

ROLE OF ATTACHMENT OF *XYLELLA FASTIDIOSA* TO GRAPE AND INSECTS IN ITS VIRULENCE AND TRANSMISSIBILITY

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ABSTRACT

Attachment of *Xylella fastidiosa* to xylem vessels and insect vectors may be required for virulence and transmission; therefore we have individually disrupted *fimA*, *fimF*, *xadA*, and *hecA* to assess their role in adhesion to plants and in the disease process. We performed adhesion assays using each mutant and wild-type separately as well as combination of two of the mutants and observation of the phenotypes of these mutants under a scanning electron microscope is underway. Patterns of cell adhesion and aggregation of mutants on surfaces lead us to hypothesize that *fimA* and *fimF* are important in cell-to-cell aggregation while *xadA* and *hecA* are involved in the first steps of adhesion of bacteria to the plant host. Rooted grapevine cuttings were inoculated with FimA-, FimF-, XadA-, HecA-, and wild-type *X. fastidiosa* 'Temecula' or 'STL'. A higher incidence and severity of disease was observed in vines inoculated with the wild-type *X. fastidiosa* strain compared with FimA-, FimF-, XadA- or HecA- mutant strains. Similarly, wild-type strain STL strain of *X. fastidiosa* resulted in more vines with symptoms than FimA-, FimF- or XadA- mutants of this strain indicating that the process of attachment appears to involve similar genes in both the Temecula and STL strains. It thus appears that successful colonization of plants by *X. fastidiosa* requires both cell-to-cell and cell-to-surface attachment. To distinguish the various mutants from each other in mixed inoculations and to determine what factors affect attachment of the mutants we have constructed disrupted *fimA* vectors for use in a *gfp* marked *Xylella fastidiosa*. This will allow us to distinguish the FimA- cells from other cells in a mixture adhesion assay using fluorescence microscopy and to follow these cells in grape following inoculation with these mutants. Because *hecA* is a large gene, we are also disrupting various locations within the HecA gene. We will test these different HecA- mutants in inoculation experiments to determine the role of HecA in virulence of *X. fastidiosa* to grape.

INTRODUCTION

Adhesion is a well-known strategy used by phytopathogenic bacteria to initiate colonization of their plant hosts and a precursor step to invasion (Romantschuk et al. 1994). *Xylella fastidiosa* possesses many genes involved in attachment or adhesion. Simpson et al. (2000) identified 26 genes encoding proteins involved in the biogenesis and function of Type 4 fimbriae filaments (*pilA*, *B*, *C*...). We have focused on the fimbrial operon, which is composed of 6 genes (*fimA*, *ecdD*, *fimC*, *D*, *E*, and *F*). Even though the fimbrial mutant cells had less fimbriae than the wild type cells as seen in scanning electron micrographs, the cells seemed to still be able to attach to surfaces by another mechanism (Feil et al. 2003) (Figure 1A). This suggested that fimbriae are more important in cell-to-cell adhesion than in cell-to-surface adhesion. While FimA and FimF were found to be important in cell-to-cell aggregation (Feil et al. 2003) the initial attachment of *X. fastidiosa* to plants must involve other factors. The goal of this research was thus to assess the relative role of different fimbrial and non-fimbrial adhesins in the attachment process and to determine their role in the disease process. Among the afimbrial adhesins of *X. fastidiosa* we chose XadA and HecA to study because genes homologous to these in other bacteria were found to be virulence determinants.

OBJECTIVES

1. Determine the role of adhesins other than those found in the fimbrial operon, in particular of the adhesin XadA and hemagglutinin HecA in the attachment and virulence of *X. fastidiosa* in grape.
2. Characterize the behavior of the fimbrial and adhesion mutants of *Xylella fastidiosa* in grape and to compare this behavior over time via expression analysis.
3. Determine what factors affect attachment of wild-type or mutant cells to grape
4. Determine if these mutants can attach to the insect vector and be transmitted to grape.

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FUNDING AGENCIES

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

Strain Name	Host of Origin	County or state from which strain was collected
PD-1	Grape	Kern, CA
PD-2	Grape	Kern, CA
PD-3	Grape	Kern, CA
PD-4	Grape	Kern, CA
PD-5	Grape	Temecula, CA
PD-6	Grape	Temecula, CA
PD-7	Grape	Temecula, CA
PD-8	Grape	Kern, CA
PD-9	Grape	Kern, CA
PD-10	Grape	Kern, CA
PD-11	Grape	Kern, CA
PD-12	Grape	Baja, CA
PD-13	Grape	Kern, CA
PD-14	Grape	Kern, CA
PD-15	Grape	Napa, CA
PD-16	Grape	Napa, CA
PD-17	Grape	Napa, CA
PD-18	Grape	Napa, CA
PD-19	Grape	Napa, CA
PD-20	Grape	Napa, CA
PD-21	Grape	Napa, CA
PD-22	Grape (Temecula)*	Temecula, CA
CVC-1	Citrus	São Paulo, Brazil
CVC-2	Citrus	São Paulo, Brazil
CVC-3	Citrus	São Paulo, Brazil
CVC-4	Citrus	São Paulo, Brazil
CVC-5	Citrus	São Paulo, Brazil
CVC-6	Citrus	São Paulo, Brazil
CVC-7	Citrus	São Paulo, Brazil
CVC-8	Citrus	São Paulo, Brazil
CVC-9	Citrus	São Paulo, Brazil
CVC-10	Citrus (9a5c)*	Brazil
ALS-1	Almond	Tulare, CA
ALS-2	Almond	Contra Costa, CA
ALS-3	Almond	San Joaquin, CA
ALS-4	Almond	San Joaquin, CA
ALS-5	Almond	San Joaquin, CA
ALS-6	Almond (Dixon)*	Solano, CA
OLS-1	Oleander	Riverside, CA
OLS-2	Oleander	CA
OLS-3	Oleander	CA
OLS-4	Oleander (Ann-1)*	Riverside, CA
OLS-5	Oleander	CA

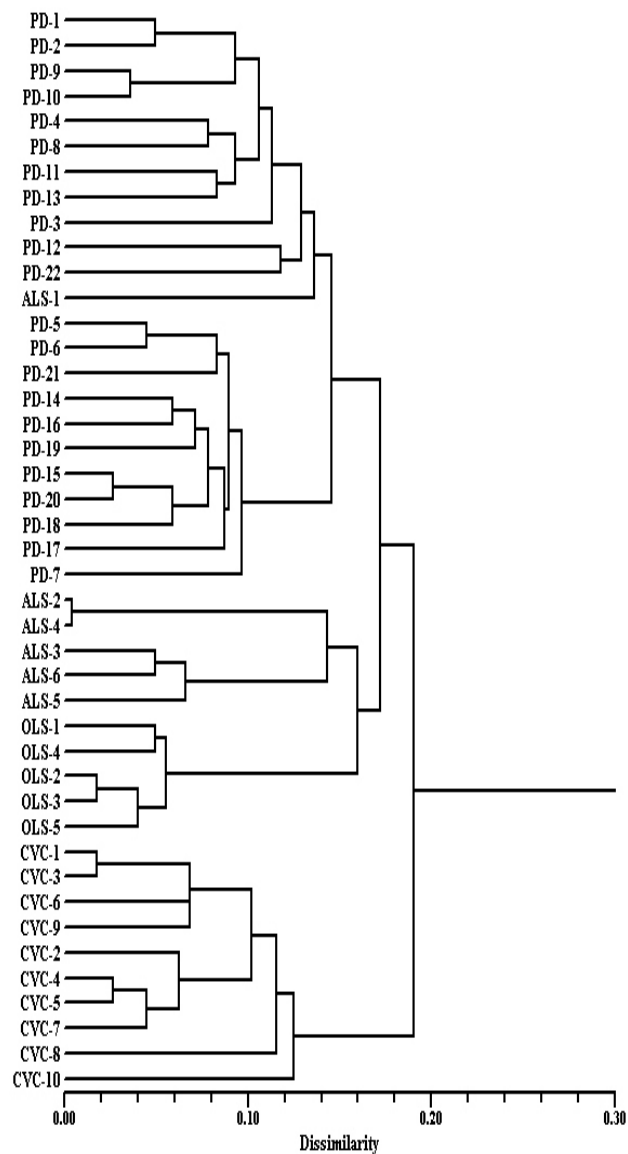


Figure 3. Dendrogram shows genetic distance among the 43 isolates in table 1. Data was compiled from 356 alleles generated by 34 SSR loci.

Table 1. 43 *X fastidiosa* isolates were used for this study. *Labels in bold are the strains used for genome sequence.

3. Sequence alignment was then performed to remove redundant loci and to identify conserved flanking sequence regions across four strains for priming sites between 100-200 bp up/down stream of each repeat locus. This step ensures that primers designed will work for all *Xf* strains.
4. BLAST analysis was performed to examine each selected locus against more than 300 microbial genomes in GeneBank to ensure selected loci are unique. No significant hits were found (E value $< e^{-30}$).
5. All SSR primers were designed using the same parameters (50% GC, $T_m=60^\circ\text{C}$, primer length $\approx 20\text{bp}$, and self dimer/cross dimer $\Delta G = -5$ kcal/mol). This facilitated SSR primer validation and should facilitate scaling up to multiplex PCR formats in future.
6. Based on the criteria and conditions above, 50 primers passed the *in silico* validation test.
7. We further evaluated 50 SSR primers using 43 *Xf* isolates collected from grape, citrus, almond and oleander hosts (see Table 1). In this study, we used thirty-four primers. The results of 34 SSR markers analyses are illustrated in Figures 2 and 3.

CONCLUSION

Repetitive DNA is ubiquitous in microbial genomes. It has been shown to be a useful tool for genetic study in prokaryotes (Belkum, et al 1998). Data from our preliminary study demonstrates that this technique works well for discriminating *Xf* strains. This project will provide an accurate and reliable marker system for genotyping, quarantine purposes, genetic diversity analyses, epidemiological analyses and risk assessment studies.

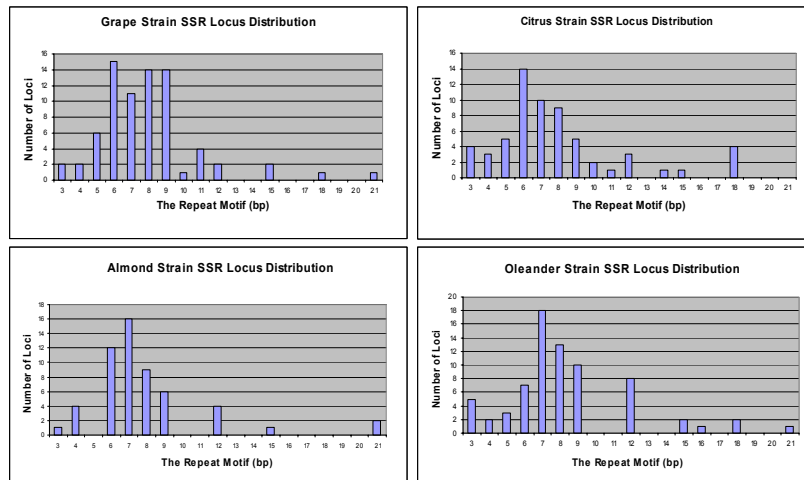


Figure 1. Summaries of SSR loci distributions in each strain of *Xylella fastidiosa*. No mono- and di-repeats occur among these four strains. The above illustrates perfect and imperfect simple repeats with repeat unit length = or > 5 .

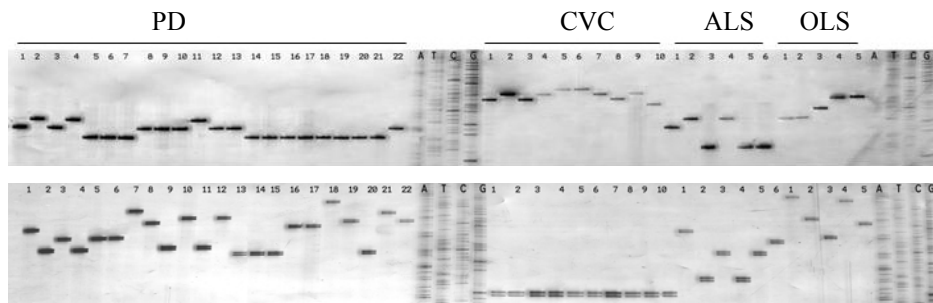


Figure 2. Examples of SSR markers with primers CSSR6 (above) and OSSR9 (below) among 43 *Xylella fastidiosa* isolates separated by 5% of polyacrylamide gel. A, T, C and G are molecular size markers.

DEVELOPMENT OF SSR MARKERS FOR GENOTYPING AND ASSESSING THE GENETIC DIVERSITY OF *XYLELLA FASTIDIOSA* IN CALIFORNIA

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ABSTRACT

Recently available genomic sequences of four *Xylella fastidiosa* strains (PD, CVCD, ALSD and OLSD) facilitate genome wide searches for identifying Simple Sequence Repeat (SSR) loci. Sixty SSR loci were selected for SSR marker development. We designed and validated 34 SSR primers with good reliability and specificity. These SSR primers showed various levels of polymorphism with average 11.3 alleles per locus among 43 *Xylella fastidiosa* isolates. These multi-locus SSR markers, distributed across the entire genome, are a useful tool for pathogen genotyping, population genetics and molecular epidemiology studies.

INTRODUCTION

Xylella fastidiosa (*Xf*) causes economically important diseases that results in significant losses in several agricultural, horticultural and landscape crops, including grape Pierce's disease (PD), almond leaf scorch disease (ALSD), citrus variegated chlorosis disease (CVCD) and oleander leaf scorch disease (OLSD). Recent introduction and establishment of the invasive and more effective vector, the Glassy-winged Sharpshooter (*Homalodisca coagulata*, GWSS) has had a great impact on the California grape industry. Host plant resistance is a critical component of integrated crop management. If this insect becomes widely established, the use of resistant varieties may become the most reliable and effective way to control PD. However, the durability of resistant grape plants depends upon the variability and adaptability of the pathogen population and its interaction with the resistance genes of plants. Most resistance studies are performed by screening against a subpopulation of a given pathogen, and neglect that fact that changes in pathogen population structure that may lead to resistance breakdown.

It is clear that pathogen populations with a high evolutionary potential are more likely to overcome host genetic resistance than pathogen populations with a low evolutionary potential (MacDonald and Linde, 2002). The risk becomes even greater with the recent establishment of a more effective vector, the GWSS, which dramatically increases the dispersal of *Xf* genes/genotypes. In California, information regarding the population structure and genetic diversity, as well as the genetic evolutionary and epidemiological relationships, among *Xf* strains in agricultural populations is not clear. In order to develop effective management strategies, it is critical to understand pathogen population structure and genetic diversity in the agricultural ecosystem. A tool is needed that is capable of precisely, powerfully, easily analyzing *Xf* diversity and genotyping strains. We developed multi-locus DNA markers to fill this need.

OBJECTIVES

1. Perform genome-wide sequence analysis to identify Simple Sequence Repeat (SSR) loci from four *Xf* genomic sequencing databases (PD, CVCD, ALSD and OLSD). Design and develop multi-locus SSR markers.
2. Analyze genetic diversity and population structures of PD *Xf* statewide. Compile a large *Xf* allele frequency database for strain identification.
3. Use the SSR Marker system to examine interactions between hosts and *Xf* including adaptation, host selection and pathogenicity of *Xf* strains

RESULTS

SSR Locus Identification and Primer Design

1. A genome wide search was performed to identify SSR loci among all four *Xf* strains (CVC 9a5c 2.68Mbp, PD Temecula 2.52Mbp, ALS Dixon 2.67Mbp, and OLS Ann-1 2.63Mbp). Figure 1 shows the distributions of SSR loci among four strains of *Xf*.
2. We used the following criteria to select SSR loci for primer design; a) each locus has single hit per genome and b) each selected locus contains at least 5 or more of repeat unit lengths.

Objective 3

Six months after inoculation (see objective 1), we also noticed an unexpectedly high percentage (35%) of inoculated vines that did not develop typical PD symptoms. One might have expected no more than 5% or so of the mutants to be non pathogenic. We sequenced the *Xf* DNA, flanking the Tn5 element in order to determine the specific location of the Tn5 insertion in each putatively “avirulent” mutant. Table 1 summarizes the categories of the genes that were knocked out in the avirulent *Xf* mutants. We then chose to further characterize insertions in open reading frames (ORFs) that code for proteins that have possible roles in *Xf* virulence/colonization or ORFs with no known function. Tn5 insertions in known “house-keeping” genes were not screened further. Three new Chardonnay grapevines growing in pots in the greenhouse were inoculated with each *Xf* mutant of interest as well as the appropriate controls. The experiment was done in duplicate. The rate of symptom development or lack thereof, is being monitored as we described in objective 1. After 14 weeks, petiole samples at the point of inoculation (poi) and 12 inches above the poi will be taken from each mutant and control vines. *Xf* cells will be cultured from those samples in order to assess bacterial population and colonization. The insertion sites will be further confirmed by PCR.

Objective 4: Develop a *Xf/E. coli* Shuttle that is Stable in planta.

A plasmid DNA fraction was isolated from the UCLA strain of *Xf* and subjected to *in vitro* mutagenesis using the transposome technology that was previously used to create our Tn5 *Xf* library. This DNA was electroporated in the UCLA strain and 4 kan^R colonies were obtained. These were sequenced and found to be insertions in the small 1.3kb plasmid that we previously attempted to develop as a *Xf/E. coli* shuttle vector. These Tn5 insertions were in different areas of the native plasmid so we tested the relative stability of these plasmids by culturing the transformants on PD3 medium with and without kanamycin. After 3 passages on non-selective media the colonies were transferred to PD3 media containing kanamycin and no colonies were observed on the plates. This indicates that the plasmids containing the Tn5 insertions were lost upon culture in non-selective medium, results that were the same as our previous attempts to engineer these small native plasmids as shuttle vectors. Future work will focus on a similar strategy to construct a shuttle vector from the 5.8kb plasmid in the UCLA strain, with the hope that this construct might be stably maintained in *Xf* without antibiotic selection.

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1995; Purcell and Saunders, 1999). The vines were grown in pots in a greenhouse using a nutrient-supplemented de-ionized drip irrigation system. The parental, Temecula strain served as a positive control and a water inoculation served as a negative control. Two months after inoculation, the vines were observed for symptom development approximately every two weeks for 6 more months (32 weeks total after inoculation). The symptoms were rated on a visual scale from 0 to 5, 0 being healthy and five being dead. Rating of 1 showed only one or two leaves with the scorching symptom starting on the margins of the leaves. Rating of 2, showed two to three leaves with more developed scorching. Rating of 3 showed all the leaves with some scorching and a few attached petioles whose leaf blades had abscised (match sticks). Rating of 4 showed all the leaves with heavy scorching and/or numerous match sticks.

We successfully identified *Xf* mutants with altered virulence, confirming for the first time, that screening a library of Tn5 *Xf* mutants in susceptible hosts can identify genes mediating *Xf* pathogenicity. We also developed a two-step procedure, direct PCR on *Xf* colony and direct sequencing of the PCR product that can rapidly identify *Xf* Tn5 insertion sites.

Objective 2

Six months after inoculation (see objective 1), 10 of the inoculated Chardonnay vines showed hyper-virulence, i.e. more severe symptoms compared to the vines inoculated with wild type *Xf* cells. This phenotype was further confirmed in Chenin Blanc and Thompson Seedless grapevines. Further analysis demonstrated that all the hypervirulent *Xf* mutants tested showed i) earlier symptom development, ii) higher disease scores over a period of 32 weeks and iii) earlier death of inoculated grapevines than vines inoculated with wild type; thus demonstrating that the hypervirulence phenotype is correlated with earlier symptom development and earlier vine death in multiple *Vitis vinifera* cultivars. The hypervirulent mutants also moved faster than wild type in grapevines. These results suggest that i) wild type *Xf* attenuates its virulence *in planta* and ii) movement is important in *Xf* virulence. The mutated genes were sequenced and their insertion sites confirmed by PCR amplification and sequencing of PCR products. None of the mutated genes had been previously described as anti-virulence genes, although six of them showed similarity with genes of known functions in other organisms. The hypervirulent mutants were further characterized for *in vitro* and *in planta* attachment. One of the hypervirulent mutants was altered in its microcolony formation and biofilm maturation within the xylem vessels (Figure 1). We are in the process of further characterizing the protein involved in *Xf* biofilm maturation.

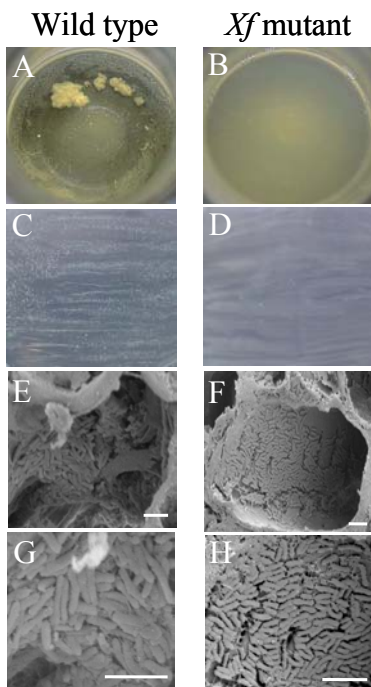


Figure 1: A hypervirulent *Xf* mutant shows a lack of microcolony formation and biofilm formation. Panels A-G are *Xf* wild type cells; Panels B-H are *Xf* mutant cells. Panels A and B wild type and mutant cells, respectively, inoculated into PD3 medium in a 125 mL flask and placed on a shaker. The degree of self-aggregation was visualized after 10 days of incubation. Panels C and D wild type and mutant cells, respectively, plated onto PD3 medium plates. The colony morphology was examined after 10 days of incubation. Panels E and F, wild type and mutant cells in xylem vessels. Note the lack of a three dimension array in the mutant compare to wild type. Panels G and H, close up of wild type and mutant cells in a biofilm. Note the wild type cells typically aggregated together side to side while the mutant cells did not aggregate in this manner. Scale bar equivalent to 5 microns in every panel.

Table 1: Function categories of *Xf* DNA flanking Tn5 transposon insertion in putatively avirulent *Xf* mutants

Putative Gene function	% of Mutants Affected
Hypothetical protein	29
House-keeping	26
Phage-related protein	20
Pathogenicity/virulence	10
Intergenic region	6
Surface protein	2
Transporter	2
Regulator of transcription	1
Mobility	1
Transposon elements	1
Cell-Structure	1
Undefined category	1

ANALYSIS OF *XYLELLA FASTIDIOSA* TRANSPOSON MUTANTS AND DEVELOPMENT OF PLASMID TRANSFORMATION VECTORS

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ABSTRACT

We screened over 1,000 random Tn5 *Xylella fastidiosa* (*Xf*) mutants in Chardonnay grapevines growing in the greenhouse in 2003. Approximately 10 of the mutants exhibited a hypervirulent phenotype, i.e. vines inoculated with these mutants developed symptoms sooner and died sooner than vines inoculated with the wild type *Xf* parental strain. The identity of the Tn5 insertion sites in these mutants was reported at 2003 PD Symposium. In 2004 we re-inoculated these hypervirulent mutants into another set of Chardonnay, Chenin blanc and Thompson seedless vines and the hypervirulent phenotype was reproduced in all 3 varieties. Movement and populations assays showed that the hypervirulent mutants moved faster and reached higher populations than wild type *Xf*. In the first Chardonnay screen, we identified an unexpectedly high number of avirulent mutants. Because some of these may have been the result of poor inoculation we sequenced the DNA that flanked the Tn5 insertion in all the mutants. Those mutants with Tn5 insertions in genes other than “house keeping” genes were re-inoculated into a new set of vines and their pathogenic phenotype is being determined. Additional small (1.3kb) native *Xf* plasmids were engineered as potential *Xf/E. coli* shuttle vectors. However, like our other similar constructs, these plasmids were not stably maintained without antibiotic selection, and not useful tools for *in planta* gene complementation studies.

INTRODUCTION

During the past 4 years one of the objectives of our research on Pierce's disease (PD) has involved the development of transformation and transposon mutagenesis systems for the bacterium that causes Pierce's disease (PD), *Xylella fastidiosa* (*Xf*). We developed a random transposon based mutagenesis system for *Xf* in 2001 (Guilhabert et al., 2001). Recently, we developed two *E. coli/Xf* plasmid shuttle vectors, one based on the plasmid RSF1010 and the other based on a small cryptic plasmid found in one of the grapevine *Xf* strains, UCLA. Both those plasmid shuttle vectors replicate autonomously in *Xf* (Guilhabert and Kirkpatrick, 2003; Guilhabert and Kirkpatrick, manuscript submitted for publication). However these plasmids are only stably maintained in *Xf* cells that are kept under selection using the antibiotic, kanamycin. Therefore, these vectors will be useful for *in vitro* studies of *Xf* gene function; however they cannot be used to study the function of *Xf* genes in the plant host. We evaluated other plasmids that can be stably maintained in *Xf* cells inoculated into plant hosts.

The complete genome sequence of a citrus (Simpson et al., 2000) and a grape (Van Sluys et al., 2002) strain of *Xf* have been determined. Analysis of their genomes revealed important information on potential plant pathogenicity and insect transmission genes. However, approximately one-half of the putative ORFs that were identified in *Xf* encode proteins with no assignable function. In addition, some of the putative gene functions assigned on the basis of sequence homology with other prokaryotes may be incorrect. For these reasons we felt that it was important to develop and assess the pathogenicity of a library of random Tn5 mutants in order to identify any gene that may influence or mediate *Xf* pathogenicity. Our group, as well as other PD researchers, is evaluating specific mutants in *Xf* genes that are speculated, based on homology with other gene sequences in the database, to be involved with pathogenicity. However, screening a random transposon (Tn) library of *Xf*, a strategy that has led to the identification of important pathogenicity genes in other plant pathogenic bacteria, may identify other novel genes, especially those that regulate the expression of pathogenicity/attachment genes that will be important in the disease process. Using Tn5 mutagenesis, there is a high probability that we can knock out and subsequently identify *Xf* genes that mediate plant pathogenesis. Proof that a particular gene is indeed mediating pathogenicity and/or insect transmission would be established by re-introducing a cloned wild type gene back into the *Xf* genome by homologous recombination, or more ideally, introduce the wild type gene back into *Xf* on the plant stable shuttle vector.

OBJECTIVES

1. Screen a library of *Xf* transposon mutants for *Xf* mutants with altered pathogenicity, movement or attachment properties.
2. Identify and characterize anti-virulence *Xf* genes.
3. Identify and characterize virulence *Xf* genes.
4. Develop a *Xf/E. coli* transformation plasmid that is stable *in planta*

RESULTS AND CONCLUSION

Objective 1

Using the transposome technology previously described (Guilhabert et al., 2001) we obtained 2000+ *Xf* Tn5 mutants, which should represent fairly random mutagenesis events throughout the *Xf* genome. During the spring and summer 2002, we inoculated 1,000 chardonnay plants with individual *Xf* Tn5 mutants using a pinprick inoculation procedure (Hill and Purcell,

outer membrane fractions using two-dimensional (2-D) gel electrophoresis with the assistance of our cooperator Linda Bisson and a graduate student in her laboratory, Paula Mara. This technique separates proteins based on their isoelectric points (pI) and their apparent molecular weights. In our initial experiments, we identified over 40 well-separated spots and have analyzed these gels using Phoretix proteome analysis software. This software has allowed us to make a tentative assignment of molecular weights and isoelectric points to many of the predominant proteins. To confirm the identification of some of the ambiguous spots, we plan to cut out these spots and identify the proteins using MALDI-TOF-MS as described above. Although we are still working out some technical details, using 2-D gels will allow us to determine the relative abundance of each of the outer membrane proteins under different environmental conditions (the focus of Objective 2). These gels will also provide us with a proteome map for *Xf* Temecula 1 outer membrane, which we can then compare to the published whole-cell protein map for *Xf* CVC (9).

CONCLUSIONS

Proteins on the bacterial cell surface play an important role in the ability of pathogenic bacteria, such as *Xf*, to induce the disease state. During the past year, we have used one-dimensional gel electrophoresis to examine the *Xf* outer membrane profile and have assigned three proteins to specific genes on the *Xf* chromosome. We have also been developing a protocol for analyzing the *Xf* outer membrane proteome using two-dimensional gels. Once these technical details have been worked out, we will be in the position to examine how different physiological and environmental signals affect the relative abundance of specific *Xf* outer membrane proteins. This information should provide valuable insights into the role of the outer membrane proteins in *Xf* virulence and identify potential new targets that may help in the development of effective strategies for controlling the spread of PD.

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FUNDING AGENCIES

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

RESULTS

The primary focus of our research during this reporting period has been to analyze the outer membrane proteome of *Xf* and to assign the outer membrane proteins to specific genes on the *Xf* chromosome. In last year's Symposium Proceedings (6), we described our protocol for analyzing the protein profile of the *Xf* outer membrane. This protocol involves rupturing the *Xf* cells with a French pressure cell and isolating the outer membrane fractions by sucrose density gradient centrifugation. The proteins in this fraction are then analyzed using SDS-polyacrylamide (PAGE) gel electrophoresis. These gels have allowed us to quantitate the amount of the different proteins in the *Xf* outer membrane and to predict the sizes of the proteins based on their migration in the gels. Figure 1 shows a series of SDS-polyacrylamide gels, which reveal the outer membrane profile of *Xylella fastidiosa* strain Temecula 1. These Coomassie-stained gels indicate that there are at least 14-16 major proteins in the *Xf* outer membrane. The sizes of the outer membrane proteins range from 130K to 18K. (Proteins smaller than 18K would not have been detected in this series of experiments.)

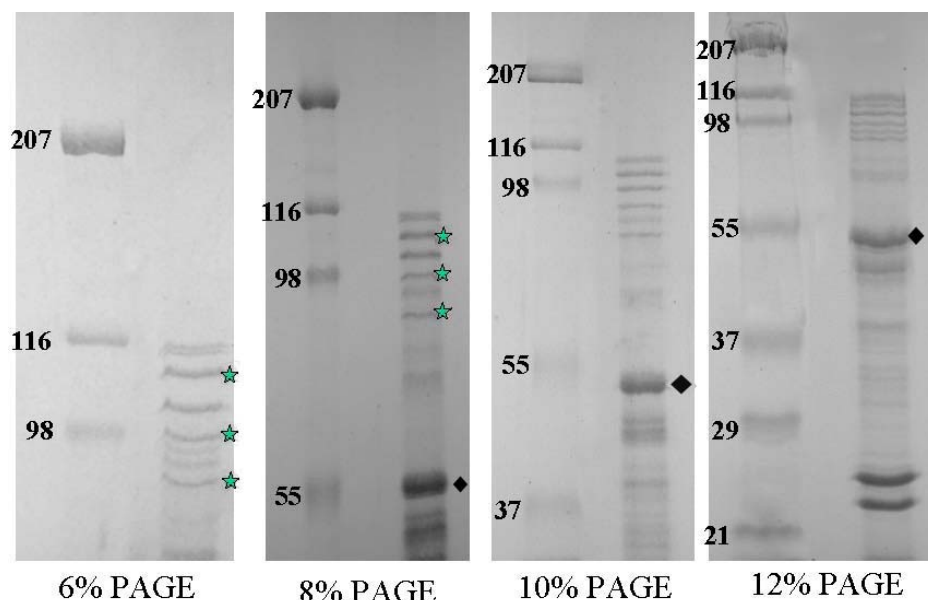


Figure 1: The outer membrane profile of *Xylella fastidiosa* strain Temecula 1.

Proteins in these gels were identified using Coomassie blue stain. The numbers indicate the size of molecular weight standards and their migration on the different percentage gels (left lane). On each gel, the outer membrane proteins from *Xf* Temecula 1 are present in the right lane. The diamonds indicate the location of the MopB protein on the different percentage gels. The stars indicate the locations of the three excised bands, which contained a unique protein based on the MALDI-TOF spectra.

The most abundant outer membrane protein is the MopB protein, which has been characterized by George Bruening and his colleagues (2). Using their purified MopB protein, we have been able to determine the location of the MopB protein relative to other proteins in our outer membrane profiles. (MopB is indicated by the diamonds in Figure 1). The next step in our analysis was to assign additional proteins to specific genes on the *Xf* genome. For these experiments, we separated the proteins in the outer membrane fractions on preparative SDS-PAGE gels and excised five distinct bands from the gels. The proteins in each band were then subjected to trypsin digestion and the resulting fragments were analyzed by MALDI-TOF-MS at the UC Davis Molecular Structure Facility. The resulting information was analyzed using MS-Fit at Protein Prospector (UCSF; <http://prospector.ucsf.edu>). Analysis of the bands at ~114K and ~104K indicated that more than one protein was present in the excised gel fragment. In contrast, the other three bands contained unique proteins. This allowed us to assign these three outer membrane proteins to specific genes on the *Xf* chromosome (10). The locations of the bands containing these proteins are indicated by the stars in Figure 1.

The largest of the three proteins is ~108K and corresponds to PD1283. PD1283 is predicted to encode a 958 amino acid protein and has been classified as a TonB-dependent receptor protein. The second protein is ~98K and corresponds to PD0326. PD0326 is predicted to encode a 784 amino acid protein and shows homology to the outer membrane protein/protective antigen OMA87. Based on this homology, PD0326 is also called the *oma* gene in some databases. The third protein is ~90K and corresponds to PD0528. Interestingly, this gene is classified in many databases as an inner membrane. However, our analysis of this protein using relatively new computer algorithms suggests that PD0528 encodes a beta barrel outer membrane protein (1). This assignment is more consistent with our fractionation results, which indicate that the PD0528 protein is a major component of our *Xf* outer membrane fraction.

Our analysis of the outer membrane fractions using one-dimensional (1-D) gels illustrates the validity and power of our approach for assigning outer membrane proteins to specific genes on the *Xf* chromosome. However, it was not possible to completely separate all of the outer membrane proteins using 1-D gels. To overcome this problem, we are analyzing our

THE *XYLELLA FASTIDIOSA* CELL SURFACE

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Reporting Period: The results reported here are from work conducted from October 1, 2003 to September 30, 2004.

ABSTRACT

A common response of Gram-negative bacteria to environmental stress is to change the composition of their cell surface, particularly the protein composition of their outer membrane. These changes are known to have a profound effect on the sensitivity of Gram-negative bacteria to detergents, antibiotics, and bacteriophages. The goal of this project is to determine how environmental changes influence the protein composition of the *Xylella fastidiosa* (*Xf*) outer membrane. Our strategy has been to isolate the outer membrane fraction from *Xf* cells grown under different environmental conditions. The proteins in this fraction are then separated by one- or two-dimensional gel electrophoresis and their identity established by peptide mass fingerprinting. In this report, I have focused on experiments that examine the *Xf* outer membrane protein profile using one-dimensional gel electrophoresis. This analysis has allowed us to assign three outer membrane proteins to specific genes on the *Xf* chromosome. These gels have also allowed us to examine how the composition of the *Xf* outer membrane changes in response to environmental signals and the physiological state of the bacterial cell.

