptible parents used for crosses in 2008 to determine if the resistance allele is unique and not present in susceptible selections. The resistance allele was unique in size, which made these markers very valuable and robust for marker-assisted screening. There are three key recombinants from the tested set of more than 900 plants. For two plants, the recombination event happened between VVCh14-78 and *PdR1a*, and other plant had a recombination event between VVCh14-81 and *PdR1a*. With the addition of new markers, the *PdR1a* locus is within a 1cM window and it completely correlates to the physical distance between the markers that were developed from Pinot noir genome sequence.

F8909-08 possesses the *PdR1b* resistance locus, which is being mapped in the 04190 population. Previously we reported that *PdR1b* maps between VvCh14-02 and VVCh14-70. Additional markers (VvCh14-28/VVCh14-29/VVCh14-30) were added to the entire set of 397 plants in the 04190 population. The greenhouse screen was repeated for key recombinants, which also helped refine the data. In addition, marker analysis identified 14 recombinants from 15 different crosses (1,000 plants) based on resistance from F8909-08. We completed the greenhouse screen on 35 recombinants (including seedlings from *PdR1b* background crosses). The screen identified four key recombinants: in two plants the recombination event occurred between *PdR1b* and VVCh14-02; and in one plant the recombination event occurred between *PdR1b* and VVCh14-70. The greenhouse screen is being repeated for four other recombinants that had inconclusive first test results. In the most updated map, we have placed the *PdR1b* locus between markers VVCh14-81 and VVCh14-78 (**Table 1**). Both of these markers are less than 200Kb apart from each other based on the Pinot noir genome sequence.

The 04191 population (*V. vinifera* F2-35 x F8909-17) has 153 progeny and a population where resistance from F8909-17 (*PdR1a*) can be examined without possible confounding effects from D8909-15 (since D8909-15 has a multigenic resistance from b42-26). The resistance locus *PdR1a* is mapped in the 9621 (D8909-15 x F8909-17) population, and the 04190 population mentioned above, and refined mapping focused only on chromosome 14. The 04191 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, and the 153 plants in 04191 will be greenhouse screened. Greenhouse results will be available in Spring 2010 and the framework map will follow.

Objective 2. Previous mapping and greenhouse screen data from the 0023 population (D8909-15 x *V. vinifera*) with resistance from *V. arizonica/girdiana* b42-26 found that PD resistance is quantitative. The 05347 population (b42-26 x *V. vinifera*) was created to better study this resistance source. A total of 337 markers were tested on a small 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. A total of 70 markers were added on a set of 64 progeny, and many will have marker data soon. The current population size is 165 and crosses were made to increase the population size this Spring. Greenhouse screening results will be available in Summer 2010, and a framework map will be developed on a core set of 165 seedlings; this population is being expanded for the future mapping of this quantitative PD resistance trait.

Vitis arizonica b40-14 is a third promising resistance source with PD resistance that seems to be homozygous and controlled by a single dominant gene. Previously, we reported that all F1 progeny from a cross of *V. rupestris* x b40-14 (the R8918 population) were resistant except three genotypes with intermediate results. Two resistant siblings were used to develop two populations: 07388 (R8918-02 x *V. vinifera*) and 07744 (R8918-05 x *V. vinifera*). We completed DNA extractions from 122 seedlings of the 07744 and 105 seedlings from the 07386. A total of 277 markers were polymorphic for one or the other parent in preliminary marker screening. One hundred fifty two polymorphic markers were completed on the entire set of 122 plants in the 07744 population. Mapping analysis was carried out on each parent separately. The framework map of R8918-05 was produced with 152 markers on 121 genotypes with JOINMAP (3.0). Only three markers were unlinked and the remaining 149 markers were grouped into the expected 19 chromosomes. QTL analysis was performed with MAP QTL (4.0)

and the Kruskal-Wallis approach was used to complete the preliminary analysis. No association with PD resistance was found on any other chromosome except 14 – the same chromosome where *PdR1a* and *PdR1b* map. PD resistance from b40-14 (which we have named *PdR1c*) also maps in the same general region between flanking markers VVCh14-78 and VVIN64 and within 1.5 cM. The LOD threshold for the presence of this QTL was very high (**Table 2**). Next, interval and MQM analysis will be carried out after the selection of markers as cofactors, to determine the level of variance contributed by this b40-14 based resistance locus.

Objective 3 and 4. Two BAC libraries (each from different restriction enzymes) created from the homozygous resistant b43-17 were developed. In the first phase of the project, library screening was carried out with markers (VVCh14-10 and VVCh14-56), both tightly linked to *PdR1*. This process identified 24 positive clones – four of the clones were positive with both markers: H23-P13, H34-B5 and H64-M16 and H45-J22. New markers (both SSR and non-repetitive) were developed from the 695Kb region from the Pinot noir genome sequence covered by markers VVCh14-56 and VVCh14-70/77/78 (see previous reports). This region overlaps two different scaffolds from the Pinot noir genome sequence (9 and 21). Currently, *PdR1* is placed between Ch14-81 and Ch14-78 at a physical distance of ~200Kb. Based on the genetic map from the 9621 population, the physical and genetic distance correlates because 1cM is equivalent to about 216Kb. The second round of BAC library screening was carried out with the Ch14-58 marker. A total of 17 clones were positive, five of them were also positive with the VVCh14-56 marker (see details in previous report). Clone H69J14 was selected for 454 sequencing. A total of 42,000 sequences were generated. Two different programs were used to assemble the sequence. The DNASTAR program seqman allowed assembly at a stringency level of 99%, which generated more than 4,000 contigs representing 38,000 sequences. A total of 79 sequences were bigger than 5 KB. **Table 3** presents the contigs that are bigger than 6 Kb and have sequence similarity to the Pinot noir genome sequence (**Figure 1**). It is important to note that the sequence similarity to scaffold 21 of Pinot noir was almost 98% identical for most of the contigs, however, the b43-17 sequences that overlap with scaffold nine of Pinot noir matched to multiple sites and level of similarity was less. This result suggests that either the b43-17 genomic region with the PD resistance gene(s) is divergent from Pinot noir, or the 8X assembly of Pinot noir's scaffold 9 has lots of gaps and errors. The 12X coverage of Pinot noir genome would be more helpful to conduct meaningful sequence comparison. In the next step, primers will be designed to fill the gaps, improve the coverage and to verify the sequences at the ends of the contigs.

CONCLUSIONS

Genetic mapping efforts have identified valuable genetic markers for marker-assisted selection and enabled rapid progress towards PD resistant winegrapes (see companion report). These mapping efforts have now identified three alleles of *PdR1*: *PdR1a* and *PdR1b* derived from *V. arizonica/candi*cans b43-17; and *PdR1c* derived from *V. arizonica* b40-14. These alleles were found to map within the same general region, but suggest that although PdR1 seems to be a single gene trait, the region may be composed of a number of tightly linked genes. BAC library sequence analysis of b43-7 is resulting in candidate genes suggestions for *PdR1* and these are being compared to the Pinot noir genome sequence and to similar regions in other plants. The genomic characterization of this region will help us determine how this form of PD resistance functions and which genes control it.

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Table 1. Key recombinants from the 9621 (*PdR1a*) and 04190 (*PdR1b*) populations. The genotypes in bold red font are key recombinants with a recombination event between the marker and the resistance locus. "0" indicates a susceptible allele and "1" indicates a resistant allele.

Genotypes with *PdR1a* background	A010	VVCh 14-56	VVCh1 4-81	*PdR1a*	VVCh 14-78	VVCh 14-77	VVCh 14-70	VVCh14 -29	VMCNg2 b7.2
-416	0	0	0	0	0	0	0	1	1
-426	0	0	0	0	0	0	0	1	1
-470	0	0	0	0	0	0	0	1	1
-554	0	0	0	0	0	0	1	1	1
-1064	1	1	1	1	1	1	0	0	0
-8	0	0	0	**0**	**1**	1	1	1	1
-194	0	0	0	**0**	**1**	1	1	1	1
-38	0	0	**0**	**1**	1	1	1	1	1
-15	1	1	1	1	1	1	1	0	0
-23	1	1	1	1	1	1	1	0	0
-901	1	1	1	1	1	1	1	0	0
-915	1	1	1	1	1	1	1	0	0
-919	0	0	0	0	0	0	0	1	1

Table 1. Cont'd.