INTRODUCTION

Pierce's disease (PD) is a devastating disease of grapevines that is caused by the Gram-negative, endophytic bacterium *Xylella fastidiosa* (*Xf*). Although the specific details of the disease process are not fully understood, an important feature is the ability of this pathogen to colonize the xylem tissue of plants and the foregut of insect vectors (for a recent review, see 5). As with most pathogenic bacteria, successful colonization is dependent on the ability of planktonic *Xf* cells to adhere to the host cell surface and to form a microcolony (3, 4, 7). This surface-associated growth commonly leads to the formation of a biofilm. Biofilm-associated *Xf* bacteria constitute a major component of the bacterial biomass in the host tissue. In contrast, planktonic bacteria are less prevalent and are seen primarily as a mechanism for the bacteria to translocate from one surface to another.

The transition of bacteria from the planktonic to the biofilm-associated state involves profound physiological changes (3). The most obvious change is the production of an exopolysaccharide matrix, one of the distinguishing characteristics of a bacterial biofilm. However, the matrix-enclosed mode of bacterial growth requires many other changes, including changes in the protein composition of the bacterial cell envelope. In Gram-negative bacteria, these changes include differences in both the relative abundance of some major outer membrane proteins and the appearance or disappearance of specific high-affinity receptor proteins. This differential expression allows the bacteria to cope with the new environmental condition and with alterations in the nutrient supply.

Changes in the protein composition of the outer membrane are known to have a profound effect on the sensitivity of Gram-negative bacteria to detergents, antibiotics, and bacteriophages (8). As a result, strategies designed to attack planktonic cells are usually not effective against biofilm-associated cells (3). Therefore, in order to develop effective methods for controlling the spread of *Xf*, it is important to obtain information concerning the protein composition of the *Xf* outer membrane and how the composition of this membrane changes in response to environmental signals and the physiological state of the bacterial cell.

OBJECTIVES

The goal of this project is to analyze the outer membrane proteome of *Xf* and to determine how the outer membrane protein profile changes in response to various physiological and environmental conditions. Our experiments are designed to address two objectives:

1. Identify the major outer membrane proteins of *Xf* and assign them to a specific gene on the *Xf* chromosome.
2. Determine how the protein composition of the *Xf* outer membrane is influenced by environmental signals and signals from the infected grapevine.

Specific Aim 2: Identify specific phage with potentially useful properties within our collection.

2A) Screen the collection to identify virulent phages.

2B) Screen the collection to identify generalizing transducing phages.

RESULTS AND CONCLUSIONS

The first goal of this project is to generate a collection of *Xf*-specific phages that exhibit different biological properties. To increase our chances of obtaining a diverse set of phages, we have collected samples from PD-infected grapevines growing in different vineyards in Northern California. Using infected grapevines as a source seems particularly promising based on the work of Dr. Lauzon and her colleagues (5). Our strategy has been to collect sap from infected vines and samples from the tissue of symptomatic plants. We have also collected soil samples from around infected grapevines to determine if the soil is a good source of *Xf*-specific phage. The next step in our analysis will be to determine if any of these samples contain phage that can infect *Xf*. As a starting point, we will use previously published protocols that have successfully been used to isolate phages from environmental samples for other Gram-negative bacteria.

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FUNDING AGENCIES

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ISOLATION OF BACTERIOPHAGES SPECIFIC FOR *XYLELLA FASTIDIOSA*

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Reporting Period: Funding for this project was received in September 2004.

ABSTRACT

This report gives an overview of the project. The goal of this project is to isolate a collection of viruses (phages) that can infect and replicate in *X. fastidiosa* (*Xf*). This collection will then be screened to identify phage exhibiting useful biological properties.

INTRODUCTION

The causative agent of Pierce's disease (PD) is the Gram-negative bacterium *Xylella fastidiosa* (*Xf*). *Xf* is highly specialized and is capable of multiplying in both the foregut of xylem-feeding insects, such as the glassy-winged sharpshooter and in the xylem system of the host plant (for recent reviews, see 4, 6, 7). The complex nature of the bacterial-host interactions that take place during the PD infectious cycle and the fastidious growth properties of *Xf* in the laboratory present a formidable challenge to researchers working with this bacterium. At present, there are only a few methods available to perform such basic operations as genetic exchange, mutant isolation, strain construction, and complementation. Further complications of working with *Xf* arise because of its slow generation time, its tendency to form aggregates, and its poor plating efficiency. Finally, few methods are available for disrupting the interaction between *Xf* and its hosts, which is a key component of the PD infectious cycle. As a result, there are currently no effective treatments to cure infected vines.

In other Gram-negative bacteria, bacteriophages, phage derivatives and phage components have played a major role in overcoming these issues (1, 3, 8). For example, phages have been used to move genetic markers between strains, for complementation, and as cloning vectors. In addition, phages have been used as diagnostic reagents to detect pathogenic bacteria, and as therapeutic agents in bacterial infections. Unfortunately, since not all phages possess exploitable properties, it is usually necessary to isolate a collection of phages that infect the bacteria of interest and then to screen the individual phages for desirable properties.

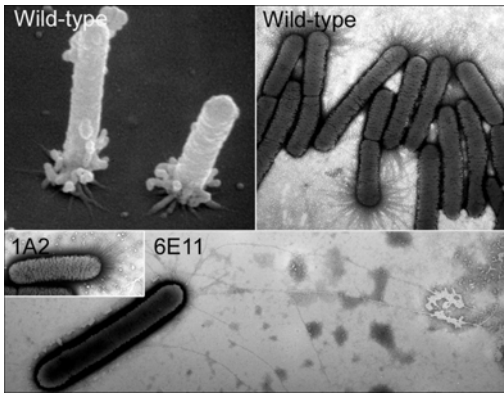
Based on studies of environmental samples, it has been estimated that there are $>10^{30}$ tailed phages in the biosphere and that phage typically outnumber bacterial cells 10 to 1 (2). These studies also revealed that phages could be found anywhere that their bacterial hosts are present. This observation has already proven true for *Xf*. Carol Lauzon and her colleagues have reported the presence of two phages associated with *Xf* from infected grapevines (5). The goal of this project is to isolate a collection of phages that are capable of infecting and replicating in *Xf* (Aim 1). These phages will then be screened individually to identify specific phages that have the potential to be used as genetic tools and for killing *Xf en planta* (Aim 2). Phages capable of moving genetic markers between *Xf* strains would give researchers in the field a powerful tool for investigating the properties of this unusual bacterium and establishing which parts of its genetic material make it such a deadly pathogen for certain varieties of grapes. Furthermore, phage or mixtures of phages capable of killing *Xf* would provide the tools necessary to determine the feasibility of using phage therapy to control the spread of PD.

OBJECTIVES

The primary goal of this project is to isolate a collection of phages as pure stocks and to screen this collection for phages that exhibit useful biological properties for studying and controlling the growth of *Xf*.

Specific Aim 1: Generate a collection of pure phage stocks that infect *Xf*.

- 1A) Collect environmental samples that potentially contain *Xf* specific phages.
- 1B) Isolate and obtain pure stocks of phages from the samples.



SEM and TEM of wild-type cells attached to the substratum at the pili-bearing polar ends. Mutant strains 1A2 and 6E11 depicting only short pili and only longer type-IV pili, respectively.

Electron microscopy substantiated the presence of polar pili on the wild-type and many of the mutant strains. Negative staining of TEM preparations of the wild-type strain revealed an abundance of pili, the majority of which were 0.4-1.0 μm in length with many additional filaments 1.0-5.8 μm in length. Mutant strains 1A2 and 5A7 had only the shorter class of pili, whereas strain 6E11 had predominantly long pili. The correlation between the presence of long and short pili on the wild-type *X. fastidiosa* strain, the occurrence of essentially only long pili on the twitching, biofilm-deficient strain (6E11), and the absence of long pili on the twitching-minus, biofilm-enhanced mutants (1A2 and 5A7), clearly relates to distinct functional roles for two length classes of pili.

CONCLUSIONS

Microfabricated fluidic chambers were created to mimic plant xylem vessels, in which we studied the non-flagellated *Xylella fastidiosa* bacterium. We discovered that the bacteria migrate 'upstream' by twitching motility, which explains, in part, how they are able to travel against the flow direction of xylem sap to invade non-colonized plant regions.

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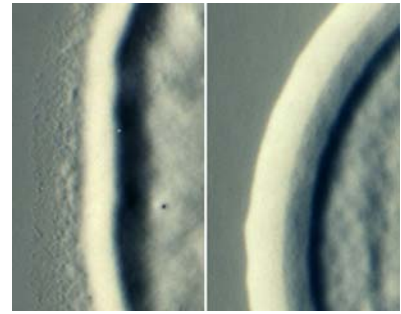
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FUNDING AGENCIES

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

Mutagenesis of *Xylella*

The EZ::TN Transposome system was used to generate *X. fastidiosa* mutants (Guilhabert et al., 2001). Two types of mutants were sought: biofilm modified mutants, and mutants deficient in ‘twitching’ (type-IV pili) movements. Ninety-six well polystyrene microtiter plates were used to screen for biofilm-modified mutants. The wild-type strain was used as a baseline control for biofilm development. Crystal violet, added to each well, served as an indicator for the presence of biofilm. Wells exhibiting either enhanced or decreased biofilm expression as compared to the wild-type strain were identified visually. Subsequently, biofilm development was assessed by dissolving similarly stained biofilms with DMSO and quantifying by absorbance (A620) in a microtiter plate reader. Screening for twitch minus mutants was performed on modified PW solid medium (Davis et al., 1981). Colonies with a peripheral fringe were designated as having a normal twitching phenotype characteristic of wild-type *X. fastidiosa*. Colonies lacking a peripheral fringe were designated as having a twitching defect.

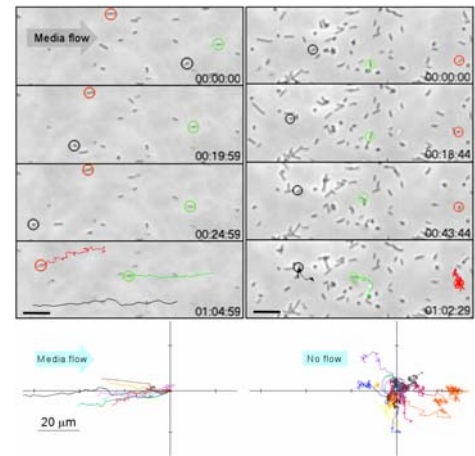


Light micrographs of wild-type and twitch-minus mutant (1A2) colonies on agar medium with and without a peripheral “fringe.”

Movement and Biofilm Development of *Xylella* Bacteria

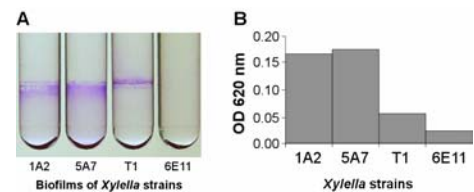
Wildtype *Xylella fastidiosa* (Temecula) exhibited a colony morphology, viz. fringed margin, consistent with twitching motility that is observed in other bacterial species. Time-lapse imaging of bacteria at the colony edge, revealed both individual bacteria and aggregates of cells that migrated between 0.01-0.32 $\mu\text{m min}^{-1}$, generally in a direction away from the colony periphery. When the bacteria were introduced into a microfluidic chamber, twitching movements propelled migration of individual cells in various directions depending on the rate and direction of medium flow. Under stagnant no-flow conditions, the cells exhibited no directional preference for migration. However, when the medium was passed through the chamber at approximately 20,000 $\mu\text{m min}^{-1}$ (volumetric flow rate = 0.20 $\mu\text{L min}^{-1}$), a rate comparable to grapevine xylem sap flow under high transpiration conditions (Braun and Schmid, 1999a; Braun and Schmid, 1999b; Lascano et al., 1992; Peuke, 2000), the bacteria migrated predominately against the direction of flow. Under both flow and no-flow conditions the cells were either prostrate on the substratum or, often they were erect and attached at one pole. Maximum twitching speed for *X. fastidiosa* cells examined under flow conditions was $4.9 \pm 1.1 \mu\text{m min}^{-1}$ ($n = 17$), a speed comparable to the observed rate of bacterial spread within grapevines assessed through destructive sampling (Newman et al., 2004).

(Also see, <http://www.nysaes.cornell.edu/pp/faculty/hoch/movies/>)



Light micrographs of time-lapse series depicting paths of three (circled red, green, black) wild-type twitching bacteria in microfluidic channels under flow (left) and no flow (right) conditions. Scale bar, 10 μm . Time (h:min:sec). Lower figure, cumulative twitching motility paths for 17 cells under corresponding conditions for 60 min, respectively.

A number of mutant strains were identified as twitching-minus mutants; two (1A2, 5A7) are reported here. Colony peripheries of 1A2 and 5A7 were well demarcated and without bacteria distinctly separated from the main colony mass (lack of peripheral fringe). Colony expansion for these two mutants occurred through repeated cell division and gradual spread as the cell mass increased. When examined in the microfluidic chambers, neither mutant strain exhibited migration, with or without medium flow. Both of these strains were biofilm enhanced. Another mutant, 6E11, was found to be biofilm deficient but still produced colonies with a peripheral fringe and exhibited active twitching, similar to that observed for the wild-type strain. Growth rates of all mutants were not significantly different from the wild-type strain. Sequence analysis of mutants 1A2, 5A7, and 6E11 indicated that transposon insertion occurred in ORFs PD1927, PD1691 and PD0062 of the Temecula genome corresponding to putative genes *pilB*, *pilQ*, and *fimA*, respectively. PilB is known to function as a nucleotide binding protein supplying energy for pilin subunit translocation and assembly, whereas PilQ is a multimeric outer membrane protein that forms gated pores, through which the pilus is extruded (Wall and Kaiser, 1999; Alm and Mattick, 1997; Strom and Lory, 1993). Mutants deficient in these proteins have smooth colony edge phenotypes, do not twitch, and are generally devoid of type IV pili (Kang et al., 2002; Huang and Whitchurch, 2003; Alm and Mattick, 1997; Strom and Lory, 1993). Disruption of *fimA* in *X. fastidiosa* (Feil et al., 2003) as well as in *E. coli* (Orndorff et al., 2004) indicates that the gene encodes for an essential protein of type-I pili that functions in surface attachment and biofilm formation.



Biofilm formation by *X. fastidiosa* wild-type (T1) and mutant strains 1A2, 5A7, and 6E11 following 7 days growth.

UNDERSTANDING *XYLELLA FASTIDIOSA* COLONIZATION AND COMMUNICATION IN XYLEM LUMINA

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Reporting Period: The results reported here are from work conducted from October 2003 to October 2004.

ABSTRACT

Microfluidic chambers were fabricated using photolithographic and soft-molding methods. The chambers were made to mimic the physical parameters of grape xylem vessels in which *Xylella* cells were studied temporally and spatially for colonization and biofilm development. *Xylella* bacteria were observed to migrate by 'twitching' motility against a rapid flowing medium in microfluidic chambers. Twitching motility is important in explaining how this pathogen is able to migrate against the flow of the plant's transpiration stream to colonize previously non-invaded xylem vessel regions. Mutant strains with insertions in *pilB*, *pilQ*, and *fimA* genes established the roles of short pili, and longer type IV pili in biofilm development and long distance migration of the bacterium.

INTRODUCTION

Pierce's disease of grape is generally recognized as being caused by restricted sap flow and resultant water stress due to plugging of xylem elements by live bacterial aggregates and associated mucilage. It is not clear whether the extracellular polymeric mucilage is of bacterial and/or plant origin. Based on the analysis of the complete genome sequence of *Xylella fastidiosa*, gums produced by the *X. fastidiosa* are similar to the 'xanthan gums' produced by *Xanthomonas campestris* pv *campestris*, although they may be less viscous (Simpson et al., 2000). In addition, tylose development in xylem vessels in response to the presence of the bacterium further restricts sap flow (Mollenhauer and Hopkins, 1976). These general concepts regarding *X. fastidiosa* pathogenicity are readily recognized; although, it is not understood how the bacteria become established in the turbulent habitat of a 'fluid conduit' i.e., xylem vessels and tracheae, to form colonies. In addition, how the bacteria are disseminated throughout the xylem vessels from insect-vector feeding sites has long been a particularly puzzling and important question. Long-distance intra-plant migration of the bacteria is even more perplexing since xylem sap flow is always down the pressure gradient, viz., with the transpiration stream that flows toward the leaf. Even under nocturnal conditions when leaf stomates are mostly closed, cuticular transpiration maintains sap flow toward the leaf, albeit at slower rates. Sap flow is seldom stagnant, and rarely, if ever, moves in a reverse direction away from the leaves. Since *X. fastidiosa* is a non-flagellated bacterium, one hypothesis for its ability to migrate against the normal flow of the plant's vascular system has been through the slow and incremental expansion of the bacterial colony through repeated cell division along xylem vessel walls. Another possibility is that occasional cavitation of the water column causes momentary reversal and short distance flow of the sap, thereby carrying the bacteria down the xylem elements. Neither of these scenarios satisfactorily explains colonization of upstream xylem regions.

Investigations conducted during the last research period concentrated on understanding biofilm development and how *Xylella* bacteria are able to colonize regions 'upstream' from their initial site of introduction. Toward this, we generated mutant strains to help answer these queries, and we used microfluidic chambers in which we were able to examine the temporal and spatial aspects of bacterial colonization.

OBJECTIVES

To understand how the physical parameters of xylem tracheae and vessels influence *Xylella fastidiosa* colonization. Toward this, we evaluated bacterial movement, colony formation, and biofilm development. Our approach has been to use microfabricated 'artificial' vessels that mimic topologies and chemistries of xylem vessels.

RESULTS

Development of Artificial Xylem Vessels (Microfluidic Chambers)

Microfluidic chambers were fabricated from polydimethylsiloxane (PDMS), supported by a microscope slide with the channel side sealed with an air plasma treated cover glass. The mold for the PDMS device was prepared in silicon wafers using photolithographic procedures. 'In' and 'out' ports and tubing were sealed to the microfluidic chamber. Flow of media through the chambers was facilitated with a syringe pump. Chamber dimensions were as previously reported, but generally were 50-100 μm in width and depth, and up to 14 cm in length.

CONCLUSIONS

Genetic resistance and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's Disease/GWSS Research Program. The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that, when expressed in grape, will disrupt infection, spread or symptom development by *Xf*. Published information from our laboratory established that specific transgenes from homologous or heterologous hosts that block programmed cell death (PCD) (1) during plant disease development (4), can arrest both symptom development and microbial growth *in planta* in a range of plant-microbe interactions (3, 4, 5). PCD is now considered as a key pathway involving many gene products in numerous diseases of animals and plants. Blockage of PCD can be achieved by expression of anti-apoptotic transgenes, RNAi suppression of endogenous genes, and by chemical inhibitors of PCD. Significantly we demonstrated that expression of the anti-apoptotic p35 gene in transgenic grape tissue blocked cell death and PD symptoms in *Xf* infected tissue. We believe that examination of the molecular basis of cell death in symptomatic tissues will be very informative in the short run in terms of PD biology and physiology. In a longer time frame these data will likely yield genetic or chemical strategies for protection of grape against infection by *Xf* in years not decades.

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FUNDING AGENCIES

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

Table 1. “Short list” of plant anti-apoptotic genes, derived from functional screen of cDNA libraries, for transformation into grape

Name	ID (putative)
404	empty vector
P35	baculovirus p35
G8	glutathione-S-transferase
G71	cytokine-like protein
P14LD	pathogenesis related gene secretory form
P14	pathogenesis related gene non-secretory form
MT	metallothionine
Y376	mycorrhiza up regulated gene
Y456	nematode up regulated gene

It is important to emphasize that this screen is not dependent on the presence or role of PCD in PD but will detect any gene that affects the integrity of the bacterium in the infected tissue or the ability of the bacterium to elicit symptoms of PD, regardless of whether the step being affected is strictly dependent on the induction of PCD.

Two of the genes (P14LD and Y456) were constitutively expressed in grape by *A. rhizogenes* transformation. The transformed roots were protected against *Xf*-induced death, as were those *Xf*-infected grape explants from which the emerging grape roots transformed with the p35 gene. This indicates a role for PCD in PD and provides optimism that novel genetic determinants of resistance can be identified using this screen. Given the strategies used it is likely the genes will function in grape by altering the effect of *Xf* infection in grape through suppression of symptoms either directly on cell death or indirectly by modifying the behavior of the bacterial in the xylem. It should be emphasized that the effect of anti-apoptotic transgenes on plants is not to induce so-called systemic acquired resistance (SAR) as no markers of SAR are induced in the presence of anti-apoptotic genes such as the p35 gene (4) nor were they observed in the case of the P14LD and Y456.

Our goal is to rapidly identify resistance genes in grape genotypes that block any one of several required steps in the Infection and spread of *Xf* in the xylem, steps which logically will include genetic factors regulating PCD induced by disease stress in grape. We have begun to evaluate the effect of experimental transgenes both from tomato and from grape on grape tissue bearing GFP-*Xf* in xylem elements with various cell death markers and GFP-marked bacteria. By using the GFP-tagged *Xf*, this also is a direct functional assay for genes that block bacterial movement or accumulation in the xylem of newly differentiated grape tissue (6). Of particular interest is the possibility that PD blocking signals initiated with transgenes may move systemically through the vascular system from transformed rootstocks to upper regions of grafted cultivated grape tissue affording protection against systemic movement or activity of *Xf* without genetically engineering the cultivated grape. To this end, the MP plants provide an excellent experimental system by which transformed roots can be initiated on untransformed shoots. The fact that these transformed roots can be formed within 4-6 weeks means that any gene that protects roots can quickly be evaluated for systemic protection; protection from a transformed root stock (in the real world) to an untransformed susceptible fruit producing scion illustrated in Figure 3 above.



RNA Induced Gene Silencing (RNAi)

This same system will enable us to explore the potential for using RNA induced gene silencing (RNAi) (7,9), the expressed silencing small RNA molecules, comprised of small (21 bp) sequences derived from the gene to be silenced, are known to move systemically throughout the plant (8) and silence transgenes from roots to scions. The RNAi from RNAs expressed in the roots have the potential to silence any gene from our project or from other labs that is induced in either susceptible or resistant responses, and deemed to have a definitive role in disease. The small mobile silencing RNAs further have the potential to move systemically in the plant (8) to silence genetic determinants of susceptibility. If either signals from the transgenic roots (from cDNA library screen) or roots expressing RNAi were to provide protection against PD, the best case scenario would be to simply graft a transformed shoot onto an existing infected plant and block the disease without transforming either the roots or the scion. To this end we have developed a plant transformation vector capable of expressing a hairpin RNA. As proof of concept we have used this vector to construct a GFP RNAi expression vector and have shown it is capable of knocking out GFP expression in transient assays. We are currently using *A. rhizogenes* to produce GFP RNAi roots on GFP-expressing transgenic grape shoots to explore the ability of transgenic roots to knock out expression in the shoot.

The research discussed herein has been reported at the Pierce’s Disease Symposium in San Diego and in annual reports to the CDFA Pierce’s Disease/GWSS Research Program. Manuscripts are being prepared on the various screens developed for the cDNA libraries and the construction of the libraries.

RESULTS

Construction of cDNA Libraries

The construction of a grape cDNA library initially proved much more difficult than we had experienced in making libraries from 4 other plant species. Isolation of mRNA was not difficult but the grape tissue contains high levels of phenolic compounds in an oxidative environment that contaminate the RNA, rendering it difficult to reverse transcribe. We now have an efficient protocol for generating full-length cDNA libraries from grape using an antioxidant cocktail during homogenization and CsCl gradient purification of RNA. The Hanes City (*V. shottworthii*) and Chardonnay libraries are completed with 300,000 members each with an average insert size of 1000 bases. The tissue source was field grown plants provided by Dr. Walker. The susceptible Chardonnay is used as a recipient host to screen cDNA libraries. We have begun screening these libraries while continuing to develop libraries from Cowart (*M. rotundifolia*) and Dr. Walker's resistant tester line 8909-15. The inserts for all libraries are cloned into the binary vector B5 for direct transformation into the *A. rhizogenes* functional screen in Chardonnay and a transient assay. The transient assay is based on a leaf infiltration approach that we have used successfully for tomato and tobacco disease assays of putative resistance genes. For transient assays, selected cDNA inserts in the B5 vector are used to transform *Agrobacterium tumefaciens* strain GV2260. The resulting GV2260 transformed bacteria are then pressure infiltrated into attached pre-symptomatic leaves of *Xf* infected MP plants. The ability of the expressed gene to inhibit symptoms is then evaluated. As potential cloned resistance genes become available they also will be used to identify homologues from the Chardonnay cDNA library that may provide resistance by simple alteration in expression level within the homologous host in a time and tissue specific manner. These full-length cDNA libraries are available to all grape researchers in this program.

Screening of cDNA Libraries

The *Agrobacterium rhizogenes*-based transformation procedure results in the induction of transformed roots from infected (or healthy) vegetative tissue sections following co-cultivation with the transforming bacteria. Each emerging root is an independent transformation event, contains a single new DNA insert from which the transgene can be re-isolated by PCR for characterization. Figure 1 (below) illustrates the successful transformation of all emerging roots from a grape stem explant



Figure 1

with the green fluorescent protein (GFP). This technique is a functional cDNA library screen (each root contains a different cDNA library member) for genes from grape libraries that block either bacterial multiplication, movement, or symptom expression. We previously determined that viable roots do not form on host tissue explants that are infected with *Xf* unless protected by transgenes. The genes that will be identified will be those that directly affect the ability of the pathogen to cause disease and are not dependent on DNA sequence relationships. The library is being screened in sets of 50,000 cDNAs to improve the efficiency in terms of handling

numbers of symptom blocking cDNAs. Based on previous experience with tomato, we expect that less than 0.01% of the cDNAs will effectively protect against PCD and/or the disease development. This underscores the need for a highly effective functional screen. In order to provide sufficient *Xf*-infected tissue of similar physiological state for transformation, we developed a micro-propagation (MP) technique for producing clones of sterile grape plants in small plastic boxes that can be inoculated with *Xf* under sterile conditions illustrated in Figure 2 at the right. The MP plants show foliar symptoms typical of infected plants under field and greenhouse conditions (See leaf in foreground). Plants produced under these same conditions also are the source of *Xf* infected stem sections used for transformation in the *A. rhizogenes* functional screen.

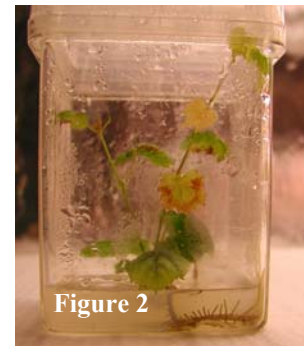


Figure 2

The major advantage of the MP plants is that they are much more efficiently transformed than the greenhouse-derived tissue, which tends to be more highly lignified and produces fewer transformed roots. As a means of fast tracking the cDNA screen while optimizing the grape transformation procedure, we have screened approximately 30,000 members of the Chardonnay cDNA library by *A. rhizogenes* transformation of tomato cotyledons. The resulting roots were subject to disease-dependent PCD induction by treatment with the pathogenic toxin FB1 (1, 2). PCR was used to amplify the Chardonnay cDNA insertion from the surviving tomato roots. The cDNA inserts were then cloned and sequenced. Using this analysis of the Chardonnay cDNA library, we so far have found several grape full-length cDNAs (encoding open reading frames) that protect tomato roots from disease-linked programmed cell death (PCD), a death process that is functionally equivalent to the death of cells in *Xf* infected grape. These grape genes are now being re-evaluated in the *A. rhizogenes*-grape system for protection of Chardonnay grape tissue against symptoms due to the presence of *Xf* in the xylem. Several potentially protecting cDNAs that protect roots are now in the queue to produce whole plant transgenics by the UCD Plant Transformation Facility (Table 1). The expression of these genes in the protected roots was confirmed by northern analysis (unpublished). Most of these genes share sequences homologous with animal genes known to block disease-linked PCD.

SCREENING OF GRAPE CDNA LIBRARIES AND FUNCTIONAL TESTING OF GENES CONFERRING RESISTANCE TO PIERCE'S DISEASE

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Reporting Period: The results reported here are from work conducted from October 1, 2003 to October 1, 2004.

ABSTRACT

Our overall objective is to identify genes from cDNA libraries of either grape or heterologous plants that, when induced in grape, will disrupt infection, spread or symptom development by the xylem-limited bacteria, *Xylella fastidiosa* (*Xf*). We are interested in the effect of the genetic disruption of PD symptoms on the movement or establishment of the bacterium in the xylem of susceptible grape plants. Specific objectives are to: a) create cDNA libraries from several different grape backgrounds, including three with PD resistance; b) develop a functional *A. rhizogenes*-based cDNA screen in grape; and c) investigate the potential of blocking PD symptom expression and disease impact with anti-PCD (anti-apoptotic) transgenes. To these ends we have created full-length cDNA libraries from resistant and susceptible grape and developed an *Agrobacterium rhizogenes*-based transformation procedure that provides a functional screen for genes that alter the disease phenotype. Transformation of grape explants with *A. rhizogenes* results in the emergence of a transformed root containing a single new DNA insert, from which the transgene can be re-isolated for characterization. The identified genes will be those that directly affect the ability of the pathogen to cause disease and is not dependent on DNA sequence relationships. Pathogenicity tests with any isolated disease-disrupting cDNA will first involve a transient expression system using micro-propagated (MP) plants that are vegetative clones of sterile grape plants in small plastic boxes that can be infected with *Xf* under sterile conditions. This ensures that these plants will have uniform physiology without confounding by stress inductions as would likely occur in the field or greenhouse grown plants. The MP plants show foliar symptoms typical of infected plants under field and greenhouse conditions. Transient assays with test genes involve infiltration of *A. tumefaciens* containing the gene of interest into MP leaf tissue. The bacteria transfer the test gene into leaf cells that are presymptomatic will determine if the expression of the transgene in the leaf can block PD symptoms.

INTRODUCTION:

Published information from our laboratories confirms that specific transgenes from homologous or heterologous plants, that block PCD during plant disease development (4), as well as chemical inhibitors of apoptotic proteases (3), can arrest both symptom development and microbial growth *in planta* in a range of plant-microbe interactions (3, 4, 5). The conserved genetically determined PCD process can be studied by biochemical, cytological and genetic techniques and can be transgenically manipulated by techniques developed in our laboratory (3, 4). Based on previous results we tested the effect of the p35 transgene from baculovirus on viability of roots, produced on *Xf* infected chardonnay and observed protection of the roots against death in the presence of *Xf*. We believe that the effect of specifically expressing anti-apoptotic transgenes in PD infected tissues on the development of death-related symptoms in grape will contribute significant information in terms of PD biology and physiology. In a longer time frame these data will likely yield genetic or chemical-based signaling strategies for protection of grape against infection by *Xf* in years not decades, perhaps similar to the effects we reported previously in tomato (4).

OBJECTIVES

1. Construct cDNA libraries from several different grape backgrounds including from lines with PD resistance and from infected and uninfected grape tissue.
2. Conduct functional *A. rhizogenes*-based cDNA screen and clone genes that give altered phenotype in grape.
3. Evaluate specific anti-apoptotic plant genes in grape for effect on *Xf* and PD symptoms.
4. Determine the potential of blocking PD symptom expression with anti-apoptotic transgenes through chemical induction of such genes in transgenic grape tissue or by tissue-specific expression in roots or vegetative tissue of *Xf* infected grapes.
5. Use a combination of genetic and signal molecule discovery tactics to elucidation of the molecular basis of susceptibility

III. Production of transgenic plants and plant tissues of grape and application of transient assay of promoters

We will employ three different but functionally related approaches to testing and characterizing the isolated promoter regions indicated above. All three of the approaches described below will be initiated simultaneously in the interest of time. Each of the promoters of the four genes will be assembled in several different configurations with the reporter gene (GFP) and will be evaluated in conjunction with a constitutive promoter (CaMV 35S or FMV 34S) giving a total number of 40 transgene constructs. Total costs will be minimized by terminating any of the whole plant transformants bearing promoter constructs that are demonstrated by the transient or *A. rhizogenes* assays to be unresponsive to the presence of *Xf*.

- A. Stable, full-plant grape transformation will be provided on a recharge basis by the Ralph M. Parsons Foundation Plant Transformation Facility at the rate of \$2,000 per construct. This facility is located at UC Davis as a service oriented facility dedicated to providing cost effective plant transformation services for the University of California system and outside academic and industrial partners.
- B. Transient and root-specific stable transformations will be used for rapid identification of promoter specificity and relative strength. The intent is to decrease the number of whole plant transformations that need to be conducted -- because whole plant transformation is labor intensive, time consuming and expensive. The transient assays using *Agrobacterium tumefaciens* and the root transformations by *A. rhizogenes*, bearing the test promoters and marker genes, will be conducted by techniques that have used successfully for several years in the Gilchrist Lab.
- C. *A. rhizogenes*-derived root transformations will be used for initial assay of the expression of transgenes in differentiated tissue with vascular connections to *Xf*-infected stem sections. *A. rhizogenes* effects stable transformation of plant tissues by transferring genes of interest to intact plants under controlled conditions. The inoculation with *A. rhizogenes* bearing a gene of interest leads directly to the formation of transformed roots, which appear within 2-3 weeks and at which point the pathogen can be introduced into the assay system. Our procedure will be to introduce the putative promoter sequence, coupled to GFP, into grape roots via transformation as indicated above. Our recent data obtained with the *Xf*-GFP indicates that the bacteria can move both up and down from the site of infection. Hence, the presence of the bacteria, either directly placed in the transformed tissue with the putative promoter constructs have a chance of responding to the direct presence of *Xf* (in the roots) or to distal signals from bacteria present in the stem. Not only will these assays indicate *Xf* responsive promoters, some information on the strength of the promoters but whether they are responsive to distance signals also. These are all procedures that have been developed in our lab with grape as recipient host tissues.

IV. Characterization of GFP expression during *Xylella* infection and leafhopper feeding to identify desired promoter specificities.

Confocal Microscopy. Real time, non-destructive images of the isolated promoters driving the expression of GFP in grape plants will be obtained using a laser activated confocal microscope (BioRad MRC1024) by excitation at 488nm with a Krypton/Argon 15 mW laser. The use of the laser allows non-destructive GFP detection in intact plant leaves and roots. For stem imaging, hand sectioning will be used. Three different fluorescent emissions can be detected simultaneously depending on the filter set used. Current configuration is with the following three filters: (emission filter 578nm-618nm); (emission filter 506nm-538nm); and (emission filter 664nm-696nm).

The first characterized promoters are expected to be available beginning in February 2006 with the final characterization and methods for expression completed by May of 2006. All promoters and characterization details will be available for research purposes at the conclusion of the two-year project.

CONCLUSIONS

The research envisioned will be accomplished by combining expertise and materials from two laboratories, active in PD research, to isolate and characterize PD-responsive promoters from grape. The current project led by Dr. Cook has already identified several genes that are expressed strongly in *Xylella*-infected tissues, but not in healthy counterparts. The project led by Dr. Gilchrist has developed both a transient leaf-based and a stable root-based grape assay and has identified putative anti-PD genes from grape. We are poised to isolate the promoters of the PD-responsive genes from BAC genomic DNA libraries of Cabernet Sauvignon in the Cook lab and functionally test them by techniques used in the Gilchrist lab.