Genotypes with *PdR1b* background	VVCh 14-10	VVCh 14-02	VVCh 14-81	PdR1 b	VVCh 14-78	VVCh 14-77	VVCh 14-70	VVCh 14-30	VVCh 14-27
06314-24	0	0	0	0	0	0	0	1	1
06328-05	0	0	0	0	0	0	0	1	1
04190-026	0	0	0	0	0	0	0	1	1
06317-50	1	1	1	1	1	1	1	0	0
04190-383	1	1	1	1	1	1	1	0	0
06317-50	1	1	1	1	1	1	1	0	0
04190-320	1	1	1	?	1	1	1	0	0
04190-065	1	1	1	?	1	1	1	0	0
04190-109	1	1	1	1	1	**1**	**0**	0	0
04190-381	1	1	1	**1**	**0**	0	0	0	0
06711A-60	0	0	0	?	1	1	1	1	1
04190-236	1	1	1	?	0	0	0	0	0
06315-49	1	0	0	0	0	0	0	0	0
06326-23	1	0	0	0	0	0	0	-	-

Table 2. The Kruskal-Wallis analysis LOD values for the PdR1c locus in the 07744 population based on resistance from *V. arizonica* b40-14.

Genetic map	Map locus	K* (df)
0	VVIN70	5.392 (1) **
3.5	VVIn94	9.323 (1) ****
9.5	ctg1025882	16.293 (1) *******
10.4	VVIP26	12.764 (1) ******
10.7	VVIS70	17.315 (1) *******
11.6	UDV025	16.160 (1) *******
15.0	VVIN64	21.081 (1) *******
16.7	VVCh14-78	22.692 (1) *******
16.7	VVCh14-77	22.946 (1) *******
17.7	VVCh14-70	19.350 (1) *******
20.3	VMCNg2b7.2	17.282 (1) *******
21.5	VVMD24	20.496 (1) *******
22.0	VMC5b3	20.631 (1) *******
22.5	VMC2a5	22.915 (1) *******
22.5	VVIV69	21.978 (1) *******
23.2	UDV033	22.857 (1) *******
28.9	VMC6c10	15.577 (1) *******
36.2	VMC2c3	8.872 (1) ****
36.5	VMC2b11	8.057 (1) ****
36.9	VChr14a	7.229 (1) ***
39.0	ctg1008359	8.772 (1) ****
39.8	VMC9f4	9.360 (1) ****
41.1	VMC2h12	8.967 (1) ****
49.8	VMC1e12	3.507 (1) *
59.2	VVIP05	1.714 (1) -
61.1	VChr14b	0.398 (1) -
65.4	VVC62	0.386 (1) -

Table 3. Contig size, level of similarity and match location to Pinot noir contigs 9 and 21. Majority of the contigs that were similar to scaffold 9 matched to different locations. The similarity of the b43-17 sequence is greater when it is closer to scaffold 21.

Contig number	Contig length (Kb)	Number of sequences	Coverage	% Match to Pinot noir	Scaffold	Start position
4154	11,020	158	5.74	98	21	15,201,490
2454	11,011	108	3.55	94	9	15,002,217
2620	10,109	87	3.01	98	21	15,165,403
2801	9,741	82	3.36	97	21	15,183,600
1824	9,375	96	3.55	93	9	15,096,377
1834	9,324	113	4.49	98	21	15,200,996
2554	8,240	78	3.60	92	9	multiple sites,
440	8,177	64	3.14	95	9	15,103,036
673	8,066	73	3.09	98	21	15,175,438
2410	7,779	84	4.04	96	9	15,078,745
3944	7,703	69	3.57	98	21	15,165,453
3654	7,463	61	3.05	98	21	15,180,883
2411	7,288	84	4.36	97	21	15,208,989
3773	7,269	157	8.19	98	21	15,179,580
2341	7,267	102	5.89	-	-	-
1658	7,247	49	2.63	-	-	-
1918	7,230	62	3.14	90	9	multiple sites,
3997	7,226	141	7.2	92	9	multiple sites,
45	7,217	66	3.59	87	9	multiple sites,
1734	6,996	170	9.68	98	21	15,180,952
1885	6,985	87	4.77	-	-	-
496	6,945	90	5.09	99	21	15,181,699
530	6,763	319	19.75	90	9	multiple sites,
3606	6,713	57	3.17	98	21	15,181,366
959	6,647	87	4.8	99	21	15,168,301
420	6,599	99	6.07	92	9	multiple sites,
1259	6,593	155	9.28	-	-	-
2510	6,585	66	3.76	-	-	-
4108	6,545	66	3.91	98	21	15,168,284
1741	6,463	43	2.24	91	9	15,002,306
1216	6,410	184	12.05	94	9	15,070,922
3562	6,354	103	6.42	90	9	15,004,525
2610	6,331	83	4.94	97	21	15,168,523