FUNDING AGENCIES

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

expression and the deleterious effect of the disease. Moreover, the recent development of *Xf*-GFP strains by Dr. Steven Lindow at UC Berkeley offers the possibility of dual labeling to simultaneously monitor pathogen spatial distribution and host gene expression. Such dual labeling experiments are made possible by the availability of multiple forms of GFP protein engineered to fluoresce with distinct spectral characteristics. It is conceivable, for example, that host genes might be induced specifically in live cells, adjacent to sites of pathogen colonization of xylem elements, and this technology would provide the means to test such hypotheses.

OBJECTIVES

1. Identify and determine sequence of promoters driving genes specifically transcribed in grape tissue or cells of plants infected with *Xf*.
2. Construct transformation-ready vectors containing *Vitis* promoter-GFP reporter gene fusions that will be used for the functional assay of putative promoters. (GFP=green fluorescent protein) identified in (1)
3. Conduct transient functional assays of the promoter-GFP fusions in stems, leaves and roots infected with *Xf*.
4. Produce stable transgenic grape plants with promoters that functioned effectively in the transient assays and characterize the strength of the selected promoters using the GFP-reporter
5. Distribute promoters to Pierce's Disease research community to facilitate characterization of cloned grape genes suspected to be involved in PD susceptibility or resistant to *Xf*. These promoters will have application in situations where the goal is to either up- or down-regulate expression of a specific gene-of-interest; the latter by localized expression of anti-sense gene constructs.

RESULTS

Since this project just began October 1, 2004, there are few results to report. We have employed a postdoctoral researcher and are currently sequencing the BAC clones indicated in the objectives.

Experimental Procedures to Accomplish Objectives

I. DNA Sequencing and promoter identification:

A. Isolation and characterization BAC clones containing the Xylella-induced genes.

Bacterial Artificial Chromosome (BAC) libraries of *V. vinifera* are available as high density filters for gene identification in grapes through the UC Davis CA&ES Genomics Facility (<http://cgf.ucdavis.edu/>). High-density filter sets of the library were used for hybridization with ³²P-labeled probes corresponding to four *Xylella*-induced transcripts. A combination of restriction enzyme fingerprinting and DNA sequencing of BAC-derived PCR products was used to determine that each probe hybridized to a single genomic locus containing the gene of interest. One BAC clone was selected for each transcript and used to prepare a sheared BAC sublibrary, which is currently being subject to random shotgun sequencing.

B. Sequence the BAC clones to completion.

Although our specific interest is in sequences immediately 5' and 3' to the candidate genes (maximum 10 kbp) we will sequence regions beyond where we believe the promoters to reside. The rationale derives from efficiencies and strategies of modern sequencing techniques; it is both faster and more cost effective to use the BAC shotgun strategy described below which automatically provides additional sequence information for less cost than if we were to attempt to focus on shorter regions immediately adjacent to either end of the candidate genes.

C. Identify 5' promoter regions in the sequenced genomic clones based on comparison to cDNA sequences currently in hand for the four genes.

We have complete cDNA sequences for each of the candidate genes that will facilitate annotation of the BAC clones and identification of regions immediately upstream and downstream of the transcription units. As described below, we will use PCR to isolate and clone these 5' and 3' regulatory sequences into transformation ready vector constructs (see below). Generally, we anticipate using conventional 3' terminators, such as that from the *Agrobacterium* octopine synthase gene (*ocs*). However, one of the candidate genes (a small auxin upregulated, *saur*, mRNA homolog) is predicted to confer post-transcriptional regulatory properties that may be involved in *Xylella*-specific RNA levels. Thus, we will clone the 3' region of this candidate gene and incorporate its structure into a subset of the transgene constructs described below.

II. Construct transformation-ready vectors systems containing *Vitis* promoters fused to GFP.

A set of plasmids has been constructed previously that allows the rapid assembly of novel binary plasmids in *E. coli*. One is a low copy backbone plasmid with elements from *Agrobacterium*; the second is a high copy *E. coli* plasmid containing a cassette of T-DNA elements; and the third is a high copy *E. coli* plasmid comprised of a linker and many unique restriction sites for ease of cloning the several classes of sequences to be recovered and tested. These plasmids will be used to construct a collection of binary vectors containing grape 5' promoters and 3' sequences for expression of GFP genes. Analysis of the sequence of the appropriate BAC clones will allow the design of PCR primers to amplify and clone the 5' promoter and 3' sequences of the transcriptionally regulated grape genes into novel binary vectors. (Details of the plasmids are available upon request.)

ISOLATION AND FUNCTIONAL TESTING OF PIERCE'S DISEASE-SPECIFIC PROMOTERS FROM GRAPE

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Reporting Period: This two-year project was initiated on October 1, 2004. Obviously, there are few results to report at this time. Only a discussion of the justification, objectives, and timetable will be presented per request by the Pierce's Disease Symposium organizers.

ABSTRACT

Among the potential solutions to Pierce's disease in grapes are approaches based on gene transfer technology that focus on understanding the underlying biochemical and molecular mechanisms regulating PD. One of the research priorities identified by the 2003 PD/GWSS project reviews and as indicated in the 2004 RFP was the need to identify, clone and characterize unique DNA sequences that specifically regulate the expression of grape genes in tissues that are infected with *Xf*. Emphasis was placed on the urgency and practical utility of isolating promoters of PD responsive genes. One of the major bottlenecks in using transgenes, either expressed as proteins or as inhibiting RNAs in grape (or any plant) is the lack of suitable promoters to specifically drive the expression of a transgene on a specific trait (susceptibility to PD) in particular tissues (e.g., vascular tissue) or in response to particular situations (e.g., sharpshooter feeding or *Xylella* infection). In the absence of tissue or response-specific promoters, transgenic strategies to either understand or control PD one can use only so-called constitutive promoters. The basic problem associated with the use of constitutive promoters is that the transgene is expressed in all cells all the time, not just in the tissue or cells where the gene is needed. Highly controlled induction is needed if the interest is in altering gene expression to avoid a cellular change (disease) that is initiated in one or a few isolated cells. The isolation and characterization of *Xf*-responsive promoters has immediate and direct application to several current PD projects that are studying the biochemical or molecular genetic basis of PD at the cellular and tissue levels in grape. It also is of practical importance that these promoters will be useful in either the up- or down-regulation of the expression of a specific gene-of-interest. The difference in presence or absence of the target gene product is determined by whether the promoter is used to drive a sense or an anti-sense construct of the gene of interest.

INTRODUCTION

The objective of promoter analysis is to identify and characterize cis-acting DNA (adjacent) sequences that, when induced, regulate PD-associated gene expression in grapes. Although regulatory sequences frequently occur just upstream of the transcription start site, they can also be found much further upstream. Transcript abundance can also be controlled post-transcriptionally, often by cis-acting sequences in the 3' untranslated region of a gene. Thus, the challenge in our studies is to demonstrate that the cis-acting sequences have a unique functional role in PD symptom development. It is not the goal of this proposal to understand mechanisms of transcriptional regulation, but rather to isolate and confirm sequences that are active in the regulation of gene expression when *Xf* is present as an inducer of a select set of genes.

To test whether a particular DNA sequence, that lies adjacent to a gene of interest, is involved in the regulation of that gene, it is necessary to introduce such putative regulatory sequences into a cell and then determine if they are activated when the inducer (in our case, *Xf*) is introduced into the system. This is done by combining a regulatory sequence with a reporter sequence (in our case, GFP) that can be used to monitor the effect of the regulatory (promoter) sequences in the presence of *Xf*.

We have identified a set of plant genes whose expression is correlated with infection by *Xylella fastidiosa* as part of a recent study of expressed sequence tags from *Xf*-infected and healthy *V. vinifera* plants in the Napa Valley. The genes are essentially off (silent) in plants that have not been exposed to the pathogen, but strongly induced in both natural field infections and greenhouse inoculated plants. Three of these genes are induced early during disease development, prior to the occurrence of symptoms, while the fourth gene is induced in symptomatic tissues only.

In addition to their utility for engineering PD resistance in grape, the advent of *Xf*-induced reporter gene expression would provide an extremely powerful tool to examine other host responses in their intact cellular and tissue context. With such tools, it should be possible to examine the chemical and/or physical cues from the insect or pathogen that trigger host gene

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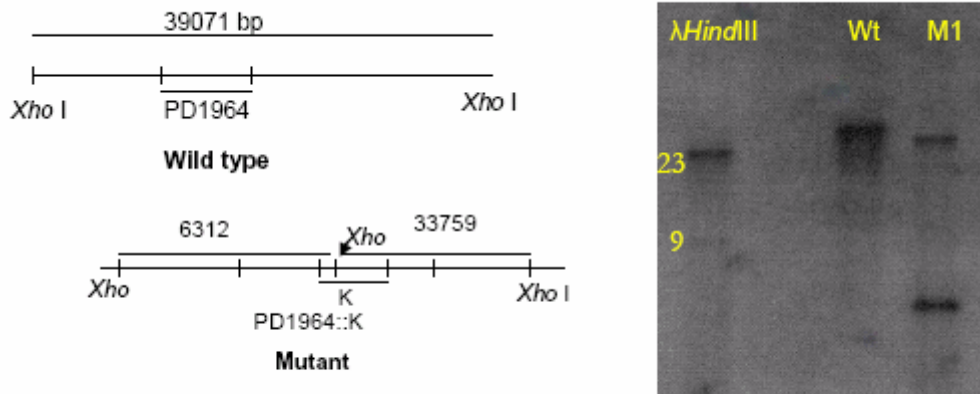


Figure 1: Southern blot of *tolC::nptII* mutant (M1) and wild type total DNA cut with *XhoI*. *XHO I* is internal to the *nptII* gene. The probe was PD1964 (wild type *tolC* from Temecula, 1459 bp).

For complementation assays, PD1964 was amplified by PCR, cloned into pGEM-T, verified by sequencing and sub-cloned into pUFR47, a wide host range replicon based on *repW* (DeFeyer et al., 1993) and pBBR1MCS-5, a wide host range replicon based on a *Bordatella* replication origin (Kovach et al., 1995). pUFR47 and pBBR1MCS-5 containing the entire *tolC* gene are referred as pJR13.2 and pJR22.2 respectively. Non-pathogenic Temecula mutant M1 was transformed with pJR13.2 and pJR22.2 independently by electroporation as described above. The cells were recovered in 1 ml of PD3 broth for 6 hours and were spread on PD3 plates amended with Gentamycin (5 µg/ml). The plates were incubated at 28 °C for 10 days and single colonies were screened for the presence of pJR13.2 /pJR22.2 and also for the integrity of *nptII* integration, by PCR assay. Grape plants (var Carnignane) were inoculated in triplicates with wild-type *X. fastidiosa* Temecula, mutant M1, M1/pJR13.2, and M1/pJR22.2 strains and are currently being monitored for Pierce’s disease symptoms. Preliminary results indicate possible complementation using both vectors. These results need to be repeated and confirmed, and these tests are currently in progress.



Figure 2: Grape var. Carnignane 90 days after inoculation with wild type Temecula (left) and *tolC::nptII* mutant M1 (right).

CONCLUSIONS

Type I secretion gene *tolC* (PD1964) of *X. fastidiosa* Temecula was disrupted by marker exchange mutagenesis. The mutant strains lost all pathogenicity, indicating a critical role of *tolC* in pathogenicity of *X. fastidiosa* on grape. Complementation assays are in progress and could result in a demonstration of a role of *tolC* in pathogenicity. If such a role can be confirmed, it would indicate several important molecular targets for potential PD control methods.

ROLE OF TYPE I SECRETION IN PIERCE'S DISEASE

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Reporting period: The results reported here are from work conducted from November 8, 2003 to October 31, 2004.

ABSTRACT

Xylella fastidiosa Temecula sequence information reveals no type III, but two type I secretion systems, both dependent on a single *tolC* homologue. Marker exchange mutagenesis using pGEM-T as delivery vector and *nptII* as marker was employed to generate *tolC* disruptions. PCR and Southern blot analyses confirmed marker exchange at the *tolC* locus. Grape (var. Carignane) plants inoculated with mutant (*tolC::nptII*) strains exhibited no symptoms of PD, indicating that pathogenic ability of PD strains may be dependant on *tolC* and type I secretion. Complementation assays using *tolC* in the mutant strains are in progress to help confirm this hypothesis.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a xylem-inhabiting Gram-negative bacterium that causes serious diseases in a wide range of plant species (Purcell & Hopkins, 1996). Two of the most serious of these are Pierce's Disease (PD) of grape and Citrus Variegated Chlorosis (CVC). The entire genomes of both PD and CVC have been sequenced (Simpson et al., 2000). Availability of the complete genomic DNA sequence of both a PD and a CVC strain of *Xf* should allow rapid determination of the roles played by genes suspected of conditioning pathogenicity of CVC and/or PD. For example, analyses of the CVC and PD genomes showed that there was no type III secretion system, but there were at least two complete type I secretion systems present, together with multiple genes encoding type I effectors in the RTX (repeats in toxin) family of protein toxins, including bacteriocins and hemolysins. RTX proteins form pores in lipid bilayers of many prokaryotic and eukaryotic species and cell types; at least one is associated with pathogenicity in plants. However, lack of useful DNA cloning vectors and/or techniques for working with either CVC or PD strains have impeded progress in functional genomics analyses. Last year we focused on attempts to perform marker-interruption in the PD strains using various suicide vectors and techniques. Although marker-interruption using suicide vectors is normally an efficient, single crossover event in many bacteria, repeated marker-interruption attempts with *X. fastidiosa* in our lab and in others have failed (Feil et al., 2003; Gaurivaud et al., 2001; Guilhabert et al., 2001). Since marker-exchange has now been reported to be successful with *X. fastidiosa* (Feil et al., 2003), we report here the utility of marker-exchange to generate *tolC* interruption in *X. fastidiosa* PD strain and the role of *tolC* in pathogenicity.

OBJECTIVES

The primary objective of this work is to determine the effect of type I secretion gene knockouts on pathogenicity of a PD strain on grape.

RESULTS

X. fastidiosa strain Temecula (Guilhabert, 2001), was grown in PD3 (Davis et al., 1981) and confirmed to be pathogenic on Madagascar periwinkle and Grape (var. Carnignane). Symptoms appeared after 2 months. Marker-exchange mutagenesis of *tolC* was performed using pJR6.3. This plasmid carries an internal fragment of PD1964 (*tolC* of Temecula) interrupted at an internal *BamHI* site by an *nptII* gene from pKLN18 (kindly provided by K. Newman and S. Lindow). One microgram of pJR6.3 DNA was used to transform electrocompetent cells (prepared by washing 10 ml of four day old PD3 broth culture of *X. fastidiosa* Temecula, serially with 10, 5, 2 ml of ice-cold deionized water and resuspending in 100 μ l the same) by electroporation (1mm gap cuvettes; 1800 volts). Electroporated cells were allowed to recover in 1 ml of PD3 broth for 24 hours at 28 °C and were spread on PD3 plates amended with kanamycin (50 μ g/ml). Plates were incubated at 28 °C for 10 days and single colonies were screened for interruption of *tolC* by PCR analysis and by Southern blot hybridization. The results (Figure 1) indicate that *tolC* gene can be disrupted and marker-exchange was efficient in generating gene-disruptions in *X. fastidiosa*.

Plant inoculation assays were performed in collaboration with Dr. Don Hopkins, at the Mid-Florida Research and Education Center, Apopka, Florida. Grape plants (var. Carnignae) were inoculated with the wild-type *X. fastidiosa* Temecula strain and the mutant (*tolC::nptII*) strain in triplicates. The plants were maintained under green-house conditions and were evaluated for Pierce's disease symptoms at 60 and 90 days after inoculation. The results (Figure 2) showed loss of pathogenicity of *X. fastidiosa tolC::nptII* mutants on grapes. All the three plants inoculated with the wild-type Temecula strain exhibited typical PD.

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with RST-31 and RST-33 was also examined, and found to detect 6.5×10^{-5} $\mu\text{g/mL}$ of DNA. In addition, an internal set of primers was developed so that nested PCR is now possible for samples appearing negative with traditional methods.

Pathogen Strain

A simple assay was conducted to categorize *Xf* by its common strains. Recent genetic and cross-inoculation studies showed that *Xf* had genetically distinct strains in different host plants (e.g., oak, oleander, grapes) (Almeida & Purcell 2003c, Chen et al. 1995, Henderson et al. 2001). Typically, *Xf* isolates from one plant species are genetically similar, despite different geographical origins. However, *Xf* isolated from almonds can be genetically separated into three distinct strains – with one ALS strain recovered in orchards in the northern San Joaquin Valley (ALS-*Xf*SJV) that is genetically more similar to grape strains than the two other ALS strains (ALS-*Xf*1, ALS-*Xf*2).

The few weeds samples that returned positive results in the winter and spring were analyzed using restriction enzyme digestion, and have so far been found to be all of the northern San Joaquin Valley (ALS-*Xf*SJV). The lack of positive results for *Xf* in vineyard weeds after mid-March prevented us from analyzing any changes (new inoculations) of *Xf* strains.

However, we were able to analyze the strain of *Xf* in the mouthparts of the GWSS tested, and found that these insects were also found to be carrying *Xf* of the PD type. These results are consistent with previous findings that strains of *Xf* tend to be host-specific (Almeida and Purcell 2003c).

CONCLUSIONS

The results of this study indicate that the winter and spring weeds may be the most important reservoirs for *Xf* in vineyards infected with Pierce's Disease. We recovered *Xf* from four species of weeds that have either not been studied in depth (*Stellaria sp.* and *Capsella sp.*) or would benefit from further investigation (*Erodium sp.* and *Poa annua*). We seem to have caught the tail end of the season where *Xf* is abundant in weeds, so the next season's sampling scheme will focus more heavily on vineyard groundcover during the winter months of December, January and February. Future research along these lines could illuminate the importance of previously overlooked alternate host plant species.

One hypothesis for the importance of winter weeds for the persistence of *Xf* is that when symptomatic leaves senesce in late fall, they land directly on the groundcover, thus greatly enhancing the likelihood that any insect feeding there will transmit the bacteria to the weeds. Conclusive evidence of this hypothesis could provide a simple and low cost method for controlling the spread of PD.

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OBJECTIVE

1. Determine the presence of *Xylella fastidiosa* in alternate host plants that are commonly visited by glassy-winged and native sharpshooters in selected ecosystems in the San Joaquin Valley; with samples representing different seasons and annual or perennial hosts.

RESULTS

Insect and Plant Samples

GWSS and native sharpshooter (Feil and Purcell 2001) visitation on common non-agricultural crops were monitored to determine the importance of the seasonal period as a component of PD epidemiology. Newly molted adult vectors need to acquire *Xf* from overwintering reservoirs in order to spread PD. GWSS displays seasonal preference for different plant hosts (Daane et al. 2003, 2004), which are often related to host plant phenology or condition (Anderson et al. 1992). We have observed that in winter and early spring, GWSS preferentially feed on perennial weeds such as stinging nettle, filaree (*Erodium* sp.) and common groundsel (*Senecio vulgaris*) in or near vineyards.

GWSS were collected in May, June, July and August from urban ornamental plants that may serve as a host for transferring *Xf* from cities to agricultural land. Insects analyzed for the presence of *Xf* in their mouthparts with the DNeasy Tissue Kit from Qiagen (Bextine 2004). Adult GWSS collected from oleander, xylosma, Chinese elm and riparian zone plants tested positive for *Xf*, while insects collected from crape myrtle tested negative for *Xf*. Nymphal GWSS testing positive for *Xf* were found only on oleander during the month of June. Nymphal GWSS testing positive for *Xf* indicate from which plant the insects are acquiring the bacteria, but will not pose a threat for long since with each successive molt, the insects lose their ability to transmit *Xf*. Adult GWSS testing positive are more of a concern, as an adult GWSS can move between many plants during its lifetime, feeding and spreading *Xf*.

Presence of Pathogen

Non-agricultural plants commonly visited by sharpshooters were screened for the presence of *Xf*. While lists of *Xf* and sharpshooter host plants are available, there are some basic questions that have not been addressed for the San Joaquin Valley: How common is *Xf* in non-agricultural plants? How often do GWSS feed on *Xf* hosts?

Vineyards with heavy infestations of PD were sampled for ground vegetation weeds in and around the crops once a month from January through September. Collections focused on the most abundant variety of weeds, and three samples were taken from each weed species on each date. Samples were processed with either the selective media scheme of PWG and PD3, or with immunocapture DNA extraction and subjected to PCR with universal primers RST-31 and RST-33 (Minsavage 1994). Some weeds collected in January and February were found to contain *Xf*, but after early March, *Xf* was not detected in any weeds collected (Table 1).

Pathogen Population Levels

For GWSS to acquire and transmit *Xf*, the titer of *Xf* within plants typically should be equal to or greater than \log_{10}^4 (CFU per g), the threshold population required for acquisition for most sharpshooters (Almeida & Purcell 2003a,b). For chronic PD and ALS to develop, *Xf* infections must survive the winter, which can vary depending on temperature and the degree of plant dormancy (Almeida & Purcell 2003c, Feil & Purcell 2001) and the plant species.

Table 1. Winter/spring weed samples tested for the presence of *Xylella fastidiosa*.

Date	Abundant Weeds	<i>Xf</i>
4 February 2004	stinging nettle	+
11 February 2004	stinging nettle	+
3 March 2004	chickweed	+
	bluegrass	+
	shepherd's purse	+
	filaree	-
	alfalfa	-
10 March 2004	tall grass	-
	bluegrass	-

Preliminary analysis of ground cover weeds was conducted using selective media PWG and PD3. However, due to the large amounts of naturally occurring bacteria in wild weeds, all samples were contaminated beyond our ability to count *Xf* colony growth. The same samples were then processed using immunocapture DNA extraction and PCR, which did detect *Xf* in some weeds. When we no longer detected *Xf* in weeds after mid-March, we then tested the sensitivity of our extraction methods and PCR. We found that using the immunocapture DNA extraction protocol for plants, we are able to detect at least 1.43×10^{-6} CFU/g of *Xf* DNA, which was satisfactory in ruling out faulty DNA extraction methods. The sensitivity of PCR to detect *Xf*

IMPORTANCE OF GROUND VEGETATION IN THE DISPERSAL AND OVERWINTERING OF *XYLELLA FASTIDIOSA*

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ABSTRACT

The purpose of this project is to determine the ability of alternate host plants, specifically “weeds,” in almonds and vineyards to serve as reservoirs for *Xylella fastidiosa* (*Xf*) and for new inoculations by the glassy-winged sharpshooter (GWSS). We collected and analyzed weed and GWSS samples in and around commercial vineyard and almond fields for the presence of *Xf* on a monthly basis. *Xf* has been recovered from weeds collected during February and March, while no collected weeds tested positive for the presence of *Xf* between April and September. Monthly ground cover sampling will continue through the winter, as this time period may prove most important in the persistence of *Xf* over consecutive growing seasons. GWSS collected from alternate host plants have also been processed for *Xf* and have shown that adults collected on many species harbor *Xf* in their mouthparts. Results from these experiments will help to identify what time of year and what ground cover species are of most concern to growers wanting to control the spread of PD with minimal environmental impact.

INTRODUCTION

The economic viability of California’s vineyards and almonds has received considerable attention of late because of the expanding range of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, which can vector the xylem-limited bacterial pathogen, *Xylella fastidiosa* (*Xf*) (Goodwin & Purcell 1992, Redak et al. 2004). *Xf* is the causal agent of Pierce’s disease (PD) and almond leaf scorch (ALS) as well as other plant diseases. The arrival of GWSS has dramatically changed the epidemiology of *Xf* and its associated diseases in California (Redak et al. 2004). GWSS may not be an “efficient” vector of PD (Almeida & Purcell 2003a,b; Purcell & Saunders 1999a,b), but it presents a more serious threat, in part, because of its wide host range (Redak et al. 2004) and dispersal abilities (Blua et al. 2003). Of importance here is that the wide host range of *Xf* commonly overlaps with plant species visited by GWSS. Our proposed research will focus on the common host range of both vectors and pathogen, with an emphasis on potential annual weeds that may provide an overwintering reservoir for *Xf* and a spring feeding site for vectors of PD and ALS.

How can this work impact control decisions? An excellent example of an overlooked insect-pathogen-host triangle is stinging nettle (*Urtica urens*), a common weed throughout the Central Valley. In our 2003 survey, we found that stinging nettle was a common host for GWSS in springtime, and recent DNA extraction showed the presence *Xf* in 60% of stinging nettle collected near a Kern County PD-infected vineyard. Whether or not *Xf* titer is high enough in these weeds for GWSS acquisition and transmission is not known, and is one aspect of the proposed study.

Regardless, management of common hosts may be a critical component of epidemiology and area wide management of PD and ALS (Redak et al. 2004). With over 145 natural or experimental host plants for *Xf* that can cause PD, the insect/pathogen relationship is far too diverse a subject for one study. For this reason, we are studying the common landscape and ground vegetation found near vineyards and almonds in the San Joaquin Valley.

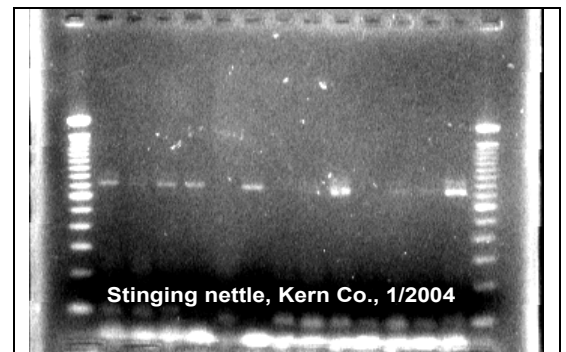


Figure 1. Stinging nettle collected with the vine rows of a PD-infected vineyard showed 9 of 12 samples positive for *Xf*.

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interactions between *Xylella* and indigenous microflora in grapevines. With the greater resolving power of the oligonucleotide fingerprinting technique proposed in our study, we expect to make considerable advances in our knowledge of grapevine microbial communities and their interactions with *Xylella* or with other endophytes being considered for establishment as biological control agents.

OBJECTIVES

1. Characterize the diversity and community structure of endophytic microorganisms in healthy and infected grapevines.
2. Compare endophytic microbial populations in different susceptible and tolerant grapevine cultivars, in different hosts that support high or low populations of *Xylella*, and in plants grown under different conditions.
3. Characterize the potential interactions of endophytic populations with *Xylella* and introduced biological control agents through experimental manipulations.

RESULTS

Several DNA extraction and PCR amplification protocols were tested over the past year. Most procedures yielded too many clones that were of plant origin. Even extracted plant sap contained considerable plant DNA, of mitochondrial and chloroplast origin, that amplified with different versions of prokaryotic-specific ribosomal DNA primers. The use of filtration with various pore sizes to remove plant material from extracted sap also did not eliminate plant DNA from the samples. Finally, we recently succeeded in selectively extracting and amplifying bacterial DNA from grapevine sap using differential centrifugation to remove DNA of plant origin (naked or in organelles). Plant sap was extracted from grapevines with a pressure pump and centrifuged at 8,000 rpm for 1 hr. The pellet was suspended in 1 ml phosphate buffered saline and loaded onto a tube containing percoll. After centrifugation for 30 min at 22,000 rpm, fractions were collected and subjected to DNA isolation. Isolated DNA was amplified with rDNA primers and cloned (Table 1). Fractions containing bacteria yielded only one plant-derived DNA clone out of 58 in the first experiment, and similar results were obtained when the experiment was repeated. A full-scale extraction and amplification from symptomatic and asymptomatic grapevines from the field is in progress.

Table 1. Bacterial species identified from rDNA sequences amplified from grapevine sap in preliminary tests.

<i>Acidovorax</i> sp.
<i>Agrobacterium</i> sp.
<i>Bacillus macroides</i>
<i>Burkholderia</i> sp.
<i>Caulobacter</i> sp.
<i>Escherichia coli</i>
<i>Escherichia fergusonii</i>
<i>Pseudomonas putida</i>
<i>Pseudomonas syringae</i>
<i>Rhizobium tropici</i>
<i>Shigella flexneri</i>
<i>Teichococcus ludipueritiae</i>
<i>Xylella fastidiosa</i>
Unidentified Acinetobacter
Unidentified Proteobacterium
Unidentified Sphingomonas

CONCLUSIONS

Most of the endophytic species that we detected through cloning of bacterial rDNA sequences were not detected in previous culture-based approaches to identify endophytes in grapevine (Bell et al., 1995; Kirkpatrick, 2003). Since the 16 species that we detected were identified among just 58 clones in our recent preliminary studies, we expect that our full surveys of endophytic bacteria in grapevine this year will yield a far greater diversity than previously known. Researchers working on biological control of the pathogen, as well as disease resistance in grapevine cultivars, will benefit from the information gained in this work. The work should enhance discovery of potential biological control agents for Pierce's disease and the implementation of biological control efforts underway.

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CULTURE-INDEPENDENT ANALYSIS OF ENDOPHYTIC MICROBIAL COMMUNITIES IN GRAPEVINE IN RELATION TO PIERCE'S DISEASE

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ABSTRACT

Culture-independent, nucleic acid-based methods of assessing microbial diversity in natural environments have revealed far greater microbial diversity than previously known through traditional plating methods. If true for grapevines, then this has important consequences for Pierce's disease management strategies that involve the establishment of introduced bacteria systemically in the grapevine xylem. Such establishment will likely be influenced by the presence of yet uncharacterized microorganisms, and knowledge of endophytic communities and their dynamics will therefore be important to the successful implementation of these strategies. In addition, analysis of microbial community composition in different hosts and conditions could lead to the identification of new biological control agents. We are employing a novel method, called oligonucleotide fingerprinting of rRNA genes (OFRG), that was recently developed by the Co-PI for analyzing microbial community composition in environmental samples.

INTRODUCTION

In recent years, culture-independent, nucleic acid-based methods of assessing microbial diversity in natural environments have revealed far greater microbial diversity than previously known through traditional plating methods (Amann et al., 1995). This is true for water, soil, the plant rhizosphere, and the plant leaf surface (Yang et al. 2001). A recent culture-independent analysis of bacterial populations inside of citrus plants in relation to *Xylella fastidiosa* also suggested that bacterial endophytic populations are much more diverse than previously realized (Araújo et al., 2002). If true for grapevines, then this has important consequences for Pierce's disease management strategies. Several strategies are being investigated to biologically control *Xylella fastidiosa* in grapevines, including the use of antibiotic-producing endophytes (Kirkpatrick et al., 2001), endophytes that disrupt cell-to-cell signaling by the pathogen (Lindow, 2002), endophytes that degrade xanthan gum (Cooksey, 2002a), and the use of nonpathogenic strains of *Xylella* for competitive exclusion of pathogenic strains (Cooksey, 2002b). These strategies have in common the need to establish an introduced strain systemically in the grapevine xylem. Such establishment will likely be influenced by the presence of yet uncharacterized microorganisms, and knowledge of endophytic communities and their dynamics will therefore be important to the successful implementation of these strategies. In addition, analysis of microbial community composition in different hosts and conditions could lead to the identification of new biological control agents.

We are employing a novel method that was recently developed by the Co-PI for analyzing microbial community composition in environmental samples. This method can be used to characterize both bacterial and fungal communities (Valinsky et al., 2002a; 2002b). Previous culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE), generate only superficial descriptions of microbial community composition (Araújo et al., 2002). A far more complete view of total microbial community composition can be achieved by amplifying, cloning, and sequencing of conserved rRNA genes from the hundreds or thousands of microorganisms present in an environmental sample, but this is prohibitively expensive for any significant number of experiments. The new methodology, called oligonucleotide fingerprinting of rRNA genes (OFRG), represents a significant advance in providing a cost-effective means to extensively analyze microbial communities. The method involves the construction of clone libraries of rDNA molecules that are PCR amplified from environmental DNA, arraying of the rDNA clones onto nylon membranes or specially-coated glass slides, and subjecting the arrays to a series of hybridization experiments using 27 different end-labeled DNA oligonucleotide discriminating probes (Borneman et al., 2001). The process generates a hybridization fingerprint and identification for each clone that is essentially like sequencing the individual clones.

The state of knowledge of the relationship between *Xylella fastidiosa* and the resident endophytic flora of grapevines is at a very early stage. Work to date has been limited to the culturing of endophytes from grapevines, but even this has led to the realization that grapevine xylem sap contains a complex community of microorganisms. Bell et al. (1995) cultured over 800 bacterial strains from grapevine xylem fluid in Nova Scotia. Dr. Bruce Kirkpatrick has also isolated several hundred bacterial strains from grapevine xylem fluid in two counties of California (Kirkpatrick et al., 2001). In citrus, the culture-independent DGGE method of microbial community analysis was compared with culturing of endophytes in relation to the citrus variegated chlorosis strain of *X. fastidiosa* (Araújo et al., 2002). It was found that DGGE detected the major bacteria that were cultured from citrus xylem, but it also detected other bacterial species that had not been cultured. In addition, this method showed differences in microbial communities in different plant varieties, and most importantly, between citrus that was infected vs. non-infected with *X. fastidiosa*. This provides support to our hypothesis that there are likely to be important

Work in Progress

We have developed whole genome arrays of *Xylella fastidiosa* and are presently analyzing gene expression levels between the wild type and *rsmA* mutant, growth with and without BSA and *in vivo* versus *in vitro* conditions. We hope to catalogue the genes whose expressions are associated with biofilm formation, *rsmA* mutation and infection. Those genes which will overlap with more than one approach will be especially interesting for further analysis. Genetic analysis of these genes therefore should open a window for us into what goes on during the infection process. The *rsmA* mutant together with its parent is also being assayed for pathogenicity on grapes. In addition, we have constructed several mutants in a select candidate pathogenicity genes and are in the process of analysis these for the effects of the mutations and hence the roles of these genes in the bacterium.

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FUNDING AGENCIES

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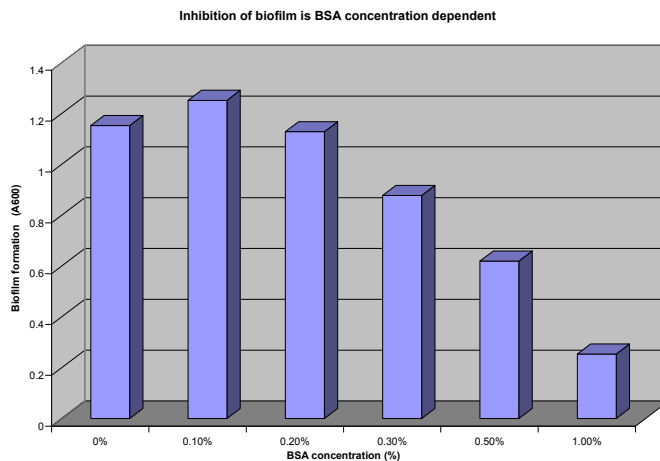


Figure 2. Inhibition of biofilm formation is BSA concentration dependent

Table 1. List of genes overexpresses at least 10-fold in RsmA19.

Gene name	Function	Volume Ratio (A19/A05)
<i>brk</i>	BrkB protein	14.4
<i>pilE</i>	Type IV pilin	10.8
<i>chi</i>	Chitinase	12.0
<i>pcp</i> or <i>lpp</i>	Peptidoglycan-associated outer membrane lipoprotein precursor	25.7
<i>pilU</i>	Twitching motility protein	10.6
<i>vacB</i>	VacB protein	14.0
<i>algH</i>	Transcriptional regulator	18.3
<i>algU</i> or <i>algT</i>	RNA polymerase sigma-H factor	21.8
<i>ccmA</i>	Heme ABC transporter ATP-binding protein	14.5
<i>colS</i>	Two-component system, sensor protein	10.7
<i>tapB</i>	Temperature acclimation protein B	69.8
<i>fucA1</i>	Alpha-L-fucosidase	10.9
<i>pilT</i>	Twitching motility protein	13.3
<i>gcvR</i>	Transcriptional regulator	12.9
<i>clpP</i> or <i>lopP</i>	ATP-dependent Clp protease proteolytic subunit	13.2

CONCLUSIONS

In conclusion, we have identified a genetic factor and an environmental factor, both of which control the important phenomenon of biofilm formation; a process that is tightly linked to pathogenicity of *Xylella fastidiosa*. *rsmA* mutants of *Xylella fastidiosa* form more biofilm than the parents and the presence of BSA in the medium suppresses biofilm formation by the bacterium. We have identified 15 preliminary genes which are coordinately regulated with *rsmA* mutation and possibly, biofilm formation. We are using high density DNA microarrays to catalogue *Xylella fastidiosa* genes which are up- or down-regulated with *rsmA* mutation and reduced biofilm formation due to BSA in the medium. This work will contribute significantly to fundamental information on the genetics and pathogenicity of *Xylella fastidiosa*. This information is essential for any attempt to design a management strategy for PD based on the disease mechanism. The identification of previously unknown virulence genes can also lead to recognition of new unforeseen targets for management strategies. In addition, the construction of a DNA microarray for this pathogen, and identification of genes differentially expressed during infection, will complement work by others on differential expression of grapevine genes during infection. This will open the door to “interactive genomic” studies that will enhance our understanding of the bacterial-plant interaction that leads to Pierce’s disease, and in the future, studies of interactions with its insect vectors.

OBJECTIVES

1. Use DNA microarray analysis to identify virulence and pathogenicity genes in *Xylella fastidiosa* through coordinate regulation with a known virulence factor or expression *in planta* during infection.
2. Clone and mutate putative virulence genes and characterize virulence defects in a bid to understand the mechanism of virulence.

RESULTS

Cloning, Characterization of *rsmA* and the Construction of *rsmA* Mutant of *Xylella fastidiosa*

As mentioned above, three observations let us to investigate the role of *rsmA* in pathogenicity and biofilm formation in *Xylella fastidiosa*: 1, the homologues of the gene are widely distributed in the prokaryotic world; 2, the gene controls pathogenicity and virulence in many phytobacteria and 3; in *E. coli*, the gene controls biofilm formation. To determine the role of *rsmA* in *Xylella*, we the cloned the gene and characterized it. The authenticity of the cloned gene was confirmed with DNA sequencing. *Xylella fastidiosa rsmA* is a small gene that encodes a predicted product is 72-amino acid with a putative RNA-binding protein. Heterologous expression of *X. fastidiosa rsmA* in a biofilm overproducing *csrA* mutant of *E. coli* resulted in reduced biofilm formation indicating that the gene does have a role in biofilm formation (Figure 1). After confirming that the cloned gene is indeed *rsmA*, we determined the effect of the mutation on biofilm formation in *Xylella*. The mutant and wild type were assayed for their ability of form biofilm *in vitro*. Observation show that, the mutant formed more biofilm that the parent (Figure 1). Since the ultimate goal is to identify virulence genes, we tested whether *rsmA* mutants are pleiotropically affected in the expression of any genes. For this, we used the nylon membrane DNA macroarrays of about 100 select pathogenicity genes based on the published genomic sequences (7, 16, 18). Hybridization of ³²P-labelled total cDNA reveal 15 genes which were more than 10-fold induced in the mutant (Table 1).

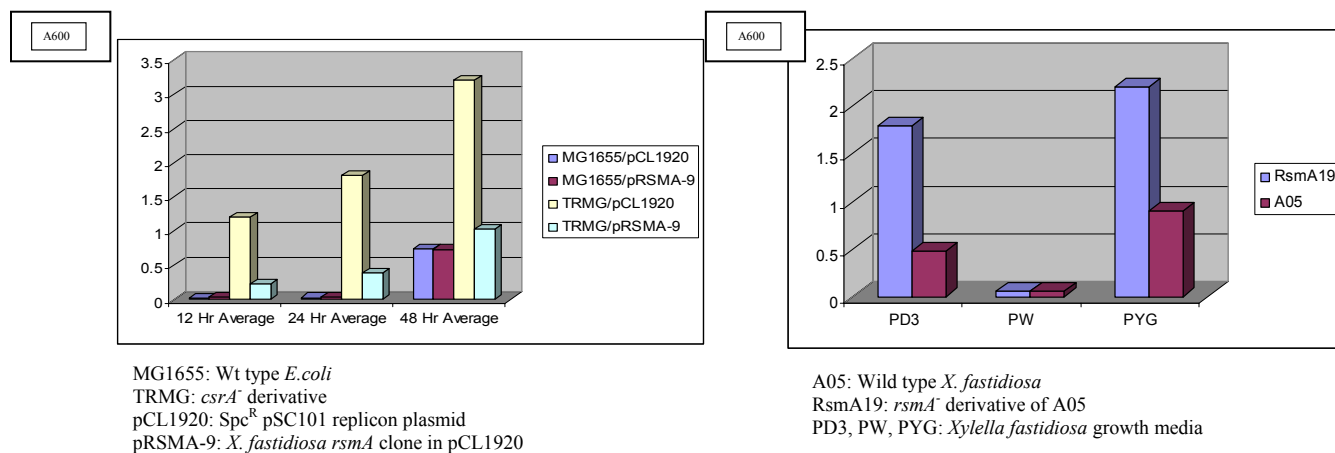


Figure 1. Left. Biofilm formation by *csrA*⁻ *E. coli* is suppressed by *X. fastidiosa rsmA* and (Right), *rsmA* mutant of *X. fastidiosa* form more biofilm than their wild type *rsmA*⁺ parents.

Identification of the PW Medium Component that Inhibits Biofilm Formation

Because of the increasing evidence of links between biofilm formation and pathogenicity in many biofilm forming bacteria (2, 13), we were interested in identifying any possible factors that control biofilm formation. We had long observed that *Xylella fastidiosa* make more biofilm when grown in PD3 medium than in PW medium. We explored this difference between the two media by adding different components of PW media to PD3 medium in order to identify the component responsible for the inhibition of biofilm formation. Our result show when Bovine serum albumen (BSA) was added to PD3 medium, biofilm formation was reduced; implying that BSA is the inhibitor. We then wanted to see if this inhibition depends on the concentration of BSA present in the medium. Different concentrations of BSA were again supplemented into PD3 basal medium and the bacterium was assayed again for biofilm formation. Our results (Figure 2) again show that the bacteria formed less biofilm with increasing concentration of BSA. These results clearly indicate that BSA is a specific inhibitor of biofilm formation. We are now utilizing this information in our full genome microarrays experiments to determine identify the genes which are coordinately regulated with biofilm as has been done for another strain of *Xylella fastidiosa* (4).

DNA MICROARRAY AND MUTATIONAL ANALYSIS TO IDENTIFY VIRULENCE GENES IN *XYLELLA FASTIDIOSA*

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ABSTRACT

The development of successful management and control strategies of Pierce's disease of grape requires the identification of virulence and pathogenicity genes and determining how they function to control the disease development process. Based on the presumption that biofilm formation is a major pathogenicity factor of *Xylella* and that it may play a major role in the disease causing process, we have been studying the factors – genetic and environmental that affect biofilm formation by *Xylella fastidiosa*. We have identified that Bovine serum albumen, a component of PW medium specifically inhibits biofilm formation in *X. fastidiosa* and that this inhibition is BSA concentration dependent. Because of its effect on the biofilm formation *in vitro*, we are studying the expression profiles of *X. fastidiosa* genes in the presence and absence of BSA in the media. We have also identified a global regulatory gene, *rsmA* (*rsm* = regulator of secondary metabolism) that control biofilm. An *rsmA*-deficient strain of *X. fastidiosa* forms more biofilm *in vitro* than the wild type. In a preliminary nylon membrane DNA macroarray experiment using about a 100 select candidate pathogenicity genes, we have determined an increased expression of 15 genes in the mutant when compared to the wild type parent. We are now using full genome microarrays of *Xylella fastidiosa* to catalogue the genes whose expressions are controlled by either *rsmA* or BSA. The results from these ongoing analyses using both approaches should help us catalogue *X. fastidiosa* genes which may be involved in pathogenicity and biofilm formation. Subsequent genetic analysis of the genes to be identified should give us some understanding of not only how pathogenicity is regulated in this bacterium but also how to tackle the problems posed by Pierce's disease.

INTRODUCTION

Although the exact mechanism of Pierce's disease is not completely understood, infected grape plants show symptoms resembling those of water-stress. Moreover, the xylem-limited *Xylella fastidiosa* bacterium produces biofilm *in vitro* and *in planta* (4, 9, 10, 12). Putting these two observations together, it has been suggested that this biofilm clogs up the vascular tissues of the plant and occlude water and nutrient transport. Because of this assumed importance of biofilm formation in the disease mechanism of *Xylella fastidiosa*, we have been studying signals and factors affecting biofilm formation in a bit to identify the regulators of pathogenicity in *Xylella fastidiosa*. *rsmA* is a post-transcriptional regulatory gene that controls pathogenicity and secondary metabolism in a wide group of bacteria including Gram positive and negative organisms (1, 3, 11, 15). In *Erwinia* spp. and other related plant-associated bacteria, *rsmA* together with its regulatory noncoding RNA pair, *rsmB* control many phenotypes including pathogenicity, extracellular polysaccharide and enzyme production, and elicitation of hypersensitive response, pigment formation, motility and antibiotic biosynthesis). And in *E. coli* and related enterobacterial human pathogens, *csrA* and *csrB*, the homologues of *rsmA* and *rsmB* regulate, among others, glycogen biosynthesis and biofilm formation (6, 8, 17, 19). Because of the role of biofilm formation on the pathogenicity of many bacterial pathogens (5, 14), and the fact that *rsmA* or its homologs control both pathogenicity and biofilm formation in different bacteria, we wanted to determine the possible role of *rsmA* on biofilm formation in *Xylella*. We found that *Xylella fastidiosa* strains vary widely in their biofilm forming abilities and this is influenced by the culture medium in which the assay is carried out.

We report that BSA is the specific inhibitor of biofilm formation in PW medium and that the amount of biofilm the bacterium forms is inversely proportional to the concentration of BSA in the medium. Further, we show that biofilm formation is regulated by *rsmA* gene as *rsmA*⁻ mutants form higher levels of biofilm than the wild type parent. We confirm this observation by showing that the heterologous expression of *Xylella fastidiosa rsmA* in *E. coli* reduces biofilm formation in this bacterium. Put together, these suggest that *rsmA* may regulate pathogenicity in *Xylella fastidiosa* through its effects on factors such as biofilm formation in the plant.

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Table 1. List of four single nucleotide polymorphisms from 38 rDNA sequences of *Xylella fastidiosa* and the related information.

Strain Name	Host	Geographic Origin	150	454	1261	1338
R116v11	Grape	Georgia	C	G	A	C
PCE-FG	Grape	Florida	C	G	A	C
PD28-5	Grape	Florida	C	G	A	C
PCE-FF	Grape	Florida	C	G	A	C
Temecula	Grape	California	C	G	A	C
GR.8935	Grape	Florida	C	G	A	C
Temecula	Grape	California	C	G	A	C
Temecula	Grape	California	C	G	A	C
Mul-2	Mulberry	Nebraska	C	G	A	C
Dixon1	Almond	California	T	A	G	C
Dixon2	Almond	California	T	A	G	C
Ann-1 1	Oleander	California	T	A	G	C
Ann-1 2	Oleander	California	T	A	G	C
PWT-22	Periwinkle	Florida	T	A	G	C
PWT-100	Periwinkle	Florida	T	A	G	C
Mul1	Mulberry	Massachusetts	T	A	G	C
Elm	Elm	Washington DC	T	A	G	C
OAK	OAK	Washington DC	T	A	G	C
PLS2-9	Plum	Georgia	T	A	G	C
PLM G83	Plum	Georgia	T	A	G	C
PP4-5	Peach	Georgia	T	A	G	C
RGW-R	Ragweed	Florida	T	A	G	C
ELM-1	Elm	Washington DC	T	A	G	C
ALS-BC	Almond	California	T	A	A	T
MUL-3	Mulberry	Massachusetts	T	A	A	T
P3	Coffee	Brazil	T	A	A	T
B14	Citrus	Brazil	T	A	A	T
SL1	Citrus	Brazil	T	A	A	T
CRS2	Coffee	Brazil	T	A	A	T
CM1	Coffee	Brazil	T	A	A	T
CI.52	Citrus	Brazil	T	A	A	T
CO.01	Coffee	Brazil	T	A	A	T
CVC93-2	Citrus	Brazil	T	A	A	T
9a5c	Citrus	Brazil	T	A	A	T
9a5c	Citrus	Brazil	T	A	A	T
PE.PLS	Pear	Taiwan	T	A	A	C
PL.788	Plum	Georgia	T	A	A	C
OSL92-3	Oak	Florida	T	A	A	C

FUNDING AGENCIES

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

16S rDNA is by far the most sequenced locus in bacteria including *X. fastidiosa* that has at least 38 sequences currently available. These 38 16S rDNA sequences from eight different sources were retrieved from the GenBank database. The sequences were aligned using CLUSTAL-W program. Nucleotide variations were examined manually. Only the variations supported by multiple sequences were considered as true SNPs. The nucleotide order in the 16S ribosomal RNA gene, PD0048, in the *X. fastidiosa* strain Temecula genome sequence was used as reference to standardize the nucleotide number (Table 1).

Currently, the microarray system is still being established. The evaluation of SNPs for strain identification was done using PCR methodology. The Primer 3 program was used to facilitate primer designs. All primers were designed with $T_m = 60 \pm 3$ C. The basic strategy of primer design was to arrange the SNPs at the 3' end of the oligo-primers. Two multiplex PCR formats were implemented. For the three primer format, primers Teme150fc - Teme478rg-XF16s1031r generated two dominant amplicons, a 348 bp band for the PD group, and a 700 bp band for non-PD group generated by A non-specific prime paired with Teme150fc. In the four primer format, two primer sets were used. The PD group specific primer set, Teme150fc-Teme454rg was the same as in the three primer format. The other primer set, Dixon454fa-Dixon1261rg generated an 847 bp amplicon for the non-PD almond leaf scorch disease (ALSD) group (Figure 1). For comparison purpose, primer set RST31-RST33 was also included. RST31-RST33 is the most commonly used primer set for PCR identification *X. fastidiosa* at the species level. Primer specificity was also compared to non-redundant GenBank database through the BLAST network service.

Efforts have also been made to obtain a comprehensive collection of *X. fastidiosa* strains in California with emphasis on grape and almond strains. Over 300 isolation attempts have been made from samples of grapes, almonds and other plants. Samples were collected from San Diego, Kern, Tulare, Kings, Fresno, Stanislaus, Butte, Alameda and Solano counties. Strains were initially confirmed by biological characters such as slow growing and opalescence colony type and then by PCR with primer RST31/33. Over 200 strains were used to evaluate the specificity of the identified SNPs. Research results obtained by far consistently indicate that SNPs in the 16S rDNA sequence have high potential for *X. fastidiosa* strain differentiations. Current design strategy for microarray experiments is to place these SNPs in the center of the oligomers. Also as shown in Table 1, a total of four SNPs can be considered for oligomer designs to cover all the known strains of *X. fastidiosa*. The advantage of such a microarray identification system becomes even more obvious when 16S rDNA primers of different specificity levels, such as universal primers, are used to generate a large amount of target DNAs from a low titer of bacterial cells.

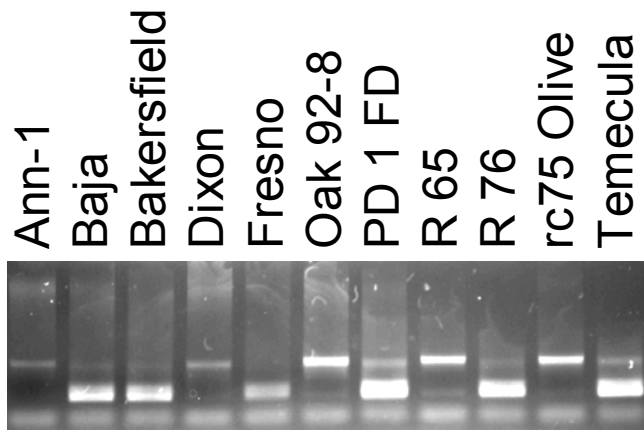


Figure 1. Representative results of multiplex PCR using the four primer format based on single nucleotide polymorphisms in the 16S rDNA sequence. The STRONG presence of the upper band (847 bp) indicates the almond leaf scorch strain group. The STRONG presence of the lower band (348 bp) indicates a grape Pierce's disease strain group.

DEVELOPING A MICROARRAY-PCR-BASED IDENTIFICATION AND DETECTION SYSTEM FOR *XYLELLA FASTIDIOSA* STRAINS IMPORTANT TO CALIFORNIA

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Reporting Period: The results reported here are from work conducted from January 2004 to September 2004.

ABSTRACT

From the analysis of the 16S rDNA sequence of *Xylella fastidiosa*, we have identified four single nucleotide polymorphisms (SNPs). The combination of these four SNPs placed all of the known *X. fastidiosa* strains into four groups. With a few exceptions, the four SNP groups are very similar to those based on other genetic analyses such as RAPD analysis, whole 16S rDNA sequence analysis, and the combination of phenotypic characterization, particularly pathogenicity tests. Of particular interest is the PD group. All eight PD strain 16S rDNA sequences from different labs clustered into the same group characterized by two SNPs. Utilizing the SNP information, primer sets, Teme150fc-Teme454rg, specific to PD strain group, and Dixon454fa-Dixon1261rg, specific to non-PD almond leaf scorch group, were designed. More than 200 *X. fastidiosa* strains isolated from California have been tested for the specificity of these SNPs and the results were quite consistent. A microarray system, initially based on the characteristic SNPs from the 16S rRNA locus, is under construction. Coupled with PCR using universal 16S rDNA primers, the microarray-PCR based system has a high potential for quick and accurate *X. fastidiosa* strain identification.

INTRODUCTION

The need to accurately identify and differentiate *X. fastidiosa* strains is becoming more apparent considering the coexistence of different pathotypes in the same crop (Chen et al., 2004a, b). This prompted us to research on improvement of pathogen detection. Polymerase chain reaction (PCR) has been a common technique for *X. fastidiosa* identification. There are, however, technical problems limiting the application of PCR. False positive amplifications can occur among related organisms in the environment sharing similar genetic sequences. Specific primers may fail to amplify DNA from a particular isolate if there is a spontaneous mutation(s) in the primer-binding site, leading to a false negative result. The sensitivity and specificity of PCR amplification tend to be inversely related.

The rationale of this project is to maximize the sensitivity of PCR technology. To increase pathogen detection specificity, microarray methodology based on the principle of DNA hybridization is applied to further confirm the accuracy of the amplified DNA fragments (Chen and Civerolo, 2003). Conceptually, the development of high-density oligonucleotide arrays allows massively parallel hybridizations to occur on the same surface, permitting high levels of probe redundancy and multiple independent detections of a diagnostic DNA sequence. Because of the taxonomic value and available large genomic sequence database, single nucleotide polymorphisms in the 16S rRNA gene are particularly useful. Other genes and intergenic regions could also be the targets due to the availability of complete genome sequences from four different *X. fastidiosa* strains.

OBJECTIVES

The overall goal of this project is to develop and evaluate a microarray-PCR-based system for accurate and quick identification of *X. fastidiosa* strains. A particular emphasis is on strains currently important in California. Two specific objectives are:

1. Using the complete and annotated genome sequence of *X. fastidiosa* Temecula strain as a guide, select appropriate DNA sequences and evaluate their potential for pathotype / genotype identification. Design and construct a DNA microarray; and
2. Evaluate the effectiveness of the constructed microarray through hybridization experiment. Using the microarray as a reference, analyze genomic variation of different pathotypes with multiple strains collected from broad geographical areas and hosts.

RESULTS AND CONCLUSIONS

Selected sequences in the genome of *X. fastidiosa* Temecula were used as preliminary queries to identify diagnostic sequences. Because of the sequence availability, most comparisons were made to the four complete genome sequences including PD-Temecula, citrus variegated chlorosis-9a5c, almond leaf scorch disease-Dixon and oleander leaf scorch disease-Ann-1. In general, the tested genome DNA sequences showed high level of similarity as expected. However, single nucleotide polymorphisms were found in most cases. Yet, the number of SNPs varied from gene to gene. Genes of evolutionary importance were particularly emphasized because they could provide a more stable and, therefore, a more consistent base for strain identification. Thus, special efforts were made on DNA sequences from *rrn* operons. In addition,

Table 5. Winter, spring and summer 2004 survey of Mexican hat and perennial (western) ragweed for colonization by *Xylella fastidiosa* near four Texas Hill Country vineyards. Results of dilution plating on PWG semi-selective medium were all negative through August 2004.

Season	Location and PD history			
	Gillespie PD	Llano PD	Gillespie No PD	Travis No PD
	----- Positive samples, % (N=total number of samples) -----			
Winter (Feb, Mar)	17% (N=30)	20% (N=40)	^z	43% (N=37)
Spring (Apr, May)	9% (N=33)	5% (N=41)	.	20% (N=41)
Summer (Jun-Aug)	0% (N=6)	10% (N=10)	20% (N=4)	83% (N=5)

^zSite not sampled.

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FUNDING AGENCIES

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Table 1. Selected plant families negative for *Xylella fastidiosa* in one or more species with ELISA and in some cases, also with dilution plating in 2003.

Family	Number of plant specimens
Cupressaceae	2
Cyperaceae	14
Euphorbiaceae	12
Juncaceae	3
Onagraceae	12
Poaceae	43
Solanaceae	16
Taxodiaceae	7
Vitaceae ^z (excluding <i>Vitis vinifera</i> , <i>V. aestivalis</i>)	31

^z*Cissus trifoliata* (L.) L., *Parthenocissus* spp., *V. mustangensis* Buckl., *V. berlandieri* Planch.

Table 2. Plant families with one or more species positive for *Xylella fastidiosa* with serology and dilution plating in 2003.

Family	Species
Apocynaceae	Oleander (<i>Nerium oleander</i> L.)
Asteraceae	[five species, see Table 3]
Fabaceae	Redbud (<i>Cercis canadensis</i> L.)
Fagaceae	Red oak (<i>Quercus</i> sp.) ^y
Platanaceae	Sycamore (<i>Platanus occidentalis</i> L.)
Sapindaceae	Western soapberry (<i>Sapindus saponaria</i> L.)
Ulmaceae	Cedar elm (<i>Ulmus crassifolia</i> Nutt.)
Vitaceae	European grape (<i>Vitis vinifera</i> L.) ^z

^yD. Appel, T. Kurdyla and M. Vest, unpublished data.

^zAlso in 2004, *V. aestivalis* Michx L. Moreno, cv. Cynthiana/Norton, by serology and immunofluorescence; M. Black, cv. Lenoir (uncertain parentage) by serology with dilution plating pending.

Table 3. Five weed species in Asteraceae collected near four vineyards and positive for *Xylella fastidiosa* with serology and dilution plating in summer and fall 2003.

Common name	Scientific name	Longevity	Percent positive			
			Serology (ELISA)		Dilution plating	
Perennial (western) ragweed	<i>Ambrosia psilostachya</i> DC.	Perennial	33%	N=54 ^y	65% ^z	N=17
Red-spike Mexican hat	<i>Ratibida colunifera</i> (Nutt.) Woot. & Standl.	Perennial	19%	N=48	89%	N=9
Hierba del marrano (slim aster)	<i>Symphotrichum divaricatum</i> (Nutt.) Nesom	Annual	21%	N=14	100%	N=3
Great (giant) ragweed	<i>Ambrosia trifida</i> L.	Annual	57%	N=7	75%	N=4
Common sunflower	<i>Helianthus annuus</i> L.	Annual	25%	N=12	33%	N=3

^yNumber of specimens tested.

^zDilution plating usually done only with samples positive or questionable positive with serology.

Table 4. *Xylella fastidiosa* c.f.u./g^u estimates for wine grape and five Asteraceae weed species at four locations in the Texas Hill Country in 2003.

Plant species	Vineyard location and history			
	Llano PD ^v	Gillespie PD ^w	Travis no PD ^v	Gillespie no PD ^x
Wine grape	10 ⁶ -10 ⁸	10 ⁶ -10 ⁷	- ^y	-
Perennial (western) ragweed	10 ⁴ -10 ⁶	10 ⁶ -10 ⁷	10 ³ -10 ⁶	0
Mexican hat	10 ⁶ -10 ⁷	10 ³	10 ³ -10 ⁶	0
Great (giant) ragweed	10 ⁶	-	. ^z	.
Common sunflower	10 ⁵	-	.	.
Hierba del marrano	10 ⁷	-	10 ⁴	0

^uColony forming units per gram of xylem-rich plant tissue.

^vNear riparian habitats.

^wNear smaller, varied, somewhat seasonal riparian habitats.

^xNot near significant riparian habitat.

^ySpecies found but not sampled, or ELISA-negative sample not dilution plated.

^zSpecies not found.

RESULTS

Some plant families had no positive serology reactions and two native grape species and two other native Vitaceae species were never positive with either technique in 2003 (Table 1). Plant samples that reacted serologically for *X. fastidiosa* in 2003 were from 12 plant families, but dilution plating (Hill and Purcell, 1995) with SCP buffer (Hopkins 1988) confirmed the bacterium in specimens from only eight families (Table 2). Identification of selected colonies was confirmed with serology.

Xylella fastidiosa was detected in and cultured from weeds at three (two with PD histories, one with no PD history) of the four vineyards in 2003 (Tables 3, 4). Three weed host species were found at all four vineyards (Mexican hat, western ragweed, hierba del marrano). Two weed host species were found only at the two vineyards with PD histories (giant ragweed, common sunflower). Near one no-PD-history vineyard (Travis County), *X. fastidiosa* was in some nearby weeds, but weed control in the vineyard blocks was good and vineyard perimeters were closely and often mowed.

Supplemental hosts of particular interest were five species in Asteraceae (Table 3). Two are perennials and three are annuals. Serological detection rates for two Asteraceae perennials were higher in summer and fall 2003 (aboveground plant parts, Table 3) and winter 2004 (belowground and soil surface-level plant parts) than in spring 2004 (belowground and soil surface, Table 5). Serology was not consistent among plant parts when petiole and root (Mexican hat) and underground stem, horizontal root and vertical root (perennial [western] ragweed) were tested separately. Overwintering *X. fastidiosa* may not be highly systemic on these species through winter and spring. Spittlebug nymphs (Cercopoidea) were frequently found on these two Asteraceae species in the spring, especially in riparian habitats. Fungal and bacterial contamination of dilution plates were much more pronounced in winter and spring from plant parts belowground or near the soil surface and *X. fastidiosa* concentrations could not be estimated.

This bacterium was also detected and cultured from certain urban trees and shrubs in urban landscape situations in Fredericksburg, Uvalde and San Antonio in summer and fall (Table 1). Colonies of *X. fastidiosa* on sap dilution plates developed earlier for grape and redbud compared to sycamore and oleander in 2003. There were either too few positive samples for us to compare colony growth rates, or results were mixed among sample dates and locations for Mexican hat, western ragweed, hierba del marrano, western soapberry, cedar elm, giant ragweed, and common sunflower.

CONCLUSIONS

Knowledge of PD epidemics in Texas increases prospects for disease control in other wine grape production regions. This work focused on surveys for supplemental *X. fastidiosa* host plants at diverse vineyard sites. Future work will utilize the bacterial isolates and plant community data at PD and non-PD vineyards to explore new control strategies.

A. H. Purcell described four requirements for a plant species to be an important source for *X. fastidiosa* acquisition by xylem-feeding insects: 1) frequently inoculated with *X. fastidiosa*; 2) attractive food host for the insect carrier; 3) *X. fastidiosa* spreads beyond the inoculation site [systemic spread]; and 4) $\geq 10^4$ c.f.u./g of *X. fastidiosa* in xylem-containing plant tissue.

Education efforts related to PD risk in European wine grapes grown in the Texas Hill Country include:

- A. Site selection. Avoid locating vineyards near riparian habitats because more weeds found there probably meet the four requirements listed above for important bacterial sources.
- B. Plant species composition. Based only on circumstantial evidence to date, presence of common sunflower and great (giant) ragweed may indicate higher site risk. This may be because of insect behavior on these two weeds.
- C. Weed control. Until Texas *X. fastidiosa* strains are characterized, broadleaf weed control within and near vineyards should remain a priority, including frequently mowed perimeters.
- D. Rogueing. Infected and symptomatic *V. vinifera* vines contain *X. fastidiosa* with high c.f.u./g. Early PD detection while incidence is still low, and immediate rogueing should be considered to help reduce vine-to-vine spread.
- E. Planting stock. Infected tolerant (few if any acute symptoms) cultivars grown in Texas and other southern states, including *V. aestivalis*, can be reservoirs of *X. fastidiosa* (L. Moreno, unpublished). Infected planting stocks of these varieties are potential sources of inoculum if planted adjacent to *V. vinifera* and in previously PD-free areas.

Results are pending from 2004 greenhouse wine grape plant inoculations with *X. fastidiosa* isolates from grape, weeds and woody ornamentals to determine pathogenicity. Work in progress includes estimating frequency of selected plant species at four vineyards to learn more about high and low risk sites, and strain characterization in this and another laboratory.

SUPPLEMENTAL PLANT HOSTS FOR *XYLELLA FASTIDIOSA* NEAR FOUR TEXAS HILL COUNTRY VINEYARDS

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Reporting period: The results reported here are from work conducted from June 2003 to August 2004.

ABSTRACT

Floras near four Texas Hill Country vineyards were surveyed for *Xylella fastidiosa* from late 2003 through mid 2004. Two vineyards had histories of Pierce's disease (Gillespie County, Llano County) and two did not (Gillespie County; Travis County). In 2003, 526 plant samples representing 49 plant families were tested one or more times with serology (DAS ELISA) and 80 specimens were dilution plated in attempts to confirm positive serology reactions and estimate *X. fastidiosa* concentrations in plant tissue. Two perennial Asteraceae species were then surveyed in winter, spring, and early summer and serological detection was lowest in spring. Bacterial strain characterizations are underway. This study has implications for site selection, weed control in and near vineyards, roguing of vineyards, and the need for pathogen-free planting stock.

INTRODUCTION

Pierce's disease (PD), caused by the bacterial pathogen *Xylella fastidiosa*, is the greatest limiting factor for growing *Vitis vinifera* in most of Texas. Associations of *X. fastidiosa*, known vector glassy wing sharpshooter *Homalodisca coagulata*, other xylophagous insects, and numerous host plant species in warmer climates of Texas are apparently ancient and complex. Widespread death of European grape plants has been a common occurrence in much of Texas, perhaps since the first of many plant introductions 400 years ago. There are numerous scientific advantages to studying a biological system where pathogen, vectors, and host plants are native and endemic. However, little is known about the diversity of plants and the bacterium, or potential biocontrol agents in warmer regions of Texas.

In the mid 90's, the incidence and severity of Pierce's disease escalated in the Texas Hill Country (west of Austin and north of San Antonio). While this area of Texas was once thought to be a PD risk transition zone, many established Hill Country vineyards have seen increased vine mortality due to PD. It is speculated that a series of warm winters allowed the pathogen to become more widely distributed throughout the native plant community, providing the initial inoculum for vine infections. While the disease is not known to occur in the northern Panhandle of the state, recent outbreaks at higher elevations in far-west Texas raise questions about pathogen survival and transport into commercial grape plantings.

Variation exists within and among strains of *X. fastidiosa* with some degree of specialization to be more pathogenic on certain plants and less pathogenic on others (Hopkins 1984, Purcell and Hopkins 1996). However, wine grape plants inoculated with "citrus strain," thought to be most different from "grape strain," PD-like symptoms developed on grape (Li et al., 2002). Questions abound regarding plasticity of bacterial strains in response to changes in insect vectors, climate, plant species composition near vineyards, and grape cultivars.

The greatest genetic variations within species of pathogens, vectors, and potential biocontrol agents typically occur where the species first evolved or coexisted. The *X. fastidiosa* center of origin probably includes the coastal areas of the U.S. near the Gulf of Mexico, including large areas of Texas. Various supplemental hosts may harbor diverse strains of *X. fastidiosa*, perhaps even mixed infections within a single plant. A non-native and highly susceptible species (e.g., *V. vinifera*) growing nearby may be repeatedly challenged by bacteria carried by xylem-feeding insects feeding on both weeds and the introduced plant. Numerous *X. fastidiosa* strains may have potential for some reproduction in European grape (Hopkins 1984, Li et al., 2002, Purcell and Hopkins, 1996), but the highly pathogenic populations that reproduced the most rapidly in wine grape xylem fluids and were vectored most efficiently quickly become predominant.

OBJECTIVES

Our objectives were to survey annuals, perennials, woody plants, and ornamentals near vineyards for colonization by *X. fastidiosa* using serology (ELISA) and dilution plating, and to collect isolates for European grape pathogenicity studies and other strain characterization.



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

GLASSY-WINGED SHARPSHOOTER IRIDOVIRUS PATHOGEN

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ABSTRACT

Pierce's disease of grapes, which is caused by the bacterial pathogen *Xylella fastidiosa*, threatens the national viticulture industry. The glassy-winged sharpshooter (GWSS) is the primary vector of Pierce's disease which if not controlled threatens to completely eliminate the ability of the U.S. to compete in world markets. Viral pathogens of leafhoppers have yet to be examined as potential microbial control agents. Herein we examined the potential of a dsDNA virus, from the Iridoviridae, the iridescent insect infecting viruses, as a pathogenic agent of the GWSS. The GWSS adults were successfully infected with whitefly iridovirus, WFIV that had been propagated in *Trichoplusia ni* larvae. Virus infection caused reduced longevity and fecundity of GWSS. Adults were infected by microinjection and sprays. Infected individuals transmitted the virus to 'healthy' cohorts when caged together, suggesting an aerosol mode of transmission. Detection of virus positive eggs suggests that WFIV may also have a transovarial mode of transmission. Leafhopper vectors of Pierce's disease, such as the glassy-winged sharpshooter, *Homalodisca coagulata*, are susceptible to infection by iridescent insect viruses.