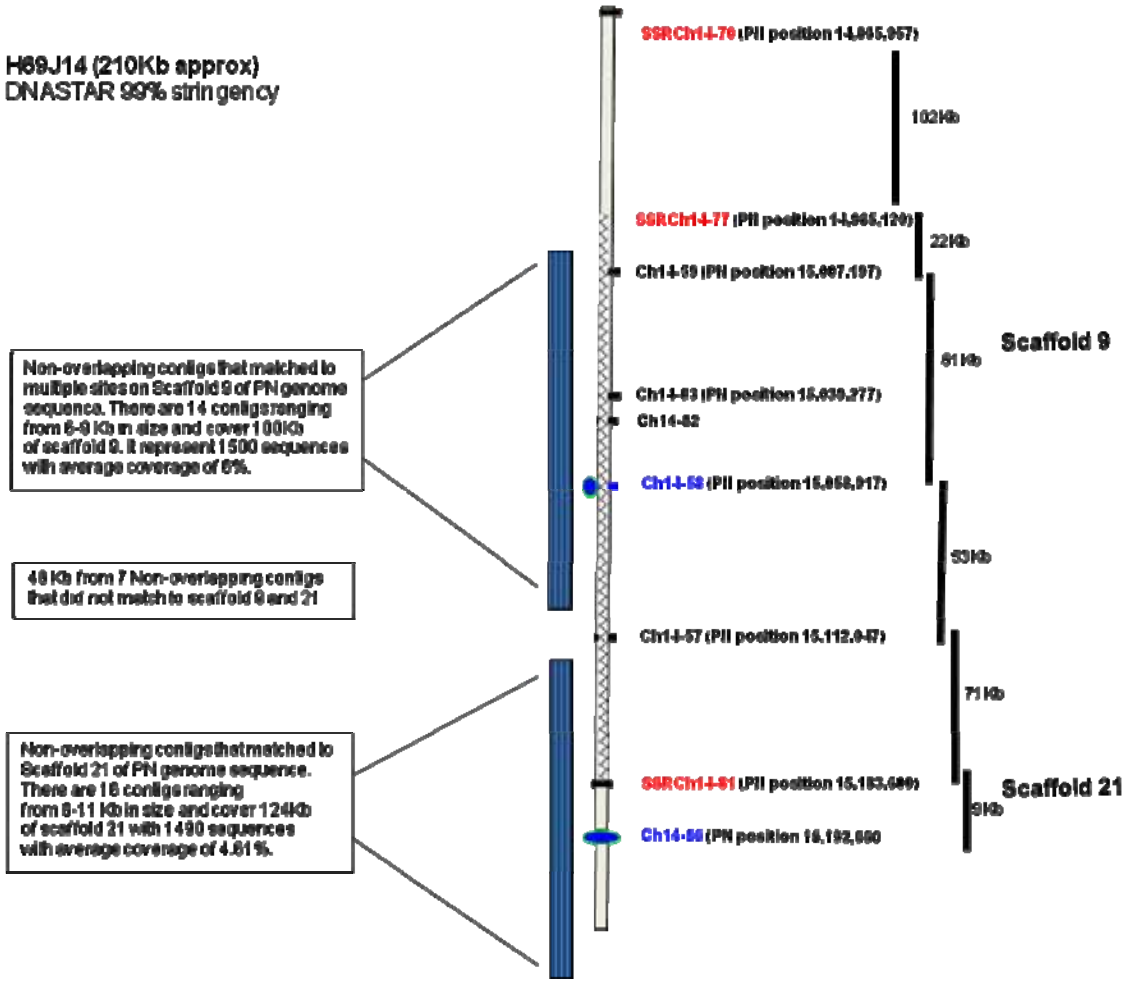

Figure 1. Assembly detail of the H69J14 BAC clone that spans two scaffolds from the Pinot noir genome (scaffold 21 and 9). Currently the PD resistance locus resides between SSR markers Ch14-81 (on scaffold 21) and Ch14-77 (on scaffold 9). The relative position and distance of all the markers that have been used in mapping and library screening are on the right. Non-overlapping contigs were grouped based on their position on the Pinot noir. Primers will be developed for the contig ends to enable BAC walking in order to fill the gaps and verify the sequences.

Section 6: Economics

THE BENEFITS AND COSTS OF ALTERNATIVE POLICIES FOR THE MANAGEMENT OF PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted October 2008 to September 2009.