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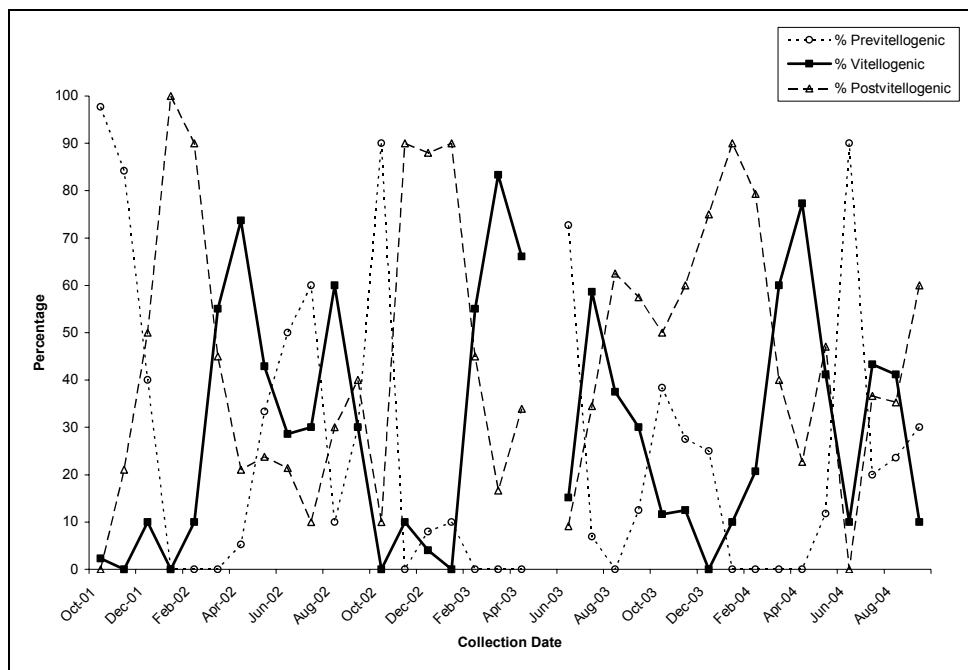


Figure 1: Percentage of previtellogenic, vitellogenic, and postvitellogenic adult female *H. coagulata* per month, according to dissections (October 2001 to September 2004), collected from citrus plants located at the University of California, Riverside, Agricultural Operations.

FUNDING AGENCIES

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(previtellogenic) females peaked in June 2004. The proportion of postvitellogenic females was highest in January 2004, followed by peaks in May and September. The patterns in percentage of previtellogenic, vitellogenic, and postvitellogenic females are similar to those observed in 2002 and 2003. These data suggest that GWSS may have two distinct generations per year. Our observations also indicate that although vitellogenic activity decreases in December, there is not a clear reproductive diapause in the population of GWSS in Riverside, California. The majority of the female GWSS that overwinter are postvitellogenic, suggesting that they have matured and oviposited before entering a reproductive rest period.

Histological studies of female oögenesis are being analyzed to verify the data collected from dissections. Morphological observations of the ovarioles are near completion, and the observations reveal that the ovarioles of the ovaries are the telotrophic type with asynchronous ovarioles.

Effect of Location on Number of Generations Per Year

We initiated sampling of GWSS populations in Tulare and Ventura Counties (California), but were unable to complete this objective due to strong eradication efforts which eliminated populations from our sampling sites.

Host Plant Study

The preliminary data of our host plant study in the summer of 2002 suggested that there is a potential difference in the female fecundity when caged on different plant species. For this study, adult female and male GWSS were caged on citrus, grape, or oleander, and allowed to mate and oviposit on the plants. We were successful in promoting GWSS oviposition and in rearing GWSS from egg to adult stage on all three host plant types. This experiment is currently being repeated with the late summer, overwintering generation of GWSS in citrus. Although the analysis is not yet complete, it appears that female fecundity patterns are different than those observed in the spring (early-summer) generation of 2002.

Scanning Electron Microscopy Studies

SEM study of the ovipositor has been carried out since September 2003. The SEM sessions have revealed sensory structures associated with the first, second, and third valvulae of the ovipositor. Many sensory hairs are also found to be located on the pygofer of the female. TEM studies are necessary to determine the exact type of sensillae present on the ovipositor. The external morphology revealed by SEM micrographs suggests that these structures include various types of mechanoreceptors and chemoreceptors.

CONCLUSIONS

It is too early this season to make any conclusions about host influences on female fecundity, but our prior data have indicated that female fecundity is influenced by host plant type. The observations suggest that it is feasible to target controls towards reproductive hosts (e.g. citrus) of GWSS in order to attempt to control future populations of GWSS. Although it appears that female fecundity varies between host plants, the fecundity may also depend on the generation (e.g. winter, spring, or early summer) being studied. Thus, it is important to avoid limiting year-long GWSS eradication efforts to those populations present on a single host plant type (e.g. citrus). In another experiment, we have successfully reared GWSS on a single host for two successive generations, under greenhouse rearing conditions. These greenhouse data suggest that multiple hosts are not necessary for the survival of GWSS. Thus, GWSS may not need to move between hosts in order to develop and reproduce. However, the pattern may change when GWSS are under field conditions where nutrients may be seasonally limiting.

More research on female host selection for oviposition is needed. Now that we have located sensilla that may function as chemoreceptors, it appears likely that there is a chemical basis for GWSS host selection. These sensilla may only function at close range, thus this knowledge may not be useful for trap development. However, the finding of chemosensilla on the ovipositor could be useful for future development of artificial media for GWSS oviposition in colonies maintained for parasitoid rearing.

Our study of the oögenesis cycle is defining the timing and number of generations of GWSS in California. This knowledge, combined with an understanding of female host selection, fecundity and offspring sex ratio, will result in a detailed understanding of host plant influences on female development and reproductive success. As indicated by somewhat conflicting results, based on the generation being studied, it is clear that the GWSS has complex reproductive patterns, and may have seasonally changing host preferences. Thus, it is important to modify eradication efforts based on the generation being controlled.

We are also beginning to understand the way in which GWSS may sense the environment and may be able to manipulate this system for monitoring trap development.

REPRODUCTIVE BIOLOGY AND PHYSIOLOGY OF FEMALE GLASSY-WINGED SHARPSHOOTERS

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Reporting Period: The results reported here are from work conducted from January 2004 to September 2004.

ABSTRACT

Female and male GWSS have been collected from July 2001 to September 2004 at monthly or bimonthly intervals from citrus hosts at UC Riverside Agricultural Operations. A sub-sample of 10 females per month was dissected to determine ovary rank of the specimens collected. Dissections of these female specimens reveal repeated patterns related to the proportion of previtellogenic females in the field. These patterns indicate two distinct generations each year with a possible third generation late in the season. Sampling will conclude in December 2004, and analysis will be completed to develop a model of female vitellogenesis cycles. A host plant study, completed in the summer of 2002, in which adult male and female GWSS were caged on grape, citrus, and oleander, has suggested differences in female fecundity and offspring survival. This study is currently being repeated. SEM studies have been completed and found a large number of sensilla on the female ovipositor. Morphology of these sensilla suggests that they may have mechanosensory or chemosensory functions. Histological studies of the female reproductive organs at varying stages of vitellogenesis are currently being analyzed.

INTRODUCTION

The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say), is a serious pest of many tree and vine crops (Turner and Pollard 1959, Nielson 1968). The GWSS is of primary concern to California growers because of its capacity to vector the bacterium, *Xylella fastidiosa*, which causes vascular disease in a number of crops, including grapes, citrus and almonds, as well as landscape plants including oleander and mulberries (Meadows 2001, Hopkins 1989, Purcell and Hopkins 1996). An adult GWSS need only acquire *X. fastidiosa* once while feeding on an infected plant to then become a vector of *X. fastidiosa* for the remainder of its life (Frazier 1965, Purcell 1979, and Severin 1949).

Little is known about the reproductive biology of the GWSS. It has been reported that GWSS has two generations per year in Southern California (Blua et al. 1999). Oviposition occurs in late winter to early spring, and again in mid-to-late summer. Adult females can live several months and lay their eggs side by side in groups of about 10, ranging from 1 to 27 (Turner and Pollard, 1959). The greenish, sausage-shaped eggs are inserted into the leaf epidermis of the host plants.

Our research is focused on the reproductive morphology and physiology of the GWSS. We are examining the seasonal differences in female GWSS reproduction between summer and overwintering populations by studying oögenesis cycles. This knowledge is important in determining how GWSS might choose plant hosts in the landscape, which host plants are particularly good for GWSS ovarian development and why they are good, and finally how control measures might best be implemented based upon season and stage of reproductive development. Better knowledge of reproductive biology might also lead to better decision support including improved choices and timing of chemical or non-chemical approaches to GWSS control.

OBJECTIVES

1. Collect and prepare GWSS specimens for studying the morphology and anatomy of females.
2. Study and describe the sensory structures located on the female ovipositor.
3. Characterize the reproductive cycle of female GWSS in Riverside, California.
4. Study the effects of location on female GWSS reproductive cycle.
5. Study the effect of host plant type on female GWSS fecundity.

RESULTS

Oögenesis study

Female and male GWSS have been collected from July 2001 to September 2004. Samples were taken on monthly or bimonthly intervals. Dissections of female specimens collected from citrus hosts at UC Riverside Agricultural Operations have revealed repeated patterns related to the proportion of previtellogenic females in the field (Figure 1). In 2004, oviposition activity began in January with peaks in oviposition activity occurring in April and July. The proportion of young

CONCLUSIONS

Nearly 90% of the BGSS trapped in this study were caught on traps at 15 feet or lower. This suggests that barriers could have a significant impact on reducing the numbers of BGSS entering vineyards. However, this may not be enough to have a major impact on reducing the incidence of PD. In addition, results from one tower indicated that BGSS may reside in some trees early in the season. This could allow for higher than normal flight activity, allowing more BGSS to enter vineyards by flying over a barrier. The effectiveness of barriers at reducing the incidence of PD will likely depend upon the nature of the adjacent riparian plant community, its mix of host plant species and the number of tall host trees.

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FUNDING AGENCIES

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Figure 2 shows the average numbers of BGSS trapped at various heights during the early season period of March-May. Figure 3 shows the average numbers of BGSS trapped at various heights during the entire trapping period of March-September. Figures 2 and 3 include results for all towers except #10, which will be discussed separately.

From March-May, each tower averaged 16.4 BGSS. Of these, 88.3% were caught at 15 feet or lower. For the entire season, each tower averaged 23.5 BGSS. Of these, 89.7% were caught at 15 feet or lower. The patterns of trap catches for the early part of the season and the full season were nearly identical.

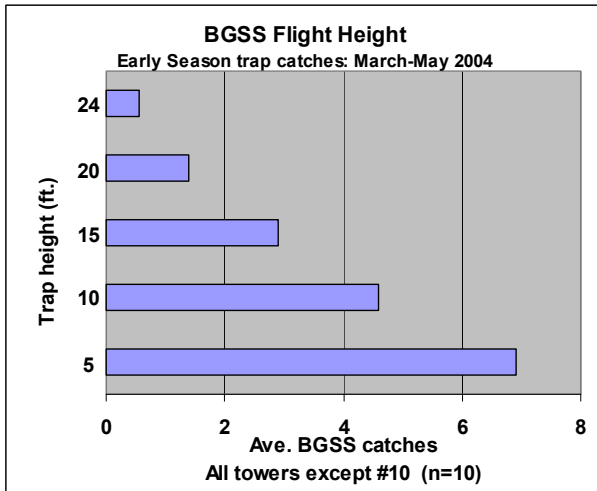


Figure 2.

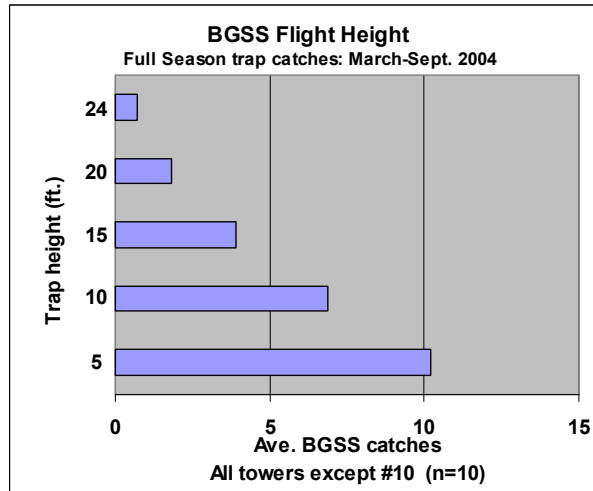


Figure 3.

These data suggest that a 15-20 foot high barrier could be effective at greatly reducing the number of BGSS entering vineyards. However, previous work with insecticides showed that even with 70-90% reductions in BGSS trap counts, the incidence of PD was not significantly reduced in vineyards planted with highly sensitive varieties (8). With a 10-15 foot screen barrier, the number of BGSS flying over the top could still result in significant amounts of PD in an adjacent vineyard.

Tower 10 had early season results very different than the others and is therefore considered separately. Figure 4 shows trap catches at Tower 10 during early March. Unlike the other towers, most BGSS were caught on the upper traps. However, for the rest of the season, the pattern of trap catches mirrored that of the other towers, albeit with greater numbers of BGSS (Figure 5).

Tower 10 was installed adjacent to a Coast Live Oak (*Quercus agrifolia*) tree, an evergreen species. Most of the other trees and shrubs in the vicinity of Tower 10 were deciduous species. In early March, these plants were still dormant or just beginning to bud out. A record heat wave in early March led to daily high temperatures of 70-85°F for nearly 2 weeks. The estimated flight threshold temperature for BGSS is 58°F (2). This unseasonable heat wave led to significant BGSS flight activity in early March as evidenced by elevated trap numbers at Tower 10 and others (data not shown).

The Coast Live oak tree adjacent to Tower 10 was apparently a preferred host plant at this time. If BGSS commonly reside in tall trees during the spring, then the effectiveness of barriers will likely be reduced. Additional studies are needed to better elucidate the early spring host preferences of BGSS in riparian zones, especially at higher elevations in the riparian canopy.

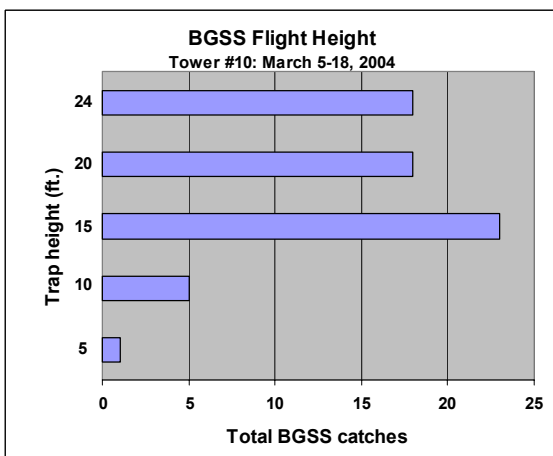


Figure 4.

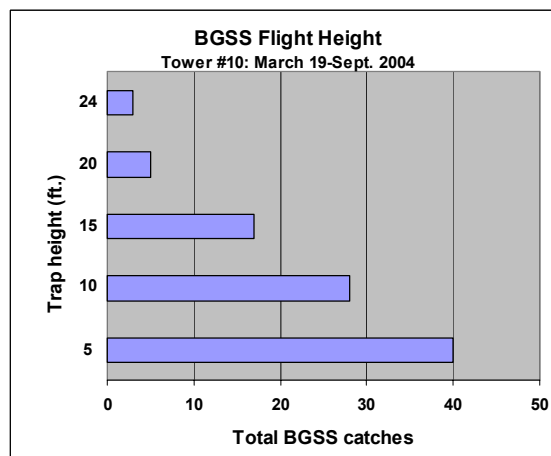


Figure 5.

EVALUATION OF BLUE-GREEN SHARPSHOOTER FLIGHT HEIGHT

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Reporting period: The results reported here are from work conducted from February 2004 to September 2004.

ABSTRACT

Flight heights of blue-green sharpshooters between vineyards and riparian zones were monitored at eleven sites in Napa Valley in 2004 using pole towers to position yellow sticky cards up to 24 feet. At 10 of the towers, nearly 90% of catches from March-September were made at 15 feet or lower. At one tower, however, a large number of BGSS were caught in the upper traps in early March. This tower's proximity to a Coast Live Oak (*Quercus agrifolia*) tree suggests that BGSS may reside at higher elevations in trees at some times of year.

INTRODUCTION

Where the blue-green sharpshooter (BGSS), *Graphocephala atropunctata*, is the primary vector of Pierce's disease (PD), control measures should be aimed at reducing the number of BGSS entering vineyards (4), especially early in the growing season. Early-season infections (March-May) are responsible for most chronic cases of PD (6, 9). Those infections resulting from BGSS feeding later in the growing season are not likely to result in PD, because most will be eliminated with normal pruning. This is unlike the situation with PD caused by glassy-winged sharpshooter (GWSS) feeding, where chronic infections may occur nearly year-round (1).

Vector control measures in the North Coast include the use of insecticides (4) as well as management of riparian plant communities to reduce the number of favorable BGSS breeding host plants (5).

Another method of reducing vector numbers is to block their flight into vineyards through the use of physical barriers. This could include the use of tall fences made with insect screening materials, as well as natural barriers created by planting dense stands of conifers or other non-host tree species. Both of these approaches are already being employed in a few vineyards in the North Coast, although there are currently no data to show their impacts. The use of barriers has also been suggested as a management tactic to keep GWSS out of vineyards (2).

For barriers to be effective, they would need to block the majority of BGSS from entering vineyards, since small numbers of insects can still lead to significant disease development (8). Unfortunately, little is known about the overwintering behavior of BGSS and its preferred winter plant hosts (7). Therefore, it is not clear how tall a barrier would need to be in order to be effective. Most trapping by both researchers and growers has been done from the ground at the 5-6 foot level. Monitoring of BGSS flight activity at higher elevations has not been reported.

This project addresses the question of BGSS flight height by installing and monitoring pole towers that can accommodate yellow sticky card trapping up to a height of approximately 24 feet.

OBJECTIVE

1. Evaluate the predominant flight height of blue-green sharpshooters entering vineyards from adjacent riparian habitats through the use of yellow sticky cards positioned at heights from 5 to 24 feet.

RESULTS

Eleven pole towers were installed and monitored in the Napa Valley in 2004. Towers were positioned along riparian zones adjacent to vineyards with a history of Pierce's disease. A diagram of a pole tower is shown in Figure 1. Towers were 25 feet in height, constructed from Schedule 40 PVC pipe. Yellow sticky cards were attached to clips on rope at the following heights: 24 feet, 20 feet, 15 feet and 10 feet. An additional trap at 5 feet was mounted on a stake.

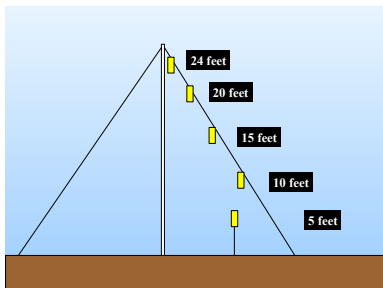


Figure 1: Pole tower diagram.

Eight towers were installed in February 2004; the remaining three were installed prior to March 9. Traps were monitored on a weekly basis through September and numbers of BGSS were recorded. Traps were replaced every two weeks or as needed.

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FUNDING AGENCIES

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

Table 1. Results of targeted dissections of internal reproductive morphology of *H. coagulata* reared under several photoperiod and temperature regimes.

Photoperiod and Temperature ^a	<i>n</i>	Ovarioles	Ova	Fat body	Brochosomes
13.5:10.5 (Aug 5)	15	2	0	2.5-3	1
23-29°C	5	3	2	2.5-3	3
13:11 (Aug 24)	18	2	0	3	1
23-29°C	6	2	0	2.5	1
Greenhouse (May19-Jun29) 13h 13m – 14h 5m (photophase) 31-37°C	15	3	2	2.5-3	3
Greenhouse (Jan6-Feb26) 10h 16m – 11h 16m (photophase) 16.7-27.2°C	15	2	0	3	1

^aPhotoperiod and date for latitude of Tallahassee, FL.

Key:

Ovarioles

- 1=not developed
- 2=fully developed; no ova
- 3=fully developed with ova

Ova

- 0=none
- 1=single ova per ovariole
- 2=two ova per ovariole

Fat body

- 1=minimal
- 2=medium
- 3=heavy

Brochosomes (within Malpighian tubules)

- 1=small; tubule translucent
- 2=medium; tubule filled opaque white
- 3=large; tubule swollen opaque white

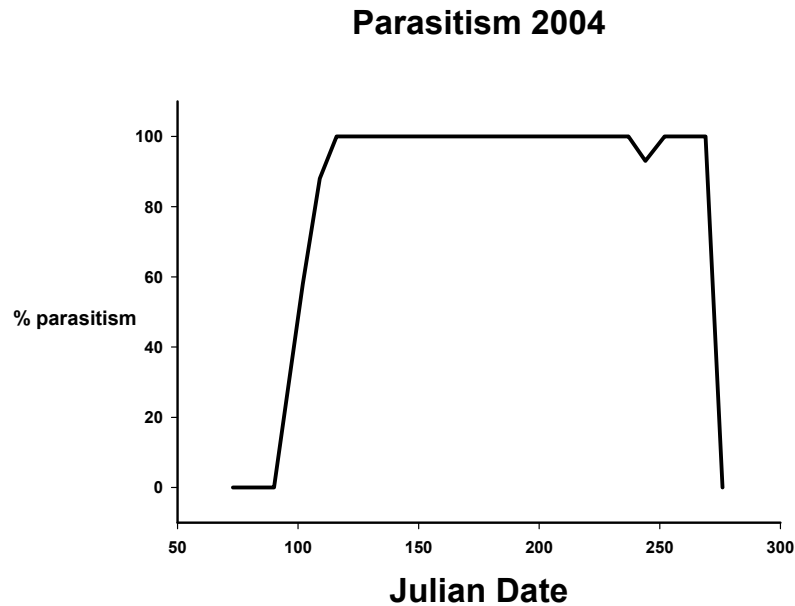


Figure 1. Seasonal parasitism of *H. coagulata* eggs by *Gonatocerus* spp. in north Florida.

Cohorts *H. coagulata* neonates were reared to adult on lemon basil, *Ocimum basilicum* L. 'Lemon', glabrous soy, *Glycine max* (L.) 'D90-9216', and cotton, *Gossypium hirsutum* L. 'Deltapine 88' in environmental chambers programmed with photoperiods of 13.5:10.5, or 13:11 at 23-29°C. Females were dissected and rated as described previously, 15-28d post eclosion. Additionally, cohorts of *H. coagulata* were reared under ambient lighting in a greenhouse during summer and winter months and dissected. Targeted dissections revealed that all female *H. coagulata* reared under the 13:11 photoperiod were in reproductive diapause when compared to individuals reared in winter conditions (Table 1). Dissections of females reared under the 13.5:10.5 photoperiod indicated that 25% (5 of 20) were reproductively active when compared with cohorts reared under early summer conditions (Table 1).

Female *H. coagulata* in reproductive diapause can be manipulated into becoming reproductively active. Cultures of overwintering *H. coagulata* were maintained in screen cages in a greenhouse at ambient light and temperatures. On January 20, 2004, cohorts of leafhoppers were placed into an environmental chamber with a programmed photoperiod of 11:13 (15-17°C) for 21d. They were then moved to a greenhouse set for summer conditions (14:10, 32°C). After 12-14d, brochosomes were observed on the forewings of many of the females. Egg masses were usually present two days later. Five cohorts of leafhoppers were treated as described previously with the same results.

A glabrous soy plant with approximately 20 *H. coagulata* egg masses was exposed to a culture cage of *G. ashmeadi* for 24h. The plant was then placed into an environmental chamber programmed with an 11:14 photoperiod (26°C). Parasites were observed emerging from parasitized egg masses after 14d. The plant was removed and egg masses evaluated for parasitism. All eggs were parasitized and all adult *G. ashmeadi* had successfully eclosed. Two additional plants with egg masses were treated as described previously with similar results. Additionally, adult *G. ashmeadi* that eclosed in the chamber were provided with a new soy plant with approximately 15 *H. coagulata* egg masses. After 14d, adults were observed emerging from the egg masses indicating short-day photoperiod had no effect on their life history.

Single potted cotton or glabrous soy plants with *H. coagulata* egg masses were placed in the field on a weekly schedule beginning the first week of March 2004. After 15d all egg masses were checked for signs of *Gonatocerus* parasitoids. Seasonal parasitism peaked sharply in early April and fell sharply in late September 2004 (Table 1).

CONCLUSIONS

Examination of the ovarioles, ova, fat body, and Malpighian tubules can provide an accurate indication of the reproductive status of female *H. coagulata*. We conclude there is a critical photoperiod important for the initiation of reproductive diapause in *H. coagulata*. However, we have not determined the sensitive life stage to these diapausing inducing cues. We have also determined the environmental conditions important for the termination of reproductive diapause. Additionally, *G. ashmeadi* does not appear to modify its life history when reared under short-day photoperiods in an environmental chamber.

Since populations of *H. coagulata* are reproductively active in north Florida for a relatively short period of four months, overwintering and diapause play a critical role in population dynamics of these insects. Understanding environmental cues critical to reproductive diapause initiation and termination are also essential for researchers attempting to rear these insects throughout the year.

The photoperiod responsible for reproductive diapause of all female *H. coagulata* corresponds to August 24 in north Florida. During this time of year and several weeks later, temperature, rainfall, and host plant availability remain adequate for an additional generation of *H. coagulata*. We propose that this early seasonal reproductive diapause of *H. coagulata* is a life-history response to predation pressure by *Gonatocerus* spp. egg parasitoids.

OVERWINTERING BIOLOGY OF THE GLASSY-WINGED SHARPSHOOTER AND GONATOCERUS ASHMEADI

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Reporting period: The results reported here are from work conducted June 2003 to September 2004.

ABSTRACT

The Glassy-winged Sharpshooter, *Homalodisca coagulata* (Say), is found throughout southeastern US and regions of California. It has 2 distinct generations per season. The majority of adult females overwinter in a reproductive diapause. Targeted dissections of female *H. coagulata* reared at a photoperiod of 13:11 at 23-29°C indicated all females were in reproductive diapause. Seventy-five percent of females reared at a photoperiod of 13.5:10.5 at 23-29°C entered reproductive diapause, perhaps indicating that photoperiod can be modified by temperature as the trigger responsible for physiological changes associated with reproductive diapause. Diapause can be broken by placing females at a 11:13 photoperiod (15-17°C) for 21d followed by exposure to mid-summer environmental conditions. Additionally, parasitism of *H. coagulata* eggs by *Gonatocerus* spp. peaked sharply in early April 2004 and remained at 100% until the last week of September 2004. Finally, short-day photoperiod did not effect development or host seeking behavior of *G. ashmeadi*.

INTRODUCTION

The overwintering biology of the Glassy-winged Sharpshooter, *Homalodisca coagulata* (Say) is an important component of seasonal population dynamics. In the southeastern US, host plant preferences of adult *H. coagulata* during spring, summer, and fall months are predictable and intimately associated with nutrition (Mizell and French 1987, Brodbeck et al. 1995). Mixed hardwoods and citrus are the preferred overwintering hosts for *H. coagulata* in its endemic and parts of its introduced range in California, respectively (Pollard and Kaloostian 1961, Blua and Morgan 2003). In most years, females break diapause during early to mid-March and begin to oviposit on a variety of plants (Turner and Pollard 1959). Presently, the physiology associated with the overwintering biology of *H. coagulata* is poorly understood.

Gonatocerus ashmeadi Girault is one of several egg parasitoids that are key natural enemies of *H. coagulata*. Little is known about their overwintering biology. Lopez et al. (2004) report *G. ashmeadi* could potentially overwinter in the eggs of their host. A greater understanding of the life history of *G. ashmeadi* is essential to maximizing their utility as classical biocontrol agents.

Diapause is loosely defined as a temporary inactivation or reduction of one or several physiological processes triggered by an environmental cue (Lees 1966). Arthropods enter diapause to survive adverse environmental conditions (Masaki 1980). Photoperiod is often the primary cue that triggers physiological changes associated with diapause, however, other environmental factors including temperature and nutrition can have a modifying effect. In the southeast US, *H. coagulata* overwinters primarily in the adult stage (Turner and Pollard 1959). However, 5th instar nymphs and viable eggs can occasionally be found in north Florida during the winter months.

OBJECTIVES

The environmental conditions that are responsible for initiation and cessation of reproductive diapause in *H. coagulata* are a major focus of this research project. Additionally, the effects of photoperiod and temperature on the development and behavior of *G. ashmeadi* were also investigated.

RESULTS

Because diapausing individuals are unidentifiable from non-diapausing individuals, we have developed and refined a protocol for targeted dissections to accurately determine the reproductive status of female *H. coagulata*. Leafhoppers were immobilized with gentle pinch to the head, placed in a paraffin filled dissecting dish and viewed under a stereoscope. The wings and telson were carefully removed with fine jewelers forceps followed by small incisions along the pleural membrane of the abdomen. The abdominal terga were then removed to facilitate examination of four Malpighian tubules, which lie dorsally in loops above the mid and hindgut. Fat body was generally concentrated in the first through fourth abdominal segments. Ovarioles were examined after portions of the gut tract were teased out of the body cavity. Ovarioles, ova, fat body, and Malpighian tubules were rated on the scale described in Table 1.

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FUNDING AGENCIES

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

Figure 1. Airflow apparatus used to induce desiccation stress in gravid female *H. coagulata*.



Table 1. Means (\pm SE) of number of eggs oviposited and or retained by stressed and unstressed gravid *H. coagulata*. Values across rows followed by different letters are significantly different; $P < 0.05$.

	Stressed	Unstressed	Pr > t
Mean \pm SE ^a			
Proportion of eggs oviposited	54.4 \pm 4.4a	28.2 \pm 5.3b	0.002
Eggs oviposited per female	13.7 \pm 2.3a	5.9 \pm 1.2b	0.015
Total oviposited + mature oocytes	194 \pm 18.9a	187.2 \pm 17.1a	0.696

^an=six replications

CONCLUSIONS

A broad ovipositional host range may not necessarily be disadvantageous to the neonates of *H. coagulata*, as we have recently documented adaptations that allow the immature stages to efficiently relocate to suitable hosts (Tipping et al. 2004). Stress-induced oviposition thus appears consistent with both the reproductive physiology and the nutritional ecology of *H. coagulata* due to the inability of females to reabsorb oocytes and the high vagility of immatures.

The phenomenon of death stress oviposition, or induced oviposition, in *H. coagulata* can be a valuable tool for researchers who require large numbers of uniform aged eggs essential for nymphal development studies. Additionally, this technique can be useful for maintaining cultures of *Gonatocerus* parasitoids. Finally, collection of many egg masses in a short period of time may also be instrumental in the creation or augmentation of existing cultures of *H. coagulata*.

A NOVEL METHOD TO INDUCE OVIPOSITION IN THE GLASSY-WINGED SHARPSHOOTER

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Reporting period: The results reported here are from work conducted from June 2003 to June 2004.

ABSTRACT

Gravid *Homalodisca coagulata* females were induced into ovipositing a significantly greater proportion of their eggs 24h after desiccation treatment with a directed flow of warm air (40°C, 5.0 meters per second for 15 m) compared to untreated females. Treated and untreated females oviposited 54.5% and 28.2% of their eggs, respectively, regardless of host plant.

INTRODUCTION

Accidental introductions of *H. coagulata* into regions of California have prompted researchers to begin a classical biological control program using egg parasitoids in the genus *Gonatocerus* (Jones 2001). Initiation and maintenance of large cultures of *H. coagulata* for egg production for culture of *Gonatocerus* parasitoids is difficult and time consuming because few host species adequately support all life stages of *H. coagulata* (Brodbeck et al. 2004). Currently, augmented releases of *Gonatocerus* parasites are an important component of long-term management of *H. coagulata* in California.

The phenomenon of death stress oviposition was first reported by DeCoursey and Webster (1952) who indicated that a variety of chemical agents, including pesticides, could produced various levels of stress to gravid female mosquitoes *Ochlerotatus sollicitans* (Walker) and gravid Angoumois grain moth, *Sitotroga cerealella* (Oliver). Individuals that were stressed deposited a greater amount of eggs than untreated controls.

One of the objectives of our research project is to determine the behavioral and physiological mechanisms associated with the overwintering of *Gonatocerus* eggs parasitoids, an important natural enemy of *H. coagulata*. Efficient acquisition of even-aged cohorts of *H. coagulata* eggs is crucial to this project. For nearly 20 years, our research group has been involved in the study of many life history characteristics of *H. coagulata*, including oviposition behavior.

OBJECTIVES

The main objective of this study was to determine and manipulate the environmental conditions conducive to inducing oviposition of gravid *H. coagulata* females.

RESULTS

Twenty gravid females were field-collected from crape myrtle, *Lagerstroemia indica* L. by sweep net. Ten females were placed immediately into a cage that was provisioned with either one three-week old cotton plant, (*Gossypium hirsutum* (L.) 'Deltapine 88'), or one glabrous soybean plant, (*Glycine max* (L.) 'D90-9216'). Ten females were stressed with a direct flow of warm air (40°C, 5.0 meters per second) for 15m (Fig 1). After airflow treatment, females were placed into a cage with a plant as described previously. Plants were examined for egg masses the next morning. Females were dissected and numbers of mature, chorionated oocytes in the lateral and median oviducts were counted. Tests with each host plant were replicated three times. Host plant effects on oviposition were analyzed by ANOVA (SAS 1990). We defined the experimental unit as total eggs per plant, as we could not accurately quantify eggs per female. Paired comparison t-tests were used to compare the differences between the total eggs, number of eggs oviposited, and of mature chorionated oocytes not oviposited between treated and control females.

Host plant had no effect on oviposition of stressed ($F = 0.84$; $df = 1, 4$ $P < 0.42$) or unstressed females ($F = 0.03$; $df = 1, 4$ $P < 0.88$). Data from the six replications were then combined for t-test analysis. Field-collected gravid *H. coagulata* oviposited a significantly higher proportion of their eggs following stress treatment compared to unstressed controls (Table 1.). Targeted dissections indicated that stressed females had fewer chorionated oocytes within reproductive structures than females that were not stressed.

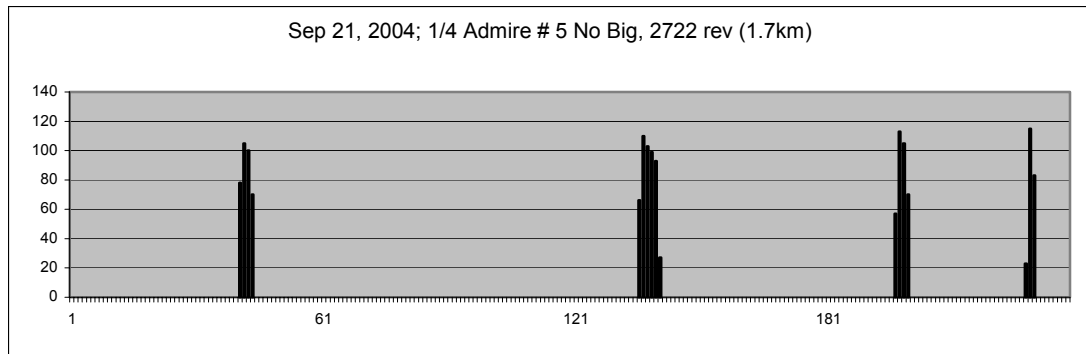


Figure 2B. Flight of a surviving GWSS fed on grape treated with 1/4 of standard dose. Note flights are fewer and shorter than untreated insects.

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FUNDING AGENCIES

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Table 2. Mortality and flight performance of GWSS males after a 24-hour exposure to untreated grape or grape with imidacloprid applied at 1/4th or 1/32nd of a standard dose (3.2 mg/500 g soil) 10 days previously.