ABSTRACT
We propose to address economic questions related to Pierce's disease (PD) by developing and applying a quantitative model of the supply and demand for California wine and wine grapes. The economic component of this model will combine existing wine market models with some entirely new elements. The biophysical component will draw on a range of technical information and, where appropriate, evidence from crop and disease simulation modeling. To begin, we have consulted with scientists the study the disease and its vectors. As a result of their advice, we have begun to model the problem of the blue-green sharpshooter in northern California. We hope that by doing so, we will gain a better understanding of how to model the statewide PD problem including the role of the glassy-winged sharpshooter and the problems it poses across the state. The end result will be a model that is designed specifically to evaluate the likely expected benefits from investments in alternative research and development projects related to the management of PD.

LAYPERSON SUMMARY
In the first year of the project we have concentrated on gathering data and other information and learning about Pierce's disease and the sharpshooters that spread it. Our progress has led us to revise some aspects of the research strategy, but the work has gone generally according to plan, albeit after a delayed start. We have laid the foundation to develop a working (but simplified) model by the end of 2009 that can be used for initial applications and as a basis for consultation to establish directions for elaboration and refinement.

INTRODUCTION
It is widely accepted that Pierce's disease (PD) with its vectors, including the glassy-winged sharpshooter (GWSS), has large current and very large potential economic consequences. However, we are not aware of other studies that have modeled and measured the economic consequences. Siebert (2001) discussed the economics and he estimated that the value of lost wine grape production—in Temecula, Riverside County, alone, in 1988 and 1989—was worth $37.9 million to California. Echoing that sense of economic importance, the National Academy of Science (2004) undertook an extensive study and published a book on California research priorities focused on PD. That book does not contain estimates of the economic consequences of PD or alternative management or control methods, but it does provide a comprehensive documentation of knowledge about the problem, as well as a useful classification of types of research and priorities for them, including economic research. Further work is needed to develop a quantitative economic understanding of PD and alternative policies to address it.

OBJECTIVES
The overall objective of this project is to develop a detailed, practical, quantitative understanding of the economic consequences of PD and alternative management strategies. More specific objectives are to quantify the current and potential economic impact of the disease, to estimate the potential economic payoff to investments in PD research and development (R&D), to evaluate alternative management strategies including alternative research investments, and to guide policy decisions, including research priorities. Additionally, we aim to study regional differences across California in PD and its associated vectors, allowing for a better understanding of the problem and a more precise evaluation of alternative

management regimes. To pursue these objectives, we propose to develop an economic model of the California wine and wine-grape sector. The model will be structured to allow us to simulate market outcomes under alternative scenarios for the prevalence of PD, and alternative technologies and policies for its management, and to assess the economic consequences of these alternatives for various stakeholder groups. The model will be designed specifically with a view to using it to evaluate the likely expected benefits from investments in alternative R&D projects related to the management of PD.

RESULTS AND DISCUSSION

Our project commenced formally on September 1, 2008. Kate Fuller has been employed as a Graduate Student Researcher to work half-time on the project. In the work to date we have emphasized investment in developing our own knowledge and information resources. One important element of this is to develop a detailed data base on the economics of wine and wine grape production in California. We have completed the data-gathering phase, and we are compiling the information into a report documenting by county and crush district for each important grape variety the area planted, yield, quantity produced (crush volume), and price over the past 30 years. We have also made some investment in learning about how to structure and use models of spatial-dynamic processes such as the spread of disease, and made significant progress in developing an understanding of the pest and disease problem, and an overview of the issues, through consulting with scientists and others and reviewing literature. We have learned that the PD/GWSS problem will be difficult to model, so we have opted to focus initially on studying the issues as they arise in the north coastal valleys where PD is spread by native sharpshooters (in particular, the blue-green sharpshooter). This approach will enable us to develop some economic data and insight into the problem, management strategies, and costs of prevention, control, and eradication strategies, which will help us in designing approaches to study the more general problem, including the role of the GWSS.

Based on this work, Kate Fuller wrote a research essay (as required by the Ph.D. program in Agricultural and Resource Economics at UC Davis), titled: “Optimal Management Strategies for Vector-Borne Agricultural Pests and Diseases: Theory and Application to Pierce’s Disease of Wine Grapes in Northern California.” This essay entailed a review of relevant literature as well as the development of the framework for a general economic model of vector-borne disease as applicable to PD. As well as providing a useful reference document for our project, it formed the basis for Kate’s oral qualifying examination, which she successfully took and passed on July 14 2009. Kate’s dissertation research plan, to be conducted over the next two years, entails elements related to the main objectives of the project. It will begin with work on the BGSS in northern California as a basis for work on the GWSS, ultimately providing a basis for evaluating payoffs to research.

As described above, we have been developing data and information but do not have any specific accomplishments to report beyond making progress as planned towards achieving the specified objectives for the first-year.

CONCLUSIONS

None to date.

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THE ECONOMICS OF PIERCE'S DISEASE IN CALIFORNIA

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ABSTRACT
The first goal of this research project is to estimate the medium to long-run economic impact to growers and consumers of California's grape and orange industries, and to taxpayers from the establishment of the glassy-winged sharpshooter (GWSS) in California. The public control program to date has managed to contain the GWSS in the southern part of the State. As part of the public program citrus growers are reimbursed their expenses for winter control of GWSS and a quarantine that regulates the movement of fresh citrus, fresh grapes, and nursery host crops to areas currently free of GWSS. In the southern San Joaquin County due to effective winter GWSS control in citrus, the cost for grape growers to treat GWSS using one soil application of imidacloprid a year ($50-$60 per acre) is offset by reductions in the use of other insecticides such as the foliar applications of imidacloprid and treatments for pests such as the grapeleaf skeletonizer. In the Temecula Valley; however, GWSS becomes active earlier, orchards and vineyards are generally smaller, and the orange/grape land interface is more complex causing growers in this area to incur additional costs. In addition to a soil application of imidacloprid, vineyards in the Temecula valley may also need an additional irrigation at $12.50 an acre at the time of the imidacloprid application, plus two additional spray treatments with Danitol at $35.50 an acre. Total costs for GWSS control in the Temecula Valley is $98 to $108 an acre. About $50-$60 of that cost is also offset by reductions in the use of insecticides needed to treat pests that are now controlled with the soil application of imidacloprid. The net increase in costs is about $48 an acre per year. The average number of acres cultivated in grapes from 2005 to 2007 in the Temecula Valley is about 200, making the total estimated annual losses to growers in the Temecula Valley about $9,600 a year. These losses could increase substantially if the public control program were discontinued as winter GWSS treatment in citrus would cease and, without a quarantine, GWSS would spread. Costs for grape growers would increase throughout grape growing regions due to higher control costs where GWSS is currently established, and the need to implement control measures in areas currently free of GWSS.

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INTRODUCTION
The establishment of newly introduced pests in California has resulted in significant costs to growers and consumers in California, and to consumers in the rest of the U.S. Infestations of the ash whitefly (*Siphonius phillyreae* (Haliday)) in the late 1980s and early 1990s caused aesthetic damages to ash and ornamental pear trees estimated between $324 million and $412 million before a biological control agent brought the infestation under control (Pickett et al., 2003). Losses to consumers and producers due to the establishment of the avocado thrips (*Scirtothrips perseae*) were estimated at $4.45 million a year (Hoddle et al., 2003). Losses from the establishment and potential spread of the red imported fire ant, first identified in California in 1997, were estimated to be about $900 million a year (Jetter et al., 2002). Should a newly introduced pest into California, the *Diaprepes* root weevil, become established throughout the state, the losses to consumers and producers of citrus and nursery crops from pest treatments and quarantines are estimated to be $100 million a year (Jetter 2007).

In 1989 a pest new to California, the glassy-winged sharpshooter (GWSS), was collected in Irvine, CA. Since then it has spread throughout southern California, into the southern San Joaquin Valley including Kern County and parts of southern Tulare County, and along the coastal counties of Santa Barbara and San Luis Obispo. Detections, mainly of egg masses on nursery shipments, have been found in many counties throughout the Central Valley as far north as Tehama County, in the Napa Valley, and in the Bay Area counties (CDFA 2007; http://max.cdfa.ca.gov/pdcp-gis/pdcp-gis.asp). The main hosts for the GWSS are citrus, grapes, almonds and alfalfa. The GWSS overwinters in citrus, avocados, in riparian vegetation and some ornamentals such as crape myrtle. As grape vines and almond trees leaf out in the springtime, the GWSS moves onto those hosts.

Governmental agencies have been involved in two control programs to manage and contain the GWSS. One program involves the control of the GWSS on citrus before it can move into vineyards and transmit the Pierce's disease (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 of a trapped vine. While some citrus growers may benefit from the control of the GWSS 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®) 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 may not have 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 it 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).