<u>Performance characteristic</u> (Males Only)	<u>Sample size</u>	<u>Range</u>	<u>Average</u> *	<u>Stand. dev.</u>	
<u>Mortality</u>	1/4 th dose	57	0- 100%	33% a	0.34
	1/32 nd dose	48	0 - 25%	9% b	0.09
	untreated	48	0 - 20%	3% b	0.08
<u>Percentage of surviving non-fliers</u>					
	1/4 th dose	38	0 - 100%	59% a	0.38
	1/32 nd dose	44	0 - 22%	7% b	0.09
	untreated	46	0 - 20%	3% b	0.08

*Numbers in a column followed by the same letter were not significantly different using chi-squared with Yates' correction and ANOVA.

The flight performance assays of GWSS exposed to 1/8th and 1/16th dilutions of the standard dosage of imidacloprid are still in progress. Preliminary indications are that the 1/8th dilution may reduce average flight activity but with some individuals flying as far as fliers from untreated plants.

Unreported Results that were Pending Last Year.

The effects of the insect-repellent kaolin clay (Surround) and Admire applied to potted grapevines were assessed in cages for possible repellency effects to GWSS and BGSS (Purcell 2003). In general Surround was repellent, whereas Admire was not. The test plants used in these behavioral experiments were saved for diagnosis for PD, as all sharpshooters used in the experiments had been exposed to plants infected with *X. fastidiosa*. Unfortunately, transmission rates in all treatments (including untreated controls) were too low (3% per plant for GWSS, 9-21% for BGSS) to be of value in assessing the effects of Admire or Surround applications on the vector transmission of *X. fastidiosa* where the insects had a choice of treated vs. untreated plants. This lower than normal transmission rate was probably due to low populations of *X. fastidiosa* in the PD-grapes used for acquisition feeding.

CONCLUSIONS

GWSS flew on flight mills for up to 4. 2 miles (6.8 km), averaging over 1.5 miles in a 4 hr period. Soil-applied imidacloprid (Admire) dosages that caused 33% mortality during a 24-hr exposure to treated plants reduced average flight performance of surviving GWSS, but some of the insects that survived this exposure flew almost normally. Dosages that caused about 10% mortality and that have been shown to drastically reduce GWSS feeding did not significantly reduce flight on flight mills. Admire treatments probably reduce long distance movements of GWSS from treated crops having sap concentrations of imidacloprid that kill at least 30% of the GWSS within 24 hours.

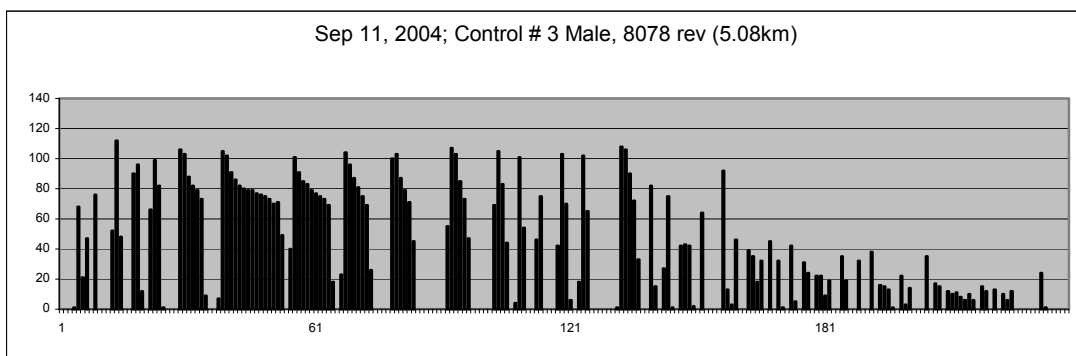


Figure 2A. Flight (flight mill rotations per minute) of a control GWSS (no insecticide); horizontal axis = minutes.

RESULTS

Objective 1. Understand the Basic Characteristics of GWSS's Flight.

Flight mills were constructed as outlined by Schumacher et al. (1997), with slight modification. The rotating flight mill arm was a 20cm plastic drinking straw rotating on a jewel bearing fitted with a steel shaft. Custom computer software counted the number of revolutions in successive 60-second intervals and generated data on flight distance, duration, and velocity. For each trial, 3 replications of 4 to 10 GWSSs per cage were allowed to feed on grape for 24 hours. The prothorax of each insect was glued to a standard insect pin using water-soluble Styrofoam glue, and the insect pin (with the insect attached) was then inserted into the arm of the mill. Flight trials lasted for 12 hours, later reduced to 4 hours, during the day. GWSS were classified as “fliers” if they flew a total distance of 100 rotations (63 m) and “non-fliers” if they failed to complete 100 rotations. Table 1 summarizes the flight mill performance of GWSS from untreated plants. Males consistently flew longer and more frequently than females (Figure 1), so data for males only (Table 2) were summarized for comparisons of GWSS from treated and untreated grape. Figure 2 illustrates a typical flight profile for GWSS males from untreated (Figure 2A) and high dosage plants (Figure 2B).

Objective 2. Examine the Effect of Various Doses of Imidacloprid on the Flight Performance of GWSS in the Context of Pierce's Disease Epidemiology

To quantify the effects of sub-lethal dosages of Admire on GWSS flight performance, we measured the flight performance of insects exposed to both treated and untreated grape vines. Imidacloprid treatments were dilutions of a standard 3.2 mg in 500 g of soil. Dilutions used were 1/4, 1/8, 1/16, 1/32 of the standard dose; controls were untreated vines. The plants were allowed one week for pesticide uptake before caging the insects on them for 24 hours and then monitoring their flight mill performance. The 1/32nd dilution caused 9% mortality over a 24-hour period, compared to controls (3%) and did not significantly reduce total distance flown. A higher dose (1/4 of standard) did kill significantly more GWSS (33%) within 24 hours and reduced the numbers of surviving insects classified as “fliers”, but some individual GWSS from the 1/4th dosage plants flew as well as those from untreated plants (Table 2). This may have been because of physiological variation among individuals or the amount of imidacloprid taken up by plants on which the insects had fed. We collected and froze xylem saps to compare imidacloprid concentrations from each plant to the flight performance of the GWSS that fed on them before flight mill assays but have not yet analyzed these samples for imidacloprid content.

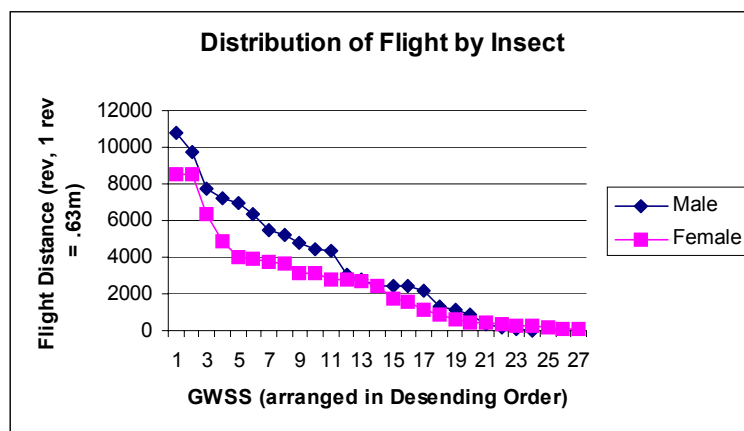


Figure 1. The flight distances of GWSS male (diamonds) and female (squares) from untreated plants.

Table 1. Flight mill performance of GWSS from untreated grape (control).

<u>Performance characteristics</u>		<u>Range</u>	<u>Average</u>	<u>Stand. dev.</u>
<u>Total Revolutions</u>	Males	12-10,826	3,853	3,085
	Females	72 - 8,557	2,537	2,410
<u>Total flight events</u>	Males	17-200	75	42
	Females	13-207	79	57
<u>Distance of longest flight event</u>	Males	6-1258 meters	358 m	359
	Females	6-495 m	149 m	140
<u>Average distance per flight event</u>	Males	6-178 m	70 m	46.9
	Females	6-151	37	40.8

EFFECTS OF SUBLETHAL DOSES OF IMIDACLOPRID ON VECTOR TRANSMISSION OF *XYLELLA FASTIDIOSA*

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Reporting Period: The results reported here are from work conducted from November 2003 to October 2004.

ABSTRACT

A computer-monitored flight mill was developed to study the effects on insect flight of sub-lethal dosages of soil-applied imidacloprid (Admire 2F, 21.4% AI) to glassy-winged sharpshooters (GWSS) in laboratory cages. Adult sharpshooters were glued to a 10 cm radius plastic arm that rotated on a pivot. The rotations per minute were recorded and tabulated by computer. The range of distances flown on flight mills by adult GWSS not exposed to insecticide treatment (negative controls) ranged from 8 m to 6,843 meters and averaged 3,853 m for males and 2,537 m for females. Over 90% of males and females flew at least 60 m ("fliers") during the 6-12 hour flight trials. More than 9 % of total distances flown by individual fliers occurred within 4 hours. Imidacloprid at sub-lethal dosages (9% mortality in 24 hours vs. 3% of untreated controls) that inhibit feeding did not reduce flight performance significantly, but dosages that killed 33% of the GWSS in 24 hours reduced flight in the surviving insects. Insects that had fed on insecticide-treated plants for 24 hours flew much less (fewer fliers), yet among those that did fly, the differences were not statistically significant. At 3.2 mg imidacloprid in 500 g soil, on average, one-third were killed after 24 hours, and less than 50 % of the survivors flew. However, there were occasional "outliers" that could fly just as well as, or sometimes more than, the control insects. Whether these individuals were resistant to imidacloprid or survived and flew as a result of uneven uptake of the insecticide by different replicate plants was not clear. There were no significant differences in flight distances of GWSS exposed to a dose of 0.1 mg in 500g soil.

INTRODUCTION

The systemic insecticide imidacloprid (Admire 2F, Bayer Co., Kansas City, MO) has been used to control glassy-winged sharpshooter (*Homalodisca coagulata*, GWSS) in citrus and grapes, mainly as a killing agent (Bethke et al. 2001). The main effect of insecticides in reducing the spread of Pierce's disease is to decrease the numbers of insects entering and remaining in vineyards. But beyond the numbers of GWSS, disease spread also depends on the level of infectivity of GWSS with *Xylella fastidiosa*, vector transmission efficiency to grape, and movements of the vector from plant to plant (Purcell 1981). GWSS movements from vine to vine should be especially important if this is the main mode by which GWSS establishes new infections of grape, as circumstantial evidence suggests (Perring et al. 2001; Purcell and Saunders 2001). Sub-lethal (low lethality) dosages may persist in treated crops longer than highly lethal dosages, as plant growth dilutes insecticide concentrations and the insecticide deteriorates to less toxic or non-toxic forms. Identifying the effects of sub-lethal dosages on the behavior of a plant disease vector is especially important because non-lethal doses of insecticide may repel some insects and increase plant-to-plant movements, leading to increased disease spread by surviving vectors. Our previous studies suggested that imidacloprid does not repel the GWSS or promote their small scale plant-to-plant movement.

Our objectives were to establish the effects of sub-lethal dosages of imidacloprid on GWSS transmission efficiency and movement. As we previously reported (Purcell 2003), systemic imidacloprid (soil applications) in grape reduced GWSS transmission of *X. fastidiosa* to grape, but the effects might have been mostly due to insect mortality rather than by affecting GWSS feeding behavior in such as way as to reduce vector transmission. Dosages that did not kill more than 10% of GWSS significantly reduced feeding by GWSS, but imidacloprid did not repel GWSS or blue-green sharpshooters in lab trials in which a documented repellent, Surround, did repel sharpshooters from plants (Purcell 2003).

We tested various dosages of imidacloprid that caused reduced GWSS feeding to determine the effects of the insecticide exposure on the flight performance of GWSS on flight mills. Computer-monitored flight mills have been used to study flight performance in other leafhoppers (Gorder 1990; Taylor et al. 1992), and we adopted a previously described flight mill design (Gorder 1990; Schumacher et al. 1997) to assess the flight performance of GWSS with or without exposure to imidacloprid treatments of grape. Flight mill performance usually requires about 30% of the power required for free flight (Riley et al. 1997), so flight mills underestimate free flight distances.

OBJECTIVES

1. Understand basic performance characteristics of GWSS flight.
2. Determine the effect of various doses of imidacloprid on the flight performance of GWSS in the context of Pierce's disease epidemiology.

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Although *Baumannia* and *Wolbachia* were the most common bacteria found, a few other 16S rDNA of bacteria not previously described from GWSS were also cloned from GWSS samples (Table 1). Some samples are still being analyzed to determine the identity (“un-id” in Table 1) or close relationship of the bacteria represented. Among those isolated were bacteria with identity similar to *Acinetobacter*, *Stenotrophomonas*, *Pseudomonas* and *Burkholderia*. All are aerobic γ -Proteobacteria, and not uncommon as environmental contaminants and nosocomial pathogens (e.g., Towner et al. 1991, Ribbeck et al. 2003). However, *Acinetobacter* and *Stenotrophomonas* have also been isolated from ticks and fleas (Murrell et al. 2003); and *Stenotrophomonas*, among other bacteria, was isolated from the guts of ants, where it was presumed to provide nutrients and to be passed to offspring (Jaffe et al. 2001). *Stenotrophomonas* was also described as an endosymbiont of a fly (Otitidae), which did not develop properly without its complement of bacteria (Wozniak and Hinz 1995). *Burkholderia*, a pseudomonad, was isolated from termite guts (Wertz et al. 2003), and was able to colonize a variety of aquatic invertebrates both externally and internally (McEwen et al. 2001).

We did not detect any bacteria in PBS buffer alone. Bacteria were detected in 4 of 12 buffer samples that were pipetted onto the outside surfaces of 12 different insects. We were only able to clone one of these DNA samples because subsequent PCRs of the other three were negative for 16S DNA. The cloned sample contained 16S DNA similar to that of *Pseudomonas*, *Acinetobacter*, and *Methylobacterium*. It is not yet possible, therefore, to determine whether *Acinetobacter* and *Pseudomonas* cloned from hemolymph samples came from the insect surface, the hemolymph, or both.

CONCLUSIONS

A wide-ranging search for secondary symbionts of the GWSS did not identify good candidates for studies on biological effects on this insect. Some bacteria we identified were possibly from insect external surfaces. The prevalence of a *Wolbachia* species, and the well-known importance of *Wolbachia* to other insect hosts make it the best candidate to pursue in further studies.

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OBJECTIVES

1. Survey glassy-winged sharpshooter and other sharpshooters in California and the southeastern United States for facultative bacterial endosymbionts and determine by DNA sequencing the identity of any bacteria discovered.
2. Depending on type of microorganism and relative frequency in surveyed insects, select candidate symbionts to determine biological effects on GWSS.

RESULTS

We collected GWSS from various locations in California and in Louisiana and Florida in spring and summer 2002. In June 2003 we collected GWSS from Louisiana, Mississippi, Alabama and Florida. Four other species of sharpshooter were also collected in California in summer 2002 and fall 2003. Some field collected GWSS from selected locations were brought back to the lab and caged together for one to several weeks in order to facilitate exchange of any potentially horizontally transmitted facultative symbionts. In several cases, long-term lab colonies were established from field populations, and could be repeatedly sampled. Laboratory-reared GWSS were also obtained from the California Department of Food and Agriculture rearing facility in Bakersfield, California on several occasions in 2003.

DNA from three types of tissue from sharpshooters collected in 2002 and 2003 were extracted: hemolymph, eggs, and bacteriocytes. Over 400 extractions have been made and analyzed for bacterial DNA. Hemolymph is known to contain bacterial endosymbionts in aphids (e.g., Chen et al. 1996) and leafhoppers (e.g., Purcell et al. 1986) and is a logical place to sample. Approximately 2- 4 uL of hemolymph was removed by puncturing the abdomen with a glass needle, and was then added to 20 uL phosphate buffered saline (PBS) and stored frozen until analysis. After extraction, we amplified the DNA of the 16S ribosomal DNA with “universal” bacterial primers, digested any bacterial DNA with restriction enzymes, and looked for different patterns that might indicate the presence of more than one type of bacteria. A subset of bacterial 16S rDNA was cloned in *E.coli*, reanalyzed with restriction enzymes (e.g., Table 1), and sequenced if deemed appropriate. This procedure was also applied to eggs (dissected from gravid females or removed from leaves after being laid) in which we expected to find any vertically transmitted endosymbionts, such as the primary symbionts, *Baumannia*, but perhaps other symbionts as well.

Forty-five percent (126/281) of hemolymph samples from all localities tested positive for bacterial 16S rDNA by PCR. Twenty-six individuals of another four species of sharpshooters from California were also tested for bacteria in hemolymph, of which five (19%) were positive by PCR. We have not yet analyzed these further. DNA from a total of 25 GWSS tissue samples from 17 individuals was chosen for cloning, and 19 produced multiple transformed *E. coli* colonies with bacterial 16S rDNA inserts. DNA from 45 of these colonies was chosen for sequencing, and others were identified by restriction digest analysis. The most common sequence was identical to that of *Baumannia*, a bacteriome-associated symbiont of the GWSS (Moran et al. 2003) (Table 1). Like other bacteriome inhabitants, *Baumannia* is presumably transovarially transmitted from mother to offspring via hemolymph (Buchner 1965). *Wolbachia*, a commonly found facultative symbiont of many insects, including GWSS (Moran et al. 2003), was also cloned or commonly found facultative symbiont of many insects, including GWSS (Moran et al. 2003), was also cloned or otherwise identified from hemolymph and eggs of California, Florida and Louisiana GWSS. In addition, we surveyed extracted DNA that was positive for 16S rDNA for *Wolbachia* by PCR. *Wolbachia* has been described from GWSS (Moran et al. 2003), but its prevalence and the existence of strain differences has not been documented. We found *Wolbachia* in 10% (8/84) of hemolymph samples and 59% (19/32) of egg samples. These figures are probably conservative, and indicate that *Wolbachia* is a very common bacterium associated with GWSS. *Baumannia* was amplified from 67% (60/89) of hemolymph samples by PCR.

Collection location (sample / no. clones sequenced or digested)	GWSS tissue	16s rDNA identity of inserts (by sequencing or restriction digest analysis)
Bakersfield	Hemolymph Eggs	Bau, Wol, Aci Wol, un-id
CDFA	Hemolymph Eggs	Bau, Wol, un-id
Louisiana State Univ	Hemolymph Eggs	Bau, Sten, Pseu Bau
Crestview FL	Hemolymph Eggs	Bau, Wol, Aci, Pseu Bau, Wol
Pearl River LA	Hemolymph Eggs	Bau, Wol, un-id Bau, Wol, Burk

EXPLORATION FOR FACULTATIVE ENDOSYMBIONTS OF SHARPSHOOTERS

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ABSTRACT

Glassy-winged sharpshooters (GWSS) were collected in California and several states in the southeastern United States in 2002 and 2003 to search for pathogenic or beneficial endosymbiotic bacteria of these insects. Various tissues were examined for the presence of bacteria by PCR: hemolymph, eggs, and bacteriomes. A subset of hemolymph and egg samples were cloned and sequenced based on unique digest patterns of their extracted 16S rDNA, or analyzed by restriction digest patterns of sample compared to known bacterial DNA. Most cloned sequences were identified as *Baumannia* (one of the primary symbionts of GWSS), and *Wolbachia* (a common secondary symbiont in a majority of insect taxa investigated). In addition, we isolated bacteria that were most closely related (by 16S rDNA sequence) to the following genera: *Acinetobacter*, *Stenotrophomonas*, *Pseudomonas*, *Burkholderia*. All are common bacteria that are found in soil, water, or plant surfaces, and also in insect guts or surfaces.

INTRODUCTION

We have surveyed populations of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, for bacterial symbionts that might be exploited to manipulate the biology of this insect vector of *Xylella fastidiosa* (*Xf*) (Purcell and Feil 2001). Pathogens or other microbial associates of GWSS have not been employed to date as biological control agents or contributors to the control of these pests largely because none are known, although some efforts to discover viruses of GWSS have been made. Although endosymbiotic bacterial associates of leafhoppers are little-understood and unexploited to date, their potential importance is well worth exploring. The first step has been to look for and identify any naturally occurring bacteria in GWSS populations from a wide geographical range.

Of particular interest to us in this study were bacterial associates that are facultative (also referred to as “secondary”), i.e., that occur in some individuals or populations but are not required by their hosts; and that could be introduced into, or augmented in pest populations. We use the term symbiont here in the biological sense of “living together” and do not imply mutual benefit (Douglas 1994). Facultative bacterial associates have been described in a variety of homopterans including leafhoppers (Swezy and Severin 1930, Schwemmler 1974, McCoy et al. 1978, Purcell et al. 1986). The only leafhopper facultative symbiont studied in some depth is BEV, a bacterium that occurs in *Euscelidius variegatus* in France, but apparently not in California (Purcell et al. 1986). Uninfected females of *E. variegatus* inoculated with cultures of BEV transmitted the bacteria transovarially (“vertically”) to their offspring, with resulting deleterious effects (Purcell et al. 1986, Purcell and Suslow 1987). This bacterium could also be transmitted horizontally between leafhoppers feeding on the same plant; hence it could persist in the population in spite of its negative fitness effects.

It is clear from our studies of facultative bacteria in aphids (Chen et al. 2000, Montllor et al. 2002) as well as from the study of BEV, that endosymbiotic associations are complex and have critically important effects, both positive and negative, on the physiology, population biology and vector potential of their hosts. Some of the most extensive studies on the effects of facultative symbionts on insect hosts involve *Wolbachia*, a transovarially transmitted bacterium that occurs in 20-76% of investigated insect species (Weeks et al. 2002) with a range of interesting effects (e.g., Werren 1994, Stouthammer et al. 1999). *Wolbachia* has recently been described from GWSS (Moran et al. 2003), though its effects remain unknown. Although *Wolbachia* has “helped raise the awareness of the potential contribution of endosymbionts...it is important not to discard other alternatives” (Weeks et al. 2002). Our approach was to investigate whether other alternatives existed for GWSS.

hosted egg masses but not the other life stages. The suitability of the host plants for these GWSS life stages may be linked to the chemical composition of the xylem fluids (Andersen et al. 1989, 1992, Brodbeck et al. 1990, 1993, 1995, 1996, 1999), data for which will be provided by the xylem analyses. Sowthistle, common groundsel, lambsquarter, cheese weed, primrose and beavertail were not hosting large GWSS numbers, if any, and may be discarded or replaced for next season.

This season, the location seems to influence the size of GWSS egg masses (larger egg masses in the south), survival (lower in the south) and parasitism (lower in the south). The underlying factors may be related to temperature and humidity which have been recorded but have not been correlated to the findings yet. The major difference between the coastal and inland locations at similar latitude is the number of second generation adults, and all life stages from the second generation are responsible for most of the location differences. Aside from the egg masses, there are no obvious differences in the other life stages recorded in the coastal and southern location.

Further conclusions cannot be drawn without the data that is still being taken in the fecundity and feeding studies and the chemical xylem composition of the host plants. For full understanding of the climatic influences behind these observations, multiple year data are needed and need to be analyzed for temporal and spatial differences, for which two additional years of funding will hopefully be forthcoming from the CDFR.

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No egg masses were recorded on oleander, sowthistle, cheeseweed, lambsquarter, common groundsel and beavertail cactus. Over all sites the mean number of egg masses recorded was largest on sycamore, cherry and grape, followed by crape myrtle and photinia (Table 1). The number of egg masses per host plant species differed significantly for crape myrtle, eucalyptus, grape, primrose and cottonwood on which fewer egg masses were found in Piru and Redlands than in Pauma (results not shown). In Piru, most egg masses were recorded on sycamore and cherry, followed by grape. In Redlands, most egg masses were recorded on grape, followed by crape myrtle and photinia, which had more egg masses than sycamore and cherry. In Pauma most egg masses were recorded on crape myrtle, grape, sycamore and cherry followed by photinia. Because of unequal variances Kruskal Wallis was used for these analyses with $P < 0.0001$ in all cases (results not shown).

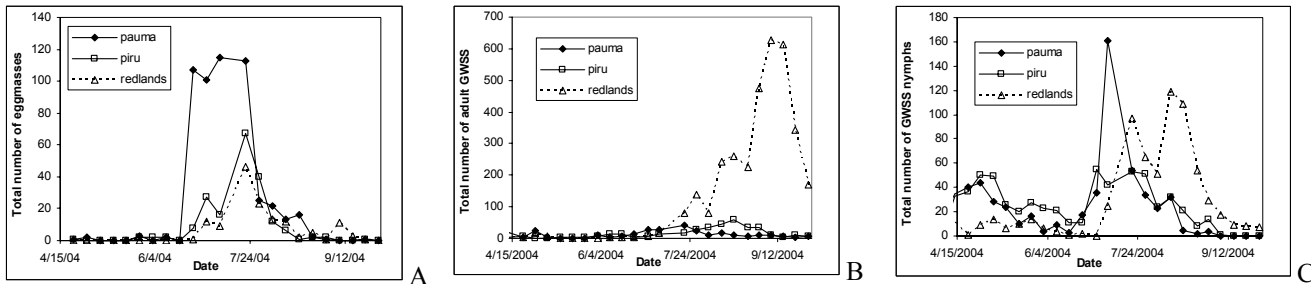


Figure 1: Total number of GWSS egg masses (A), adults (B) and nymphs (C) recorded between April and October 2004, on 100 host plants located in a citrus orchard in Piru, Redlands and Pauma Valley, CA.

When considering GWSS adults at the different locations, more were found on the host plants in Redlands between June 16 and October 1 compared to both Piru and Pauma in the same period (unequal variance: Kruskal Wallis: $t=8.4481$, $P=0.0146$) (Fig. 1b). Adults were not recorded on sowthistle, cheeseweed, common groundsel or beavertail cactus. Over all sites the mean number of adults recorded was largest on cotton wood (Table 1). In Redlands, more adults were found on hibiscus, oleander, Valencia orange, photinia, euonymus, ligustrum, cottonwood and cherry than in Piru or Pauma (results not shown). In Piru and in Redlands, more adults were recorded on cotton wood than on any other host plant species ($t=59.75$, $P < 0.00001$ and $t=72.05$, $P < 0.00001$ respectively). In Pauma, most adults were recorded on cotton wood, but these did not differ significantly from sycamore and grape ($t=63.61$, $P < 0.00001$). Because of unequal variances Kruskal Wallis was used for these analyses (results not shown).

The data on the immature GWSS were collected as small, medium and large GWSS nymphs. For the purpose of these preliminary analyses the stages were added to present one number per host plant per observation at each location. The number of GWSS nymphs at the different locations changed through the season. From April though June, significantly fewer nymphs were recorded in Redlands when compared to Pauma and Piru in the same period (unequal variance: Kruskal Wallis: $t=10.04$, $P=0.0066$) (Fig. 1c). From Late July through October, significantly fewer nymphs were recorded in Piru, when compared to Redlands and Pauma in the same period (unequal variance: Kruskal Wallis: $t=7.78$, $P=0.0204$) (Fig. 1b). No nymphs were recorded on common groundsel. Over all sites the mean number of nymphs recorded was largest on cottonwood, followed by significantly lower numbers on grape, crape myrtle, and Valencia orange (Table 1). No differences were found when comparing numbers of nymphs per host plant species between the locations (results not shown). In Piru, most nymphs were recorded on cottonwood, followed by grape and citrus ($t=70.3$, $P < 0.00001$). In Redlands, most nymphs were also recorded from cottonwood, followed by grape and crape myrtle ($t=72.49$, $P < 0.00001$). In Pauma Valley, most nymphs were found on cottonwood and grape, followed by crape myrtle and Valencia orange ($t=68.92$, $P < 0.00001$). Because of unequal variances Kruskal Wallis was used for these analyses (results not shown).

The recorded numbers of generalist predators present per location include lady beetles, spiders and lacewings. Less frequently praying mantis, assassin bugs, robber flies, scorpion flies and syrphid flies were recorded. The numbers of foraging parasitoids (*Gonatocerus* sp) were also recorded per plant. These data have not yet been analyzed. On June 30, July 1-2, August 10-12, September 28-30 xylem fluids samples were taken from all host plants except oleander, amaranthus, ivy, sowthistle, common groundsel, cheeseweed, lambsquarter, honeysuckle, primrose and beavertail. These species were omitted because experience has shown that they do not comply with the technique used for xylem extraction, rendering the sampling impossible (Brodbeck, personal communication). With the use of a nitrogen gas pressure chamber, 150-600 μ l was collected per plant and frozen for storage. The xylem samples await analyses on their chemical composition in Florida. The GWSS fecundity and feeding rate on a selection of the host plants listed in table 1 is being studied in University of Florida, NFREC-Quincy.

CONCLUSIONS

The data thus far indicates that the most eggs, nymphs and adults are not necessarily recorded on the same plant species as has been reported before (Brodbeck et al. 1999). In this study the only host plant used frequently in all life stages is cotton wood. On grape and crape myrtle nymphs and eggs are frequently recorded, while photinia, cherry and sycamore frequently

Table 1 Mean number of egg masses, adults and nymphs recorded per GWSS host plant species in Piru, Redlands and Pauma Valley, California.

Plant	Plant name	Common name	Egg masses ¹	Adults ²	Nymphs ³
1	<i>Hibiscus</i> sp.	'Mrs. J. E. Hendrey' hibiscus	3.42 ± 1.064 abc	10.50 ± 4.265 a	3.42 ± 0.908 ab
2	<i>Lagerstroemia indica</i>	Crape Myrtle	9.58 ± 1.607 de	34.25 ± 20.350 a	17.92 ± 5.113 d
3	<i>Nerium oleander</i>	Oleander (white)	O	19.75 ± 8.294 a	10.17 ± 2.925 bc
4	<i>Gardenia jasminoides</i>	'Mystery' Gardenia	1.50 ± 0.832 ab	0.42 ± 0.193 a	2.17 ± 0.842 ab
5	<i>Citrus</i> sp.	Valencia Orange	2.42 ± 1.314 abc	13.15 ± 3.175 a	11.17 ± 3.164 c
6	<i>Photinia</i> sp.	Red Tip Photinia	6.67 ± 2.021 cd	2.08 ± 0.763 a	4.92 ± 1.681 abc
7	<i>Eucalyptus cinerea</i>	Silver Dollar Tree	0.50 ± 0.167 a	0.33 ± 0.188 a	0.50 ± 0.289 a
8	<i>Vitis vinifera</i>	Thompson Seedless Grape	11.17 ± 2.49 e	14.42 ± 3.019 a	29.75 ± 6.516 e
9	<i>Euonymus japonica</i>	Silver Queen	1.92 ± 0.654 ab	0.92 ± 0.358 a	0.25 ± 0.131 a
10	<i>Ligustrum japonicum</i>	'Texanum' Wax Leaf Privet	1.58 ± 0.617 ab	1.25 ± 0.494 a	3.25 ± 0.970 ab
11	<i>Agapanthus africanus</i>	Lily of the Nile	2.00 ± 0.834 ab	1.08 ± 0.336 a	0.42 ± 0.193 a
12	<i>Hedera helix</i>	English ivy	0.33 ± 0.243 a	1.08 ± 0.763 a	0.83 ± 0.297 a
13	<i>Sonchus oleraceus</i>	Sowthistle	O	O	0.08 ± 0.083 a
14	<i>Chenopodium berlandieri</i>	Lambsquarter	O	0.33 ± 0.188 a	0.33 ± 0.256 a
15	<i>Malva neglecta</i>	Cheeseweed	O	O	0.92 ± 0.288 a
16	<i>Senecio vulgaris</i>	Common Groundsel	O	O	O
17	<i>Rhus integrifolia</i> *	Lemonade Berry	0.33 ± 0.263 a	0.58 ± 0.193 a	1.17 ± 0.767 a
18	<i>Heteromeles arbutifolia</i> *	Toyon	2.00 ± 0.872 ab	0.33 ± 0.188 a	0.67 ± 0.497 a
19	<i>Baccharis pilularis</i> *	Coyote Brush	1.25 ± 0.740 ab	0.92 ± 0.609 a	1.42 ± 0.434 a
20	<i>Lonicera subspicata</i> *	Honeysuckle	0.08 ± 0.083 a	0.17 ± 0.112 a	0.08 ± 0.083 a
21	<i>Opuntia basilaris</i> *	Beavertail Cactus	O	O	0.33 ± 0.333 a
22	<i>Oenothera speciosa</i>	Mexican Evening Primrose	0.33 ± 0.067 a	0.25 ± 0.131 a	1.42 ± 0.452 a
23	<i>Populus candicans</i>	Cottonwood	4.92 ± 1.493 bc	205.67 ± 96.643 b	54.25 ± 8.927 f
24	<i>Platanus occidentalis</i>	"Bloodgood" Sycamore	13.33 ± 3.404 e	12.75 ± 4.961 a	6.58 ± 1.694 abc
25	<i>Prunus subhirtella</i>	Akebone Ornamental Cherry	13.83 ± 4.606 e	17.08 ± 8.164 a	4.67 ± 1.689 abc

* California native plant

O life stage not recorded on host plant species

¹ Mean number of egg masses recorded on host plant species over all three locations (different letters indicate significant differences, Kruskal Wallis $t=133.69$, $P<0.0001$).

² Mean number of adults recorded on host plant species over all three locations (different letters indicate significant differences, Kruskal Wallis $t=154.54$, $P<0.0001$).

³ Mean number of nymphs recorded on host plant species over all three locations (different letters indicate significant differences, Kruskal Wallis $t=194.54$, $P<0.0001$).

When considering life stages at the different locations, more egg masses were found on the host plants in Pauma valley between June 24 and August 19 compared to both Piru and Redlands in the same period (unequal variance: Kruskal Wallis: $t=7.237$, $P=0.027$) (Fig. 1a). The numbers of eggs per egg mass was significantly higher in Pauma (ANOVA $df=2$, $F=10.93$, $P<0.001$), a larger portion of the eggs were parasitized in Pauma (ANOVA $df=2$, $F=10.67$, $P<0.001$), with no difference in emergence of eggs masses (ANOVA $df=2$, $F=3.04$, $P=0.05$). The portion survival of eggs per egg mass is lowest in Pauma (ANOVA $df=2$, $F=10.80$, $P<0.001$) (Table 2).

Of the parasitized egg masses recorded in Piru, all were *Gonatocerus* sp., but in Redlands 6% were parasitized by *Trichogramma* sp as were 4% of the egg masses from Redlands. The survival of *Trichogramma* parasitized egg masses was 0.595 ± 0.0544 significantly lower than the survival of *Gonatocerus* parasitized egg masses 0.764 ± 0.011 (unequal variance: Kruskal Wallis $t=11.89$, $P=0.000563$). No differences were found between the egg mass size and the fraction parasitized for *Trichogramma* or *Gonatocerus* (results not shown).

Table 2 The survival, fraction parasitized and fraction emerged parasitoids recorded in GWSS egg masses in Piru, Redlands and Pauma Valley, California.

	Location			df	ANOVA	
	Piru	Redlands	Pauma Valley		F	P
N	197	172	557			
#eggs/egg mass	11.56 ± 0.467 a	12.02 ± 0.499 a	13.81 ± 0.278 b	2	10.93	<0.001
Survival	0.847 ± 0.0237 b	0.795 ± 0.0254 b	0.725 ± 0.0141 a	2	10.80	<0.001
Fraction parasitized	0.666 ± 0.029 b	0.676 ± 0.031 b	0.545 ± 0.017 a	2	10.67	<0.001
Fraction emerged parasitoids	0.804 ± 0.0288 a	0.848 ± 0.0312 a	0.762 ± 0.0187 a	2	3.04	0.051

POPULATION DYNAMICS AND INTERACTIONS BETWEEN THE GLASSY-WINGED SHARPSHOOTER AND ITS HOST PLANTS IN RESPONSE TO CALIFORNIA PHENOLOGY

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Reporting period: The results reported here are from work conducted from November 2003 to October 2004.