OBJECTIVES

The first objective of this study is to 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. 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 due the changes in the costs of production.

The remaining objectives are to address the following: 1) estimate the benefits and costs of the public program, and 2) to use those results to estimate check-off rates to the different industries that are protected by the public program. To determine the benefit of the public program the industry costs with respect to GWSS management need to be determine. A two-step process is currently being done to obtain these data and includes 1) a survey of growers to determine the economic impacts before Admire was recommended to treat GWSS and 2) using that information to discuss control alternatives for grape growers that would be most likely now. Surveys of grape, orange, and nursery producers are currently being developed.

Objective 1 will be completed through the use of economic market models. Market models are used to estimate the losses to both producers and consumers when changes in the costs to grow and market a crop are significant enough to affect market prices, production and supply. 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. 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® 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®.

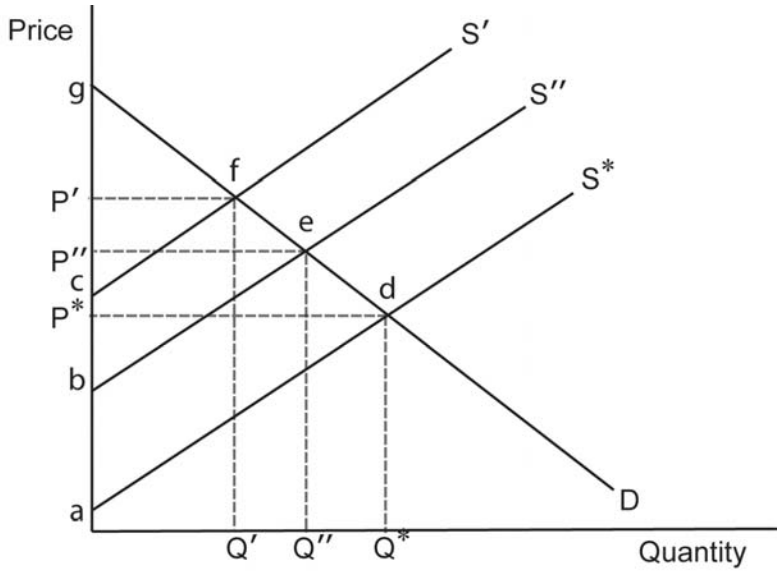

Figure 1. Market effects for grapes produced in GWSS infested counties.

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.

RESULTS

Changes in treatment costs due to the establishment of the GWSS and the public control program.

For Objective 1 data are needed on the changes in the costs of production for affected growers due to the establishment of the GWSS in California. For Objective 1 data are also needed on grape, citrus and nursery production, prices, revenues and trade data from 1998 through 2007 (the last year for which data are available); current costs of production; and elasticities (elasticities measure the percentage change in a quantity variable for a one percent change in a price variable – for example it could measure the percentage change in production for a one percent change in the farm price.)

How the GWSS affects current production was determined through meetings held with UCCE farm advisors and growers to discuss how the establishment of the GWSS affected their pest control programs for grapes. The meetings were held in November and December 2008 in the southern San Joaquin Valley in November and December 2008. The results of these meetings were compared to University of California Cooperative Extension Budgets to determine how all pest control treatments changed as a result of the treatments required for GWSS (Hashim-Buckley 2007; Peacock et al. 2007; Peacock et al. 2007b; Vasquez et al. 2007). Production and price data for grapes were collected from the National Agricultural Statistics Service.

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 taxpayers. While there is currently a low incidence of PD in Kern and Fresno counties, the incidence can rapidly increase should GWSS not be controlled.

The first line of defense against the spread of PD by the GWSS is the public control program whereby citrus is treated during the winter months to prevent the build up of GWSS populations. 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 about once every three years based on monitoring of GWSS populations. Under the public program citrus growers are reimbursed for their treatments of GWSS and participation in the public program is currently voluntary for the citrus grower.

The second line of defense against the spread of PD is to treat grape vines for GWSS. A majority of grape growers apply imidacloprid once annually to control GWSS and prevent the transmission of PD. Applications of Admire Pro are typically at the maximum rate of 14 fl oz an acre (4.6 lb ai/gal formulation) through the irrigation system. The cost of applying Admire Pro is currently about $50-$60 an acre. The patent for Admire expired in 2005. As a result the initial cost to control the GWSS was higher. Growers from the southern San Joaquin Valley will provide the costs for earlier treatments with Admire.

The treatments with imidacloprid also provide some cost savings as the GWSS also controls the variegated grape leafhopper, grape skeletonizer, and is a suppressant of the grape vine mealybug. The cost savings by growers is $62 an acre based on UCCE budgets, or about the same amount as the current costs to apply Admire Pro. No quarantine costs are incurred by grape growers, as mature fruit destined for the fresh market is hand harvested and field packed.

Total costs of production for citrus growers are also affected by the public control program and quarantines against moving citrus out of infested areas. Treatments with 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 butoxide). Turbocide has also been mentioned as a material that can be used as a fumigant. If GWSS are found in a grower's orange shipments, the grower bears the cost of treating for GWSS in his or her grove if the grower did not participate in the area wide program. This aspect of the public control program is believed to encourage greater participation by citrus growers in the control of GWSS.

Economic Effects in the Temecula Valley

In the Temecula Valley there is also a public program to control GWSS. In contrast to the program in the southern San Joaquin Valley, individual groves are treated following identification of an outbreak. Area wide coordination of treatments has been more difficult in the Temecula Valley. Many groves are being carved up into rural homesteads and cultural

procedures are completed by farm management companies instead of a grower/owner. With a lower proportion of groves being treated in the Temecula Valley than in the southern San Joaquin Valley, GWSS pest pressure in greater in the Temecula Valley.

Private treatment of GWSS in the Temecula Valley also consists of an annual treatment of Admire. However, because there is greater GWSS pest pressure, higher costs of production for grape growers in the Temecula Valley are being realized as the application of Admire is being supplemented with annual sprays of Danitol in some areas. For vineyards located near citrus groves about two applications of Danitol are needed a year. Growers in the Temecula Valley would also no longer be required to treat for the grapeleaf skeletonizer. There is no problem with leafhoppers in this area.

The Temecula Valley has a drier climate than the San Joaquin Valley. In order for growers to apply Admire when it can do the most good, a separate irrigation may be required. Farm managers with whom meetings were held estimate that half the time they need to complete a separate irrigation in order to apply Admire. The extra irrigation costs are estimated to be $12.50 on average. Danitol is applied at a rate of 11 oz per acre, with the cost per ounce equal to $1.62. With two treatments a year the cost to treat GWSS with Danitol is $35.86. The total additional cost to grape growers to treat GWSS in the Temecula Valley is about $48 a year when rounded.

Market effects of GWSS control and PD to date.
Grape growers in the southern San Joaquin Valley produce about 87% of all grapes in California while growers in the Temecula Valley account for only 3.4%. Given the low share of production in California that would be affected by higher costs of production due to the GWSS, there is no shift up in the supply curve and no market effects. Consequently, market prices would not be affected and growers in the Temecula Valley incur all losses due to the additional treatments for GWSS.

DISCUSSION
The public control program to date has managed to contain the GWSS in the southern part of the State. Due to the effective control of GWSS in citrus in the southern San Joaquin Valley, the cost to grape growers of GWSS control has been offset by cost reductions in the use of other insecticides. The area with a net increase in the costs of production for grape production, the Temecula Valley contributes a very small share to California, and U.S. production of grapes (2.8% of U.S. production). Given the low percentage of grape production in the area with the increase in costs and no net change in costs in the areas with the greatest percentage of U.S. production, there is no shift up of the U.S. grape supply curve due to GWSS control in the southern San Joaquin Valley. Market prices do not change and market demand is unaffected.

One implication of the success of the public program with regards to the absence of widescale changes in the costs of production is that consumers to date have been unaffected by the spread of the GWSS and the increase in the incidence of PD that would have happened had the program not been successful. Based on NHANES data, grapes are the fourth most widely consumed fruit in the U.S. after oranges, apples and bananas. Because grapes are so widely consumed, even small changes in price (say less than 10%), would result in large losses due to the number of people who consume grapes.

While the public GWSS control program has managed to keep the change in the costs of production to levels that do not affect market demand, the consequence for growers in the Temecula Valley is that their extra treatment costs are not partially offset by changes in market prices. The increase in changes in the costs of production then result in a decrease in profits for a grower.

If the public control program were discontinued winter GWSS treatment in citrus would cease and, without a quarantine, GWSS would spread. Costs for grape growers would increase throughout grape growing regions due to higher control costs where GWSS is currently established, and the need to implement control measures in areas currently free of GWSS. The net effect would be an increase in market prices and lower market supply, making consumers worse off in additional to producers.

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