INTRODUCTION

The focus of this research is to determine the relative phenology (the timing of biological events as influenced by the environment and intrinsic biological phenomena) of host plant use by glassy-winged sharpshooter (GWSS), other leafhopper vectors and natural enemies, and *Xf* in ornamental, agricultural and CA native host plants in key CA locations in climatically different regions: Coastal (Piru, Ventura County), Inland (Redlands, San Bernadino County), and South (Pauma Valley, San Diego County). As year 1 of a 3 year study, we plan to replicate this years' observations (only if continued CDFa funding is reinstated and received) using fresh host plants at the same locations, and full analyses of results will not be available until after all data is collected. The findings of this first season are therefore presented as preliminary results.

This research will be used to develop a GWSS performance database on the host plant species that are identified as truly critical to GWSS survival, which is needed to fully support decision making, and to supplement what is observed in the field. Currently, no quantitative data is available on the relative suitability of single or multiple hosts most relevant in Southern California's agriculture, landscape or native vegetation, to GWSS growth and development. This project will provide this baseline information, identify host plant limitations at different life stages and will ultimately identify key nutrients responsible for this phenomenon.

OBJECTIVES

Use 25 different host plant species in 4 replicates per location at three locations: Coastal (Piru, Ventura County), Inland (Redlands, San Bernadino County), and South (Pauma Valley, San Diego County) to:

1. Determine the age structure and utilization of GWSS on the host plants throughout the season
2. Determine the GWSS egg parasitization and mortality, together with the presence of general predators on the host plants throughout the season
3. Determine GWSS fecundity and feeding rate on selected host plants
4. Determine the presence of *Xf* in host plants at three times during the season
5. Determine the chemical composition of the host plant xylem fluids at tree times during the season.

RESULTS

From April onwards, the GWSS age structure and resident generalist predators on 25 different host plants were observed weekly. In four replications, 25 potted (5gal) host plants were used to test the preference of resident GWSS at 3 Southern California locations within unsprayed citrus orchards. For each replication 25 plant pots were placed in a completely randomized block design within the rows. Each block was enclosed in a 5x5ft square pen made with chicken wire. Plants were hand watered 2-3 times per week. The plant species were selected for their common ornamental or agricultural use or their status as orchard weeds or their occurrence in foothill and riparian environments in Southern California (Table 1).

Batch samples from each of the host plant species were tested for the presence of *Xf* on three occasions between April and July. With the exception of one *H. helix* batch sample in May, all batch samples tested negative. In follow-up tests of single *H. helix* plants, no individual plant tested positive for *Xf*.

technology is being used to screen the more than 250 DNA samples extracted from mycosed GWSS collected from throughout the southeastern US. This work will be summarized and submitted for publication in December 2004.

Analysis of the Dynamics of the *Hirsutella* in GWSS Populations

A field plot containing 14 cultivars of crape myrtle (total 224 trees) was established at the NFREC. Four subplots, each containing 40 trees, were established within this stand. Two subplots were fitted with an overhead mist irrigation system that was operated 15 minutes every hour, 24 hours a day. Throughout the summer, trees were sampled by counting both the live GWSS and number of mycosed GWSS. Mycosed GWSS were flagged and their positions on the trees were noted. It should be noted that throughout the season the species of *Hirsutella* accounted for virtually 100% of the disease on the GWSS. Preliminary analysis demonstrated a non-uniform distribution of live GWSS and mycosis GWSS in the plot. In part this could be related to both the cultivar and/or to the presence the misting irrigation system. The cultivars attractive to GWSS ('Osage', 'Miami', 'Tonto') contained higher levels of mycosed GWSS. Irrigated crape myrtle, regardless of the cultivar, contained significantly higher mycosed GWSS than did the non-irrigated trees. Currently, the field data from this season is being combined with the positional (cardinal orientation) data and will be subjected to additional statistical analysis

CONCLUSIONS

We have identified and have in culture several isolates of a primary pathogen and potential GWSS biological control agent, *Hirsutella sp.* Molecular methods have been established and are being used to diagnosis GWSS collected from sites throughout the southeastern US. This past field season the dynamics of *Hirsutella* has been examined in replicated crape myrtle plots.

FUNDING AGENCIES

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

MYCOPATHOGENS AND THEIR EXOTOXINS INFECTING THE GLASSY-WINGED SHARPSHOOTER: SURVEY, EVALUATION, AND STORAGE

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Reporting Period: The results reported here are from work conducted from December 2003-October 2004

ABSTRACT

A species of *Hirsutella*, the primary pathogen of GWSS in the southeastern US, has been the major focus of our research this past year. Due to the fastidious growth requirements of this fungus and the presence of numerous saprobic fungi associated with mycosed GWSS, a major effort has been made to design a series of gene-specific primers to be used to detect these diseases in field collected samples. Molecular-based diagnosis is being used to examine the hundreds of mycosed insects collected during the 2003 and 2004 regional surveys. A second effort has been directed at examining the seasonal incidence of this disease in an experimental crape myrtle plot. A number of parameters such as crape myrtle variety, host density, mist irrigation (humidity) have been found to influence the onset of *Hirsutella* in GWSS populations. Current laboratory research is being directed at examining transmission of the lab culture to both GWSS and to alternate insect hosts. In addition, culture filtrates of all of the fungi collected from GWSS are being assessed for the presence of active metabolites.

INTRODUCTION

We are not aware of any studies that have examined the insect pathogens associated with populations of GWSS. In general, the lack of pathogens (viral, bacterial, or protozoa) in leafhopper populations may be related to their piercing-sucking feeding behavior. In most cases, these pathogen groups are transmitted orally and would likely need to inhabit the xylem tissue to infect leafhoppers. Pathogens that are transmitted *per os* are typically affiliated with insects with chewing mouthparts. Thus, entomopathogenic fungi, which do not need to be ingested in order to infect insects, are considered to contain the primary pathogens of sucking insects. Indeed, the primary pathogens operating against insects such as whiteflies, scales, aphids, spittlebugs, plant hoppers, and leafhoppers are insect fungi (for listing see USDA-ARS Collection of Entomopathogenic Fungal Cultures at <http://www.ppru.cornell.edu/mycology/catalogs/catalog>). We commonly observe all mobile stages of GWSS exhibiting mycoses in north Florida and we are identifying them and assessing their impact.

OBJECTIVES

1. Identify and archive all the major pathogens affiliated with GWSS populations.
2. Estimate the distribution, frequency and seasonality of the major diseases of GWSS.
3. Screen the pathogens for exotoxins with potential toxicity to GWSS and other arthropods.
4. Confirm infectivity of the isolates and the exotoxins and determine which if any pathogens may serve as microbial controls of GWSS and other leafhopper vectors.

RESULTS

Pathogen Distribution

In the past field season we continued to survey the incidence of disease in GWSS populations in the Southeast. The purpose of this survey was twofold: first, to piece together a better picture of the distribution of the Glassy-winged Sharpshooter in the area. Secondly, it gave us the opportunity to investigate the varieties and incidence of fungal pathogens associated with this host. The survey area encompassed four states, Mississippi, Louisiana, Alabama, and Texas. A series of live GWSS and a total of 95 mummified GWSS were collected from sites in these states. In most cases, the external characters mimicked those observed on the cadavers collected from sites in Georgia, South Carolina, and Florida in 2003. The presence of various opportunistic fungi on field-collected samples has limited our abilities to culture the more fastidious slow growing species of *Hirsutella*, *Sporothrix*, and *Pseudogibbellula*. The aforementioned fungi were identified last year to be key entomopathogens isolated from GWSS populations. After multiple cycles of isolation we were able to isolate target fungi from only about 10% of these insects, the vast majority of cultures contained saprobic fungi. In order to confirm the presence of the *Hirsutella* (the primary pathogen) we have developed and optimized PCR primers within unique intron motifs of both the actin and tubulin genes that have been matched with primers from the open-reading frame. Control reactions have demonstrated that these primer combinations are able to specifically amplify the GWSS *Hirsutella* from DNA extracted from mummies. This

CONCLUSIONS

Our work in untreated citrus groves has enabled us to explore what happens to uncontrolled GWSS populations. After an additional year of data, the GWSS densities on valencias and lemons are sufficient to allow us to tentatively forecast the time at which the GWSS will attain their minimum densities on each host cultivar. The analyses show that GWSS are decreasing at a rate that, if sustained, may drive GWSS populations to very low levels. The first technique used predicts minimum densities for GWSS to be achieved during the next three to six years. The second technique, the phase diagram, indicates that an extinction of GWSS is unlikely, and that the populations on valencias and lemons are each cycling around an equilibrium point. During periods when populations are above their equilibrium density, we are likely to see GWSS densities above 1000 adults per tree. In addition, we have shown that GWSS populations manifest different dynamics in different places. As the populations become less dense, their dynamics will bring stability, allowing GWSS to recolonize areas where densities are low when GWSS adults move from areas where GWSS densities remain high (see figure 4, grapefruits as an example). This type of behavior, called metapopulation dynamics as it is known to bring stability in a wide range of biological systems where animals can readily move from one place to another. This appears to be the case for the GWSS and we expect to see these type of dynamics to emerge in the next few years.

FUNDING AGENCIES

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

A more interesting analysis using the population samples from Valencia and Lemon trees is presented in Figures 5 and 6. We plotted the total adult and the newly emerged (red-veined) adult density using a logarithmic scale. We then used a forecasting technique on these data for Valencia and Lemons separately, i.e. the lines in Figures 5 and 6 which show what would happen if the current trend is extrapolated until it reaches zero. Although it is unlikely that GWSS will ever reach zero, we use these plots to estimate a minimum and a maximum date when we expect these populations to reach their minimum. These two dates are estimated by the lines crossing the X-axis in each graph and encompass the time period during which we estimate that GWSS adult populations will reach their minimum.

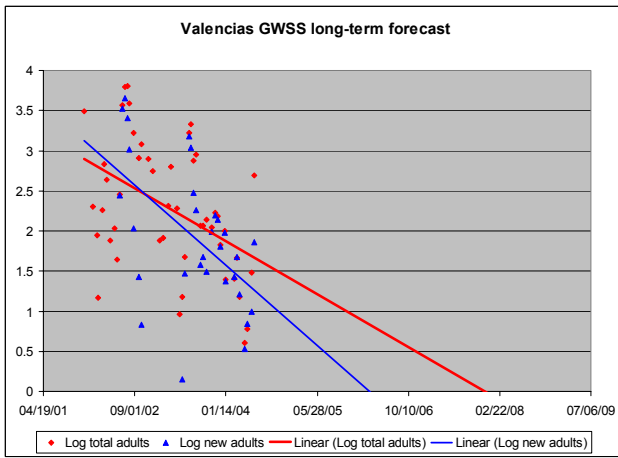


Figure 5. Logarithm of total and new adults in Valencias with trend lines showing expected “zero density” dates.

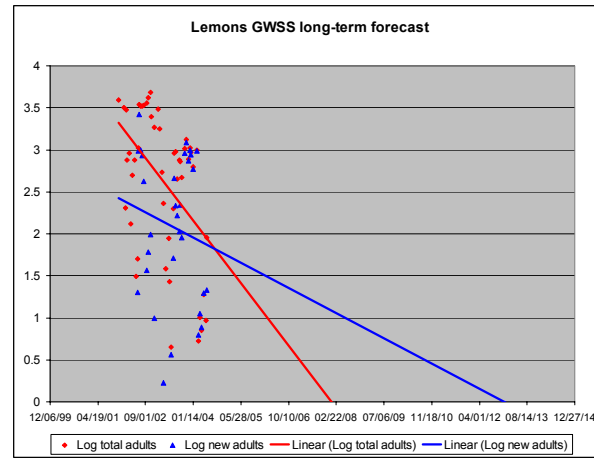


Figure 6. Logarithm of total and new adults in lemon with trend lines showing expected “zero density” dates.

If the current trend continues for several years the adult GWSS will reach their minimum densities within the next three to six years. However, as new data are collected and plotted on these graphs a more refined minimum density will be obtained but it is extremely unlikely that the GWSS densities will become extinct. A second and even more powerful technique can be used to analyze the GWSS dynamics (figures 7 and 8). These figures need some explanation. What they show is a plot of GWSS adult densities at any a specific date, as a function of the density at a previous time interval. In our case, it is the density of adult GWSS at a given week, as a function of the density two weeks previously. In a sense, it explores the effect on a given date’s density, of the density two weeks prior. When plotted in this manner, we get a phase diagram that shows whether the GWSS population density is cycling and, if it is cycling, it shows the density around which the population is likely to be cycling. Figure 7 shows the phase diagram for Valencias. The point, at which the two diagonal lines cross, shows the density around which adult GWSS population cycles, generation after generation. This does not mean that the population will reach an equilibrium density at exactly that density. Rather, it indicates the density around which the population will cycle. For Valencias, this equilibrium density is about 600 adults per tree, and for lemons, it is about 950 adults per tree. Thus, this analysis suggests that GWSS will never reach “zero density,” but will alternatively reach densities above and below the cycling density at different times of the year and in different years. The data sets for tangerines and grapefruit do not encompass a sufficient enough period of time to allow this kind of analysis. We will need at least another year of GWSS data before we can conduct this analysis using the forecasting technique. At the same time, a longer dataset for Valencias and lemons will likely improve the accuracy of this analysis.

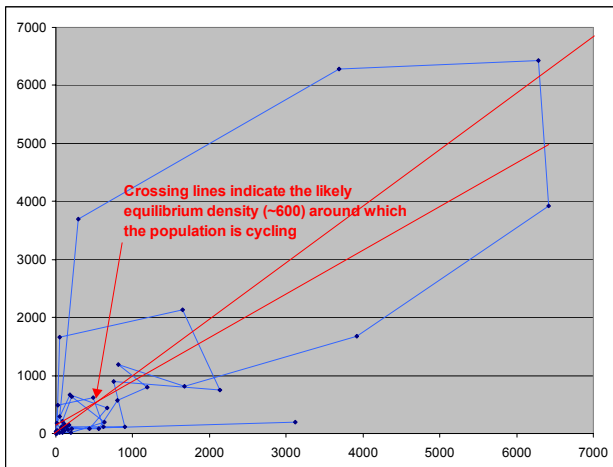


Figure 7. Phase diagram for adult GWSS dynamics in GWSS Valencias (see text).

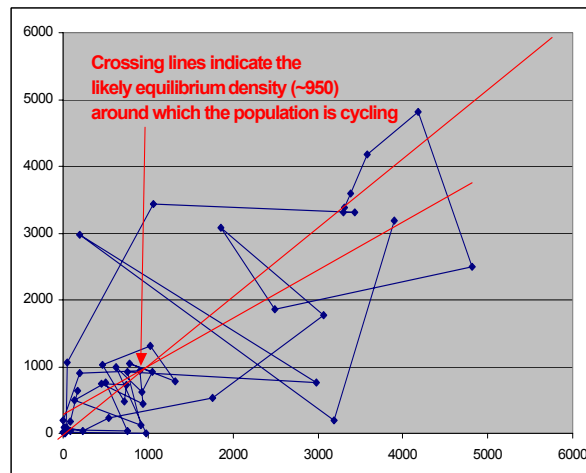


Figure 8. Phase diagram for adult dynamics in Lemons (see text).

timed might drive the GWSS population below its critical density, thus leading to its local extinction. To fulfill this goal, we propose the following objectives:

- 1- Expand our current studies to follow GWSS population dynamics at a landscape level, including urban areas, using our whole host plant sampling technique.
- 2- Determine the relative contribution of the principal host plants to the adult GWSS production in each generation.
- 3- Determine whether correlations exist between GWSS' population dynamics on a given host tree and the host's xylem chemistry and whether this correlation explains GWSS' variable performance seasonally on different host plants.
- 4- Use this information to identify critical periods during GWSS' annual population cycle where selective control strategies might drive its local populations nearly extinct.

RESULTS

The number of adult GWSS in untreated Valencia and lemon trees at the Agricultural Operations fields, University of California, Riverside has declined during the two and a half years of our study (Figure 1 through 4). GWSS densities on Tangerines and Grapefruit trees involves one and a half GWSS generations and, thus, is too short a period for a meaningful analysis of GWSS on these citrus varieties. Figures 1 and 2 show the mean number of adult GWSS obtained from three Valencias and three lemons per sampling date, during the two and a half year sampling period. It is clear that a significant downward trend has occurred in the number of GWSS adults during the two and a half years. Peak densities have decreased by 67% for Valencias and 75% for lemons between 2002 and 2003. At the time of this report, we had not reached the peak adult densities for 2004, which typically occur in late August to early September. The GWSS samples from Tangerines and Grapefruit also show a decreasing trend. The average number of new adults produced in the three Valencia and the three lemon trees per sampling date also declined during the two and a half year study (Figure 1 & 2).

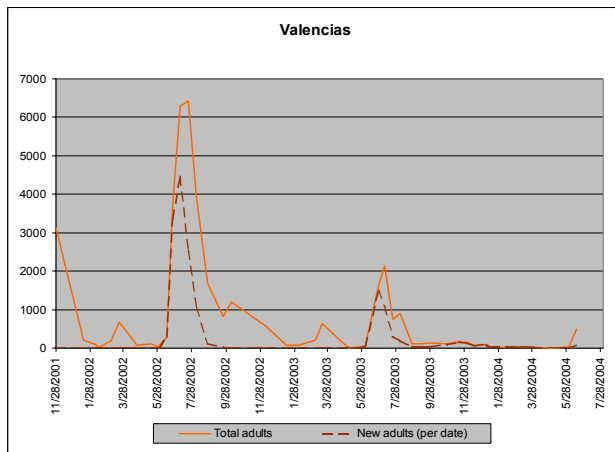


Figure 1. Actual adult GWSS densities (solid line) and newly produced adults per date (dotted line) in an untreated Valencia grove.

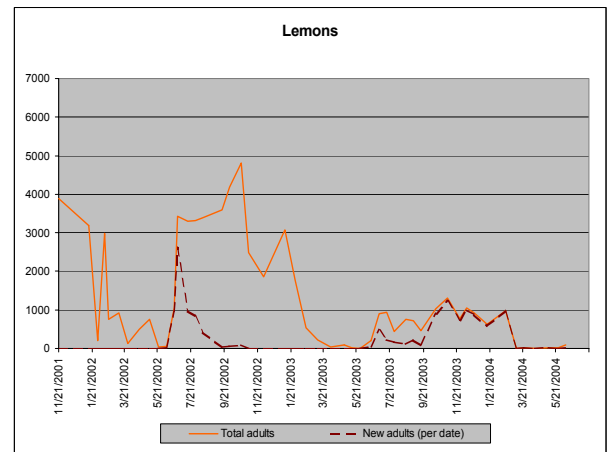


Figure 2. Actual adult GWSS densities (solid) and newly produced adults per date (dotted) in an untreated Lemon grove.

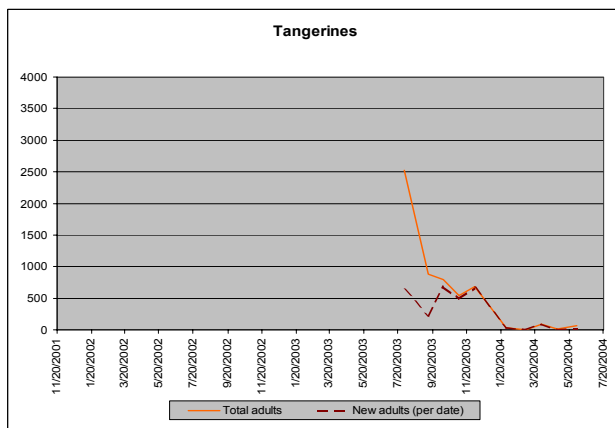


Figure 3. Actual adult GWSS density since Fall 2003 in an untreated Tangerine grove.

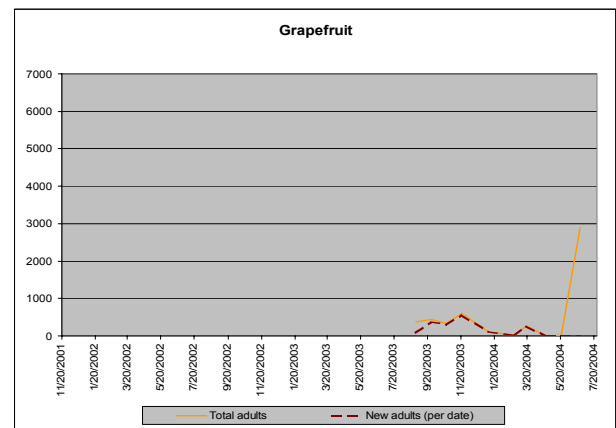


Figure 4. Actual adult GWSS density since Fall 2003 in an untreated Grapefruit grove.

GLASSY-WINGED SHARPSHOOTER'S POPULATION DYNAMICS AS A TOOL FOR ERADICATING GLASSY-WINGED SHARPSHOOTER POPULATIONS

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ABSTRACT

Our results indicate that **1)** GWSS populations in untreated areas have been declining steadily during the last three years. Current populations are only 10 to 20% as dense as those during 2001-2002. **2)** Forecast analysis indicates that, if the current trend is extrapolated, GWSS populations in untreated areas should decrease to negligible numbers some time after winter, 2008, and before summer 2013, depending on *Citrus* species. However, **3)** analyses of the data sets currently available, show that adult GWSS densities are cycling around a possible equilibrium level of 600 adults in Valencias and 950 adults in lemons, when left untreated. The period encompassed by the data sets for Tangerines and Grapefruit is still too short for this type of analysis. **4)** Overall, less than 30% of the first instar nymphs survive to the fifth instar nymphs, and less than 15% of these nymphs survive to become adults. **5)** During this last winter (2003-2004), overwintering adult densities declined in grapefruit, tangerines, and oranges but they increased in lemons, in the absence of any significant production of nymphs. The latter suggests that adult GWSS were moving among trees and cultivars due to changes in the nutritional and/or moisture status of these trees. We will use the xylem fluid samples currently being analysed, to test this hypothesis.

INTRODUCTION

It is widely recognized that disrupting *Xylella* transmission and preventing Pierce's disease (PD) epidemics requires Glassy-winged sharpshooter (GWSS) population levels to be exceedingly scarce. Recognizing critical points in GWSS' annual population cycles will allow us to identify the spatial and temporal scales during which GWSS populations are vulnerable to control measures timed to coincide with critical densities in its populations that can drive its local populations nearly extinct. In addition, determining whether GWSS populations will continue to decrease and eventually stabilize in the absence of pesticides but in the presence of parasitoids is of the utmost importance. Currently, almost all citrus groves infested with GWSS in California are treated. The groves at Agricultural Operations, University of California Riverside, are an exception. Our work in these untreated groves provides a means of exploring the dynamics of GWSS populations in untreated citrus groves exposed to egg parasitism. The results from these studies might also suggest the expected dynamics of GWSS populations inhabiting urban environments where GWSS is under little or no control except by egg parasitoids.

Our results to date suggest that GWSS has a major reproductive period during the spring and a second reproductive period during autumn. This autumn generation involves a dense egg population laid by the GWSS arising from the spring generation but very few of these eggs mature to become adult GWSS. Furthermore, nymphal mortalities are quite high, only about 30% of the first instar nymphs reach the last nymphal stage, and less than 15% of these first instar nymphs survive to become adults, but this varies between Citrus varieties. Although the source of this egg and nymphal loss still needs to be explored, we have measured egg parasitism ranging from 78% to 92% during the second half of the year. It is at this point that the GWSS may be vulnerable to a selective control measures. Our studies also showed an 80 to 90% decline during the last three years in valencias and lemons. The period of one year during which we have been sampling tangerines and Grapefruit is still too short to conduct a worthwhile analysis for these varieties (See figures 1 to 4). Next year's samples from the four citrus varieties will be crucial in testing whether the pattern in GWSS' dynamics continues or is transient.

OBJECTIVES

This project seeks to characterize GWSS' spatial and temporal dynamics involved in its annual population cycles on its dominant host, i.e. *Citrus sp.* We seek to identify periods in this cycle during which selective control measures, appropriately

Table 4. Development time of *G. ashmeadi* within *H. coagulata* eggs of different ages when parasitized at varying densities.

Density	Development time (SE) at age:				
	1d	3d	5d	7d	9d
10	15.9(0.6) d	21.0(2.1) a	17.9(1.6) c	15.7(1.4) e	17.6(1.4) c
20	16.5(0.8) a	18.5(1.6) c	18.0(1.1) c	18.3(0.9) a	18.3(0.8) b
30	16.5(0.7) b	18.6(2.2) c	18.8(1.3) b	18.1(0.9) ab	19.1(1.2) a
40	16.1(0.8) c	18.3(2.2) c	17.8(1.5) c	18.0(1.0) bc	16.9(1.6) d
50	16.0(1.4) cd	18.6(1.5) c	19.5(1.2) a	17.8(0.6) c	17.4(1.4) c
60	15.5(0.7) e	19.4(1.0) b	17.4(1.0) d	17.2(1.2) d	18.1(1.2) b
	$F = 45.39$	$F = 37.00$	$F = 88.13$	$F = 84.08$	$F = 73.93$
	df = 5,1434	df = 5,995	df = 5,1180	df = 5,960	df = 5,1253
	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

Means in a column followed by different letters are significantly different ($P < 0.05$, GLM) in ANOVA (Duncan).

Table 5. Number (mean \pm SE) of *G. ashmeadi* eggs per host egg, percentage of emergence and development time at different parasitoid-to-host egg ratios.

Parasitoid-host ratio	No. parasitoid / host		% Emergence		Development time	
	N ₁	Mean \pm SE	N ₂	Mean \pm SE	N ₃	Mean \pm SE
1:1	50	10.40 \pm 4.86 a	NA	NA	NA	NA
1:5	100	3.02 \pm 1.69 b	11	97.6 \pm 1.7 a	141	18.02 \pm 0.07a
1:10	100	2.24 \pm 1.16 c	15	98.9 \pm 0.6 a	136	18.20 \pm 0.06 b
1:15	100	1.66 \pm 0.89 d	77	93.6 \pm 1.5 b	490	18.30 \pm 0.04 b
1:20	100	1.20 \pm 0.59 d	70	91.7 \pm 1.0 b	263	18.25 \pm 0.04 b
1:25	100	1.15 \pm 0.58 d	71	90.0 \pm 1.7 b	833	18.77 \pm 0.03 c

Means in a column followed by different letters are significantly different ($P < 0.05$, GLM) in ANOVA (Duncan). N₁ represents the number of dissected host eggs, N₂ represents the number of egg masses observed, and N₃ is the number of parasitoid emerging from host eggs.

FUNDING AGENCIES

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

Table 1. Parasitism by *G. ashmeadi* on *H. coagulata* eggs of different ages at varying densities.

Density	Mean No. Parasitized (SE)				
	1d	3d	5d	7d	9d
10	9.5(1.3) a	8.7(2.2) a	8.9(1.6) a	9.0(2.2) a	9.1(1.1) a
20	18.1(1.6) b	15.5(3.2) b	14.8(3.4) b	14.6(3.9) ab	14.7(3.3) ab
30	22.9(3.0) c	17.9(8.4) b	22.0(5.8) c	19.8(7.7) bc	18.7(4.1) b
40	26.5(4.7) cd	22.2(9.8) bc	25.1(7.3) cd	22.7(5.6) c	25.8(6.2) c
50	30.3(7.5) d	25.6(10.0) cd	29.4(5.1) de	23.9(11.9) cd	29.5(13.1) c
60	34.8(4.7) e	30.7(6.9) d	32.2(4.5) e	29.9(7.3) d	30.1(3.4) c
	$F = 43.12$	$F = 11.02$	$F = 31.69$	$F = 10.59$	$F = 16.96$
	df = 5,59	df = 5,59	df = 5,59	df = 5,59	df = 5,59
	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.001$

Means in a column followed by different letters are significantly different ($P < 0.05$, GLM) in ANOVA (Duncan).

Table 2. Coefficients of determination for functional response regression models of *G. ashmeadi* to *H. coagulata* eggs of different ages.

Age of Eggs (d) ^a	Type I (r^2)	Type II (r^2)	Type III (r^2)
1	0.7776	0.9729	0.9727
3	0.4979	0.8993	0.8992
5	0.7260	0.9607	0.9608
7	0.4783	0.9038	0.9036
9	0.5872	0.9280	0.9280

^a *G. ashmeadi* targeted host densities ranged from 10 to 60 sharpshooter eggs per experimental container. Type I functional response model was evaluated using SAS PROC GLM whereas Type II and III models were evaluated using SAS PROC NLIN to generate r^2 values indicating best fit.

Table 3. Type II and III functional response parameters of *G. ashmeadi* when parasitizing *H. coagulata* eggs of different ages.

Functional response model	Host age (d)	Instantaneous attack rate ($a \pm SE$) ^a	Handling time ($T_h \pm SE$) ^a
Type II	1	0.5782 ± 0.0626 a	0.0300 ± 0.0004 a
	3	0.4544 ± 0.0959 a	0.0315 ± 0.0105 a
	5	0.5013 ± 0.0640 a	0.0286 ± 0.0058 a
	7	0.5064 ± 0.1088 a	0.0377 ± 0.0099 a
	9	0.4831 ± 0.0849 a	0.0296 ± 0.0082 a
Type III	1	2.8131 ± 2.2011 a	0.0342 ± 0.0056 a
	3	1.0137 ± 0.5410 b	0.0333 ± 0.0117 a
	5	1.4394 ± 0.6301 b	0.0316 ± 0.0067 a
	7	1.3858 ± 0.9508 b	0.0403 ± 0.0113 a
	9	1.2495 ± 0.6620 b	0.0322 ± 0.0094 a

^a Instantaneous attack rate (a) and handling time (T_h) estimated by SAS PROC NLIN and pairwise compared among host ages using indicator variable (0 or 1) for age.

from type II functional response models varied slightly but were not significantly different among host ages (Table 3). The a value for 1-d-old eggs was slightly higher than that for other ages when data were fit to a type II functional response model. The estimate for handling time (time spent on eggs) by the wasps for all host egg ages did not vary significantly. When the data were fitted to a type III functional response model, the a value estimated for 1-d-old eggs was significantly higher than that for host eggs of 3-, 5-, 7- and 9-d-old. However, the handling time of *G. ashmeadi* for all egg ages was similar, ranging from the value of 0.032 to that of 0.040.

Effect of Host age on Parasitoid Development Time

The development time of *G. ashmeadi* within host eggs varied significantly with host density and host age (Table 4). Within the 1-, 3-, 5-, 7- and 9-d-old host eggs, the mean development time (\pm SE) of the parasitoid was 16.0 \pm 1.0 d ($n = 1435$), 18.9 \pm 1.8 d ($n = 996$), 18.3 \pm 1.5 d ($n = 1181$), 17.6 \pm 1.2 d ($n = 961$) and 17.8 \pm 1.5 d ($n = 1254$), respectively. The parasitoid within 1-d-old sharpshooter eggs developed significantly faster than that within other ages ($F = 766.41$, $df = 5$, 5826, $P < 0.0001$). A two way ANOVA further showed that host age ($F = 999.47$, $df = 4$, 5826, $P < 0.0001$) and density ($F = 58.26$, $df = 5$, 5826, $P < 0.0001$) contributed significantly to the development time of *G. ashmeadi*. The significant interactive effect on development time occurred between host age and density ($F = 62.82$, $df = 20$, 5826, $P < 0.0001$).

Super-parasitism.

Maximum number of parasitoid eggs in one host egg was 18. The level of super-parasitism of *G. ashmeadi* (Table 5) varied significantly with increasing host density ($F = 225.17$, $df = 5,549$, $P < 0.0001$). The mean number of parasitoid eggs per sharpshooter egg at 1:1 parasitoid-to-host ratio is significantly greater than that at other ratios. When the parasitoid-to-host ratio increased to $> 1:15$, host eggs pooled from each host density were almost all parasitized. There was a significant positive correlation between the number of parasitoid eggs per host egg and parasitoid-to-host ratio ($F = 1231.69$, $df = 548$, $r = 0.8319$, $r^2 = 0.692$, $P < 0.0001$). *G. ashmeadi* is a solitary parasitoid and normally only one wasp emerges from each egg of its host. In treatments with high host densities such as at 1:1 and 1:5 parasitoid ratios, the percentage of parasitoid eclosion was significantly higher than in low-density treatments ($F = 3.996$, $df = 4,243$, $P = 0.004$)(Table 5). However, there is no correlation between parasitoid-to-host ratio and percentage of parasitoid eclosion ($F = 3.29$, $df = 242$, $r = 0.1140$, $r^2 = 0.013$, $P = 0.071$). Although there was a significant statistical difference in development time of the parasitoid within the host egg among different parasitoid-to-host ratios ($F = 46.851$, $df = 4$, 1862, $P < 0.0001$), the maximum difference was only about 0.7d.

For *G. ashmeadi*, χ^2 goodness-of-fit analyses of parasitoid egg numbers per host egg revealed that frequencies of super-parasitism were significantly different from the expected Poisson distribution over all host densities ($\chi^2 = 231.291$, $df = 4$, $P < 0.0001$). The relationship between the variances (S^2) and means (m) was described by Taylor's power law (Taylor 1961) as: $\log S^2 = -0.4384 + 1.0288 \log m$ ($r^2 = 0.604$, $df = 28$, $F = 42.78$, $P < 0.0001$, where $b = 1.0288 > 1$, indicating an aggregated distribution of super-parasitism for *G. ashmeadi* over all experimental parasitoid-to-host ratios.

CONCLUSIONS

The studies on the functional responses of *G. ashmeadi* to GWSS eggs of different ages and densities in the laboratory have improved our understanding of the interactions between the parasitoid and host egg. Because this parasitoid fits the II and III functional response models in relation to different host ages, it further confirms that the wasp has the capacity of effectively parasitizing eggs throughout most of the embryonic development of the GWSS. Further, studies on super-parasitism of *G. ashmeadi* provide valuable information for the mass-rearing and field release of this parasitoid. Our results indicate that super-parasitism occurs when the parasitoid-to-host ratio is greater than 1:15. Super-parasitism results in a waste of the reproductive potential of this species because *G. ashmeadi* is a solitary-developing wasp and usually only one parasitoid emerges from one GWSS egg.

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PARASITISM OF THE GLASSY-WINGED SHARPSHOOTER: FUNCTIONAL RESPONSES AND SUPER-PARASITISM BY THE EGG PARASITOID *GONATOCERUS ASHMEADI*.

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ABSTRACT

The functional responses and super-parasitism by the egg parasitoid, *Gonatocerus ashmeadi*, on *Homalodisca coagulata* eggs were related to host age and density when studied under laboratory conditions. Parasitism of Glassy-winged Sharpshooter (GWSS) eggs, 1-, 3-, 5-, 7- and 9-d-old, was measured at $22 \pm 1^\circ\text{C}$ and under 10L:14D regime. For each host age, 10-60 eggs were exposed to an individual parasitoid for 24 h. The functional responses for the parasitoids to host eggs of all age groups most closely fit the type II and III models of Hollings (1959) and Hassell (1978) which relate to the elapsed time for accomplishing the behavioral events associated with parasitism of the host as modified by host density. The instantaneous attack rate by parasitoids on 1-d-old host eggs, as specified in the type III model, was significantly greater from that of the other ages. This rate was also greater in the type II model but was not statistically significant. The total number of host eggs parasitized varied significantly with host density and age of the eggs, but not when analyzed by a host x density interaction. Host age and density, as well as the host x density interaction, contributed significantly to the differences found in length of development time of *G. ashmeadi* within host eggs. The wasps exhibited a tendency towards super-parasitism at relatively high parasitoid-to-host ratios. The maximum number of parasitoid eggs found in a single host egg was 18. The development time and eclosion of the parasitoids had no correlation with parasitoid-to-host ratios. Frequencies of super-parasitism for *G. ashmeadi* displayed an aggregated distribution over all observed host densities.

INTRODUCTION

The effectiveness of parasitoids in regulation of a pest population is highly dependent on their ability to search for and handle hosts in a varying ecosystem. This effectiveness has been traditionally related to the functional response of a parasite or predator (Hassell 1978, Fujii *et al.* 1986). The functional response is defined as the relationship between the numbers of prey taken by the predator as a function of prey density (Holling 1959). The functional response is an essential component of the dynamics of host-parasitoid relationship, and is an important determinant of the stability of the system (Oaten and Murdoch 1975). Functional response analyses are commonly used to help predict the potential for parasitoids to regulate host population (Solomon 1949, Oaten and Murdoch 1975). Successful parasitoids have the ability to discriminate among parasitized eggs, avoid super-parasitism and minimize the waste of time and energy associated with their searching and parasitizing behaviors (Godfray 1994). However, under certain circumstances, superparasitism might be adaptive (van Alphen & Visser 1990). Further, when mass-rearing solitary parasitoids for use in an augmentative release program, super-parasitism represents a waste of the production colony's potential output. This report presents the progress on investigations determining certain aspects of the functional responses and super-parasitism by the parasitoid, *G. ashmeadi*.

OBJECTIVES

1. Investigate the response of *G. ashmeadi* to GWSS eggs of different ages and determine the effects of host egg age on functional response parameters and parasitism.
2. Determine effect of host densities and ages with respect to developmental time of wasps.
3. Investigate relationship between super-parasitism by the wasp at different host densities and effect of super-parasitism on wasp emergence and development time.

RESULTS

Functional Responses

There was a significant increase in the numbers of *H. coagulata* eggs of different ages parasitized by egg parasitoid, *G. ashmeadi*, with an increase in host density (Table 1). At the host densities of 40, 50, and 60, the numbers of eggs parasitized were significantly higher than that of relatively low densities of 10 and 20 over all host ages. The number of 1-d-old eggs parasitized was slightly greater than that of 5-, 7- and 9-d-old-eggs. A two-way ANOVA, with age and density as factors, revealed that the number of eggs parasitized varied significantly with host age ($F = 3.64$, $df = 4, 299$, $P = 0.007$) as well as host densities ($F = 88.43$, $df = 5, 299$, $P < 0.0001$). There was no significant effect of age x density interaction on the number of host eggs parasitized ($F = 0.44$, $df = 20, 299$, $P = 0.899$).

The functional responses of *G. ashmeadi* parasitizing host eggs at the various ages showed that the shape of the functional response curves were affected by differences in the parasitization rates of *G. ashmeadi*. At all host ages, the *G. ashmeadi* functional response data most closely fit the type II and III models. Coefficients of determination (r^2 values) for type II and III curves were very similar (Table 2). The instantaneous attack rates (a) and handling time (T_h) estimated

Table 2. Parasitism and emergence by *G. ashmeadi* on the GWSS eggs exposed to the daily stepwise temperature regime (10, 11, 12, 13°C - changing at 6h intervals) for 15 to 140 d.

Storage time	No. egg masses	No. eggs	Parasitism (mean % ±SE)	Emergence (mean % ± SE)
15 d	7	88	74.99 ± 3.20 a	68.25 ± 3.11 a
20 d	11	106	76.98 ± 9.26 a	67.18 ± 9.23 ab
25 d	6	69	77.76 ± 6.58 a	57.15 ± 13.49 ab
50 d	21	226	47.75 ± 8.15 b	41.89 ± 8.05 bc
60 d	23	208	37.27 ± 7.49 b	28.69 ± 6.61 c
65 d	13	126	44.36 ± 8.69 b	22.58 ± 7.11 c
80 d	31	253	11.79 ± 4.68 c	7.31 ± 3.84 d
95 d	17	193	4.25 ± 2.40 c	1.90 ± 0.89 d
140 d	9	96	2.02 ± 1.38 c	2.02 ± 1.38 d
			$F = 14.934$	$F = 13.661$
			$df = 8,137$	$df = 8,137$
			$P < 0.001$	$P < 0.001$

Means within a column followed by different letters were significantly different at the significant level of 0.05 (SAS Proc GLM with LSD). Data were square-root transformed before analysis.

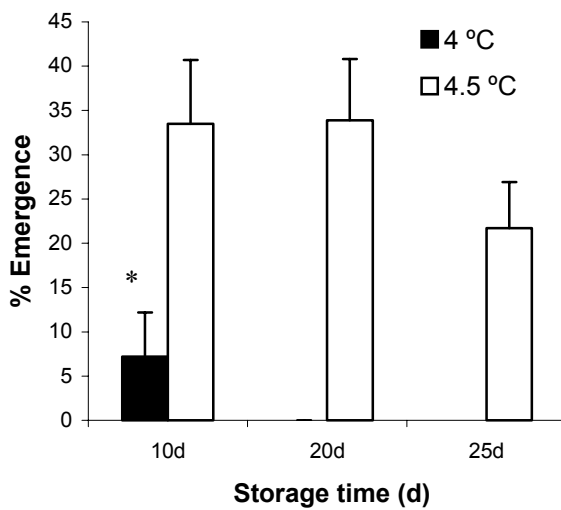


Figure 3. Percentage emergence of *G. ashmeadi* from GWSS eggs stored at constant temperatures for 10-25 d. Bar marked by an asterisk represents a significant difference ($P < 0.05$).

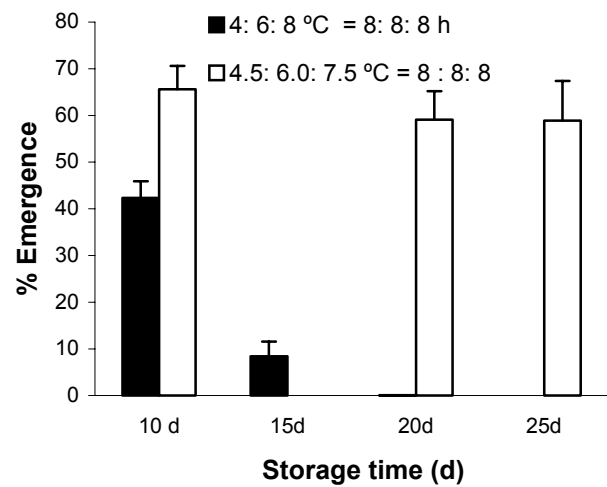


Figure 4. Percentage emergence of *G. ashmeadi* from the GWSS eggs stored at stepwise temperatures for 10-25 d. Bar marked by an asterisk represents a significant difference ($P < 0.05$).

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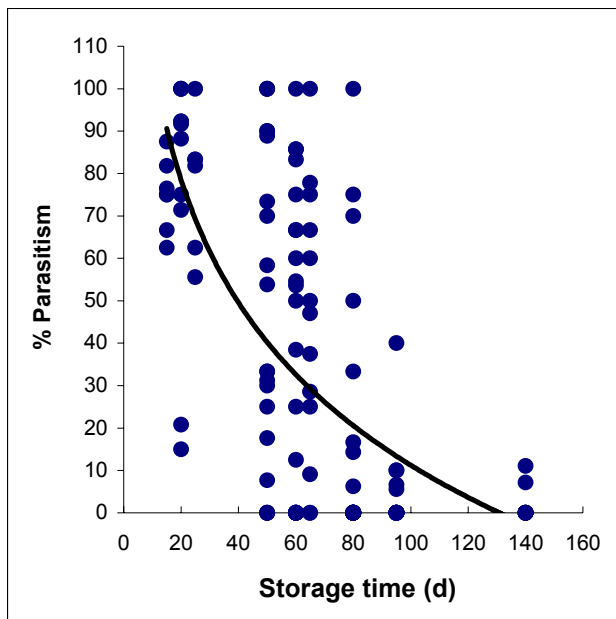


Figure 1. Relationship of the % parasitism (y) of *G. ashmeadi* to storage time (x) of the GWSS eggs at stepwise temperatures (10~13°C) ($y = 5.10 + 1393.18/x$, $r = 0.58$) ($F = 68.24$, $df = 136$, $P < 0.001$)

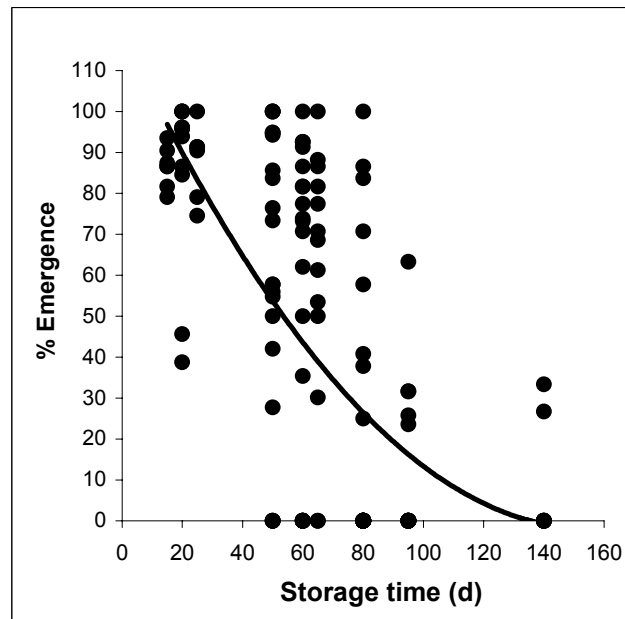


Figure 2. Relationship of the % emergence (y) of *G. ashmeadi* to storage time (x) of the GWSS eggs at stepwise temperatures (10~13°C) ($y = -0.35 + 1286.50/x$, $r = 0.59$) ($F = 79.01$, $df = 136$, $P < 0.001$).

Table 1. Egg hatch, development time of nymphs and reproduction of adults for GWSS eggs stored under different temperature conditions (mean \pm SE).

Storage conditions	Egg hatch (%)	Development time of nymphal stage			Adult reproduction	
		% survival	Male (d)	Female (d)	No. eggs/female	Ovipositional period (d)
Control (25°C)	82.9 \pm 7.4	80.2	35.9 \pm 0.5 a	35.3 \pm 0.6 a	1068.8 \pm 187.7	113.4 \pm 49.6
12°C for 20 d	52.7 \pm 10.2	50.0*	43.9 \pm 0.9 b	42.5 \pm 0.7 b	589.3 \pm 81.9	65.7 \pm 26.0
10-13°C for 15 d	73.9 \pm 5.4	50.0*	43.0 \pm 0.7 b	41.0 \pm 1.2 b	662.7 \pm 111.1	65.0 \pm 11.2
10-13°C for 20 d	62.6 \pm 10.3	40.0*	43.0 \pm 3.5 b	41.9 \pm 0.4 b	In progress	In progress

Only 1 replicate. Means within a column followed by different letters were significantly different at the significant level of 0.05 (SAS Proc GLM with LSD). Data for egg hatch were square-root transformed before analysis.

62.6 ± 9.1% (n = 98) and 44.6 ± 9.1% (n = 104), respectively. There was a significant difference in percentage hatch of 1-d-old GWSS eggs between the control eggs (83.0 ± 7.4%, n = 317) and those eggs stored for 25d ($F = 3.939$, $df = 3,45$, $P = 0.014$), but no significant differences were found between those groups stored in the cold for 15 or 20 days and the control. After storage for 80d under the daily cycled regime, no hatching was observed.

To determine effect of cold storage during GWSS egg stage on nymphal development and adult reproduction, newly hatched nymphs from eggs stored at 12 °C for 20 days, and at the daily cycled temperature regime for 15 and 20 days were reared on sunflower plants until they emerged as adults. When the characteristic patch of brochosomes was observed on the forewings of the adult females (brochosomes were considered as the sign that females had mated), they were then individually maintained on sunflower plants and their egg mass output recorded until death occurred. Our preliminary data (Table 1) shows that 50% of nymphs from eggs stored at 12°C for 20 d and 50% and 40% of nymphs held under the stepwise temperature regime for 15 days and 20 days, respectively, successfully developed into adults. In comparison with the control groups, GWSS males and females from those eggs that had been exposed to either cold storage regime took significantly longer to complete their nymphal stages (Table 1). There were no differences in male and female developmental times among the nymphs that hatched from GWSS eggs that had undergone cold storage. The number of eggs produced/female and the ovipositional period was considerably greater for the control groups and approached 2-fold differences.

Effects of Cold Storage of GWSS Eggs on Parasitism and Emergence by *G. ashmeadi*

Following storage in incubators set at a constant 12 or 12.5°C and also at the stepwise cycled regime as described above, GWSS egg masses were exposed to caged *G. ashmeadi* colonies for 2 days at room temperature (ca. 22 °C) and under an 10L:14D photoperiod. Before statistical analysis, the data recorded for parasitism and emergence were square-root transformed to correct non-normality because the number of eggs/mass was not constant.

After storage at 12°C for 30d, 69.6 ± 11.7% of the 3-d-old GWSS eggs (n = 90) and 47.7 ± 11.7% (n = 106) of the 1-d-old eggs were successfully parasitized by *G. ashmeadi*. The percentage wasp emergence was 68.5 ± 11.3 for 3 day-old eggs and 35.3 ± 10.0% for the 1 day-old eggs. There were no significant differences in the incidence of parasitism, as determined by egg dissection, ($F = 4.034$, $df = 1,14$, $P = 0.066$) and emergence ($F = 1.728$, $df = 1,14$, $P = 0.211$). Further, *G. ashmeadi* successfully parasitized about 77% of the 4-d-old, 52% of the 5-d-old, and 45% of the 3-d-old GWSS eggs stored at 12.5°C for 30d, and 46% of 3-d-old eggs stored for 50d. As above, there were no significant differences between parasitism and emergence in any of the comparable groups (data not shown).

When stored under the cycled stepwise temperature regime (10-13 °C), the parasitism ($F = 14.934$, $df = 8,137$, $P < 0.001$) and emergence ($F = 13.661$, $df = 8,137$, $P < 0.001$) of 1-d-old GWSS eggs by *G. ashmeadi* varied significantly with storage time (Table 2). More than 75% of GWSS eggs stored up to 25d were successfully parasitized and there was no significant difference in the incidence of parasitism between the control (92.1 ± 9.9%, n = 172) and the eggs stored for 15, 20 or 25d ($F = 1.764$, $df = 3,35$, $P = 0.172$). However, percentage emergence for the eggs stored for 25d was significantly lower than that for the control (91.7 ± 2.7%, n = 172) ($F = 3.250$, $df = 3,35$, $P < 0.033$). Further, there were no significant differences in percentage emergence between the control eggs and the eggs stored for 15 or 20d ($P = 0.099$). After storage for 65d, < 44% eggs were parasitized by *G. ashmeadi*, and about 23% of wasps emerged, which was significantly lower than for eggs for stored for 25d or less. When stored for over 80d, the percentage parasitism and emergence were less than 12% and 7%, respectively. When these data were analyzed via a regression analysis, the percentage parasitism and emergence vs. storage time was found to be inversely correlated (Figures 1 and 2).

Cold Storage of GWSS Eggs Parasitized by *G. ashmeadi*

The experimental conditions for this study consisted of a constant 4 or 4.5°C storage temperature and 2 daily cycled stepwise-increasing regimes (4, 6, and 8°C or 4.5, 6, and 7.5°C - each temp. changing at 8h intervals) under an 8 L: 16 D photoperiod. After the parasitized eggs were stored at 4 °C for 10d, only 7.2 ± 5.0% (n = 85) of the wasps emerged, which was significantly lower than those parasitoids similarly stored at 4.5°C (33.5 ± 7.2%, n = 280), 20 days (33.9 ± 6.9%, n = 114) or 25 days (21.7 ± 5.2%, n = 125) ($F = 11.962$, $df = 4,66$, $P < 0.001$). No parasitoids (n = 164) emerged from host eggs stored at 4°C for 20d (Figure 3). When parasitoids were stored within hosts under the cycled stepwise temperature regime starting at 4 °C, percentage emergence was 42% (n = 126) at 10 d, 8 % (n = 420) at 20d and 0% (n = 184) at 25d (Figure 4). However, for parasitized eggs stored at the other cycled regime starting at 4.5°C, the wasp emergence was at or above 60% throughout the 25d of storage. Thus, the percentage emergence for the parasitoids stored under the stepwise regime starting at 4.5°C for 10-25d was significantly higher than that for the eggs stored for 15d under the regime starting at 4°C ($F = 48.237$, $df = 5, 114$, $P < 0.0001$). Parasitoids within GWSS eggs did not emerge after storage for 80 days, but further research is needed to ascertain if maintenance of the *Euonymus* cuttings that bear the egg masses during the storage period is causing a problem.

EFFECTS OF USING CONSTANT AND CYCLICAL STEPWISE-INCREASING TEMPERATURES ON PARASITIZED AND UNPARASITIZED EGGS OF THE GLASSY-WINGED SHARPSHOOTER DURING COLD STORAGE

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Reporting Period: The results reported here are from work conducted from December 1, 2003 to October 1, 2004.

ABSTRACT

Glassy-winged Sharpshooter (GWSS) egg masses, deposited on *Euonymus japonica* cuttings, were stored 1d after oviposition at either a constant temperature of 12°C or under a regime that cycled daily, stepwise, (10, 11, 12, 13°C @ 6h intervals) under an 8L:16D photoperiod. After storage under the cycled temperature regime for 15 and 20d, the hatch was 74 and 63%, respectively. Control hatch at 20d was about 80% and 50% after storage at a constant 12°C. The survival to adulthood, length of the nymphal stage, and the fecundity of the adult females were all affected by cold storage during the egg stage, regardless whether the temperature was held constant or cycled. Survival to adulthood was reduced 30 to 40% and the time required to complete the nymphal stages was significantly longer than the control. The number of eggs oviposited by females and length of the ovipositional period after being held at 12°C during the egg stage was about one-half that of the control group, while the values for the 20d cycled group are yet to be determined. The rates of parasitism and emergence by *Gonatocerus ashmeadi* decreased with the length of time that 1-d-old unparasitized GWSS eggs were stored under the cycled regime. When held up to 25d in storage, parasitism by wasps and emergence of their progeny remained statistically similar. After 50d of storage, parasitism and progeny emergence dropped 30% and 20%, respectively. After a storage period of 25d, parasitoid emergence from parasitized eggs stored at a constant 4.5°C was significantly higher than those stored similarly at 4°C. The cycled stepwise-increasing temperature regime of 4.5, 6.0, and 7.5°C changing at 8h intervals yielded a significantly higher parasitoid emergence than a cycled regime of 4, 6, and 8°C. When stored under the regime starting at 4.5°C, for 10, 20 and 25d, the emergence of wasps was 66%, 59% and 59%, respectively. Parasitized eggs stored under this regime for 80d produced no wasps.

INTRODUCTION

Studies on cold storage of insects and their eggs have shown that developmental age, storage temperature, time in storage, and inherent species tolerance are the factors which influence survival after a cold storage period (Leopold 1998). The most effective temperature for storage of GWSS eggs was determined to be 12°C (Leopold et al. 2003). Storage of 1-d-old GWSS eggs at 10°C resulted in no survival after only 8d period. Storage at 13 and 14°C resulted in high survival and parasitism by *Gonatocerus ashmeadi* and *G. triggutatus* at 20d, but in-storage hatching of the GWSS eggs occurs after 30d and successful parasitism by the wasps decreases under these constant temperature regimes. The within-host cold tolerance of the *Gonatocerus spp.* is significantly greater than that of the unparasitized GWSS eggs. Emergence of the wasps occurs at temperatures $\geq 5^\circ\text{C}$ when the parasitized eggs are stored $< 20\text{d}$. Since certain conditions, such as temperature variation and fluctuation and high or low humidities have been reported to enhance survival of insects and their parasites during cold storage (Iacob and Iacob 1972, Gautum 1986, Liu and Tian 1987, Leopold et al. 1998), the present study was initiated to determine whether we could lengthen the survival time of GWSS eggs and the egg parasitoid by varying the temperature while in storage. We were especially interested in determining whether any latent damaging effects of chilling would be expressed, beyond diminished emergence, that might affect the quality of previously cold-stored insects.

OBJECTIVES

1. Compare the cold tolerance of GWSS eggs stored at a constant temperature with eggs stored under a cycled stepwise temperature regime and evaluate the post storage developmental time of nymphs and reproduction of adults.
2. Compare the effects of cold storage of unparasitized GWSS eggs under constant and cycled stepwise low temperatures regimes on the subsequent parasitism and emergence of *G. ashmeadi*.
3. Determine whether a cycled stepwise cold temperature regime enhances the shelf-life of parasitoids while in host eggs.

RESULTS AND CONCLUSIONS

Cold storage of Unparasitized GWSS Eggs

GWSS egg masses deposited on *Euonymus* cuttings were stored in incubators set at constant (12°C) and cycling stepwise-increasing temperatures (10, 11, 12, and 13 °C @ 6h intervals) under an 8L:16D photoperiod for varying lengths of time. After removal from storage, the cuttings bearing GWSS egg masses were incubated at room temperature (ca. 22 °C) to record egg hatch. After storage at 12°C for 30d, $52.7 \pm 10.2\%$ of 1-d-old eggs ($n = 102$), $50.7 \pm 7.1\%$ of 3-d-old eggs ($n = 87$) and $44.7 \pm 5.1\%$ of 5-d-old eggs ($n = 61$) hatched. However, no hatching was observed after 30d storage. When stored at the stepwise cycling temperature (10-13 °C) for 15, 20, and 25d, the hatch of 1-d-old eggs was $73.9 \pm 11.1\%$ ($n = 142$),

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FUNDING AGENCIES

Funding for this project was provided by the USDA Agricultural Research Service.

Table 1. Mean^a developmental duration and size of three biometric parameters of immature stages of GWSS reared on excised cowpea leaves.

Instar	Immature duration ± SE (days)			Biometric parameter ± SE (mm)			
	<i>N</i>	Female	Male	<i>N</i>	HCW	BDL	HTL
1	90	10.8 ± 0.9 a BC	10.1 ± 0.9 a BC	76	0.63 ± 0.004 g	2.30 ± 0.03 f	0.86 ± 0.01 g
2	76	6.1 ± 0.5 a C	5.8 ± 0.8 a C	21	0.82 ± 0.015 f	2.98 ± 0.09 f	1.19 ± 0.02 f
3	57	8.2 ± 0.9 a BC	8.9 ± 1.2 a BC	27	1.33 ± 0.034 e	5.61 ± 0.24 e	2.03 ± 0.08 e
4	40	12.1 ± 0.7 a B	12.9 ± 0.9 a AB	27	1.87 ± 0.013 d	8.63 ± 0.14 d	3.01 ± 0.04 d
5	32	23.7 ± 2.5 a A	14.6 ± 1.8 b A	40	2.13 ± 0.031 c	9.58 ± 0.15 cd	3.19 ± 0.04 d
Total	32	60.9 ± 2.9 a	53.0 ± 1.5b	-	-	-	-

^a Means followed by the same small case letter within each row and by the same capital letter within each column are not significantly different ($P > 0.05$), Ryan-Einot-Gabriel-Welsch multiple range test (REGWQ). *N*, represents the sample size.

Table 2. Fecundity and life table parameters of GWSS reared on excised cowpea leaves.

Parameter	<i>n</i>	<i>Fecundity</i> *	r_m	R_o	<i>G</i>	<i>DT</i>	λ
Mean	15	193.7	0.044	33.6	79.3	15.6	1.045
95% LCI		154.2	0.040	22.38	74.7	14.1	1.041
95% UCI		233.2	0.049	44.75	83.8	17.0	1.050

* Mean fecundity of gravid females only, i.e., 13 females; *n*, number of pairs included in analysis; r_m , jackknife estimate of the intrinsic rate of increase; R_o , net reproductive rate; *G*, mean generation time (in days); *DT*, population doubling time (in days); and λ , finite rate of increase; LCI = lower confidence limits and UCI = upper confidence limit

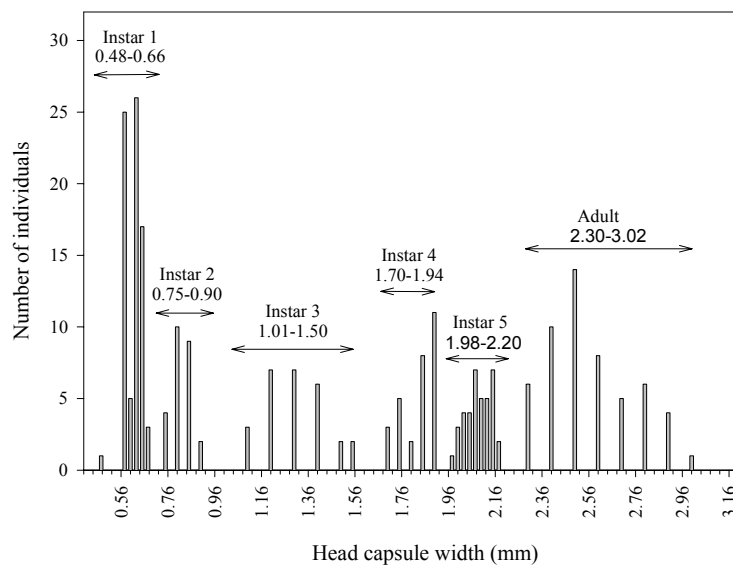


Figure 2. Distribution of head capsule widths of GWSS nymphs and adults.

Adult longevity was comparable for males (47 d) and females (52 d). For both males and females, no mortality occurred until 20 d after adult emergence. There was a 5 d pre-oviposition period (3 - 9 d) and a 3 d post-oviposition period (0 - 7 d).

A high proportion of females (88%) deposited eggs, with a mean total of 194 eggs per female. The eggs were deposited in clusters under the epidermis layer of cowpea leaves and were mostly in even numbers (93%). Most of the eggs incubated (92.6%) were fertile, and took from 5 to 8 d, with a mean value of 7.1 d, to emerge at 27 °C.

Life table statistics of GWSS on cowpea are presented on Table 2. Populations of GWSS could multiply at a rate of 33.6 times per generation on cowpea, thus doubling in 15.6 d. Analysis of natality pattern of GWSS revealed that the number of offspring per female was independent of female age, suggesting that food availability might determine the fecundity potential of females.

The successful completion of GWSS life cycle on cowpea suggests that the xylem fluid of this plant has a nutrient profile suitable for both immature and adult stages. The rearing approach used here is quite simple and allowed us to follow each individual GWSS during its development.

Biometric analysis

Values of the three biometric parameters, BDL, HTL, and HCW, varied significantly with the developmental stage (Table 1). Only the grouping for the HCW did not overlap between nymphal stages as indicated by the mean comparison and the distribution of frequency analysis (Table 1, Figure 2). Thus, the HCW could be used as a reliable parameter for distinguishing the five nymphal stages of GWSS.

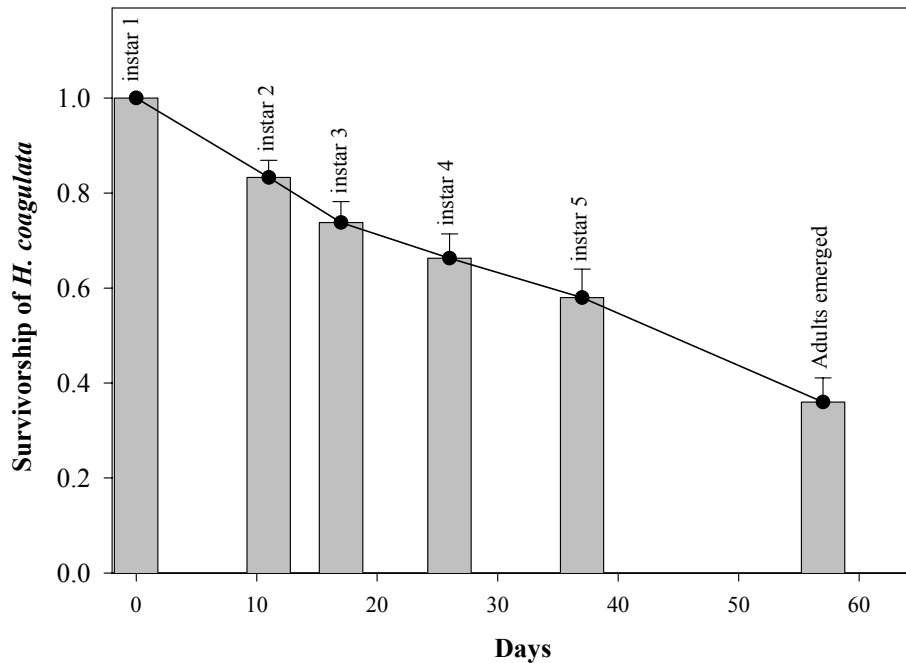


Figure 1. Survival of *H. coagulata* nymphal stages on excised cowpea leaves maintained at 27 °C.

BIOLOGY AND MORPHOMETRIC ANALYSIS OF GLASSY-WINGED SHARPSHOOTERS REARED ON COWPEA

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Reporting period: The results reported here are from work conducted from October 1, 2003 to September 30, 2004.

ABSTRACT

Stage specific survival, growth, developmental biology, and morphometric analysis of individual glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say), were studied in the laboratory at 27 ± 1 °C, 65 ± 5 RH and 14:10 L:D photoperiod regime, on excised cowpea leaves and stems. Embryonic development of eggs was completed in 7.1 days with 92.6% of the eggs incubated being fertile. The total nymphal period for females (61 ± 3.0 days) was significantly longer than that of males (53 ± 1.5 days). Significant differences were observed between the duration of the 5 nymphal stages, with the 2nd being the shortest and the last (5th) the longest for both sexes. Stage specific mortality was similar between instars, $\approx 36.4\%$ of the nymphs reached adult stage, and adult sex ratio was not different from a 1:1 ratio. Based on a cohort of 15 pairs, analysis of life table parameters indicated that populations of *H. coagulata* increased at a rate of 1.045 per day and doubled within 15.6 days. Biometric data comprising body length, head capsule width and hind tibia length were recorded on a total of 276 individuals. The different growth stages were well described by the three biometric parameters. However, analysis of frequency distribution showed that head capsule width was the most suitable parameter for distinguishing the immature developmental stages of GWSS.

INTRODUCTION

The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say), is a highly polyphagous xylem-feeder that is indigenous to the southern United States, from Florida to Texas, and northeastern Mexico (Turner and Pollard 1959). Other than being a minor nuisance in urban environments, the glassy-winged sharpshooter itself causes relatively little direct economic damage or plant loss except for the cosmetic damage to citrus fruits from egg masses deposited into fruits when populations of *H. coagulata* are high (Hix et al. 2003). The most destructive characteristic of GWSS lies in its ability to transmit a plant bacterial pathogen, *Xylella fastidiosa*, one of the causal agents of Pierce's disease (PD) (Redak et al. 2004). However, the recent invasion and establishment of *H. coagulata* in California has dramatically changed the ecology of *X. fastidiosa* and the epidemiology of Pierce's disease (Almeida and Purcell 2003).

Despite the importance and vector status of GWSS, few studies have evaluated its reproductive biology. Little is known about its life table statistics, as published biological studies have not covered the entire life cycle of GWSS. The reasons of the paucity of knowledge on the reproductive biology of GWSS might be the lack of artificial diet-based rearing method for GWSS, as well as the different nutrient requirements of nymphs and adult (Brodbeck et al. 1996).

The present study is focused on developing a simple rearing method for following the development of individual GWSS from egg to adult emergence. We also recorded the longevity and fecundity of adults, and determined the life table statistics of GWSS. Life tables and fertility tables are powerful tools for analyzing and understanding the impact that an external factor has on growth, survival, reproduction, and rate of increase of an insect population (Bellows et al. 1992). As the GWSS undergoes five ecdyses during its development (Turner and Pollard 1959, Brodbeck et al. 1999), it is of significant importance to develop reliable morphological criteria for distinguishing the various nymphal stages.

OBJECTIVES

1. Develop a simple method for rearing individual GWSS from egg to adult on cowpea.
2. Determine the survivorship, egg to adult development time, and reproduction potential of GWSS on cowpea.
3. Examine the growth pattern of this sharpshooter based on three selected biometric parameters that could be used to distinguish the different developmental stages.

RESULTS AND CONCLUSIONS

Biology and Life Table Statistics

The ultimate survivorship of *H. coagulata* on cowpea was 36.4% (Figure 1). The duration of the five instars ranged from 6 to 24 d and was significantly affected by nymphal stage, sex and the sex by developmental stage interaction (Table 1). Within each sex group, the first three instars had the shortest development time, while the last instar (5th) took the longest time to complete for females only (Table 1). The mean total nymphal period of *H. coagulata* on cowpea was 8 d longer for females (61 d) than males (53 d) (Table 2). Out of the 32 *H. coagulata* adults that emerged, 18 were females but the sex ratio was not different from a 1:1 ratio.

will be introduced onto potted plants placed in cages and populations monitored monthly throughout the winter period and in the subsequent spring. At each location, four caged replicates of host plant species including the plant species navel orange, grape, and peach will be evaluated individually and in combination. A detailed record of adult GWSS feeding and resting preference will be observed twice monthly throughout the 20 week duration of the experiment beginning November and lasting through March.

CONCLUSIONS

We believe that this recently funded project has a high probability of success both in terms of generating significant new information regarding the overwintering population dynamics of GWSS in California and in providing practical guidance towards management of this pathosystem. This information will further be useful in accurately identifying specific regions of the Central Valley where GWSS overwintering survivorship is greatest and a significant threat of reinfestation is posed. Our research will expand on previous work that has characterized the role of climatic factors in the distribution of *Xf* diseases by defining the specific environmental constraints that influence GWSS population dynamics. Moreover, results from these experiments will be coupled with climatological data in an effort to spatially define those locations where GWSS populations may be unable to successfully overwinter or conversely where populations may find overwintering refuges from extended periods of temperatures that limit adult feeding (Figure 3).

Combined with our findings in laboratory bioassays, high resolution (i.e., 1 km scale) raster-based data can be queried to generate predictive maps revealing areas within the Central Valley that may function as “thermal islands”, which could favorably support GWSS overwintering populations compared to adjacent agricultural landscapes. As an example, Figure 3 illustrates results of a raster file generated from data collected in January 1993 portraying the number of occurrences where daily maximum temperatures never exceeded 10°C (50°F) for periods of 48 and 96 hours, respectively.

With an improved understanding of the climatological limits of GWSS overwintering survivorship, these data can help to spatially define where GWSS can be expected to persist in the agricultural landscape and identify where continued management efforts should be directed to limit introductions into currently non-infested areas. The proposed research will generate critical new information about GWSS spatial population dynamics, thereby contributing towards the development of long-term, economically, and environmentally sustainable management solutions that will directly benefit agricultural producers, crop consultants, and other stakeholders.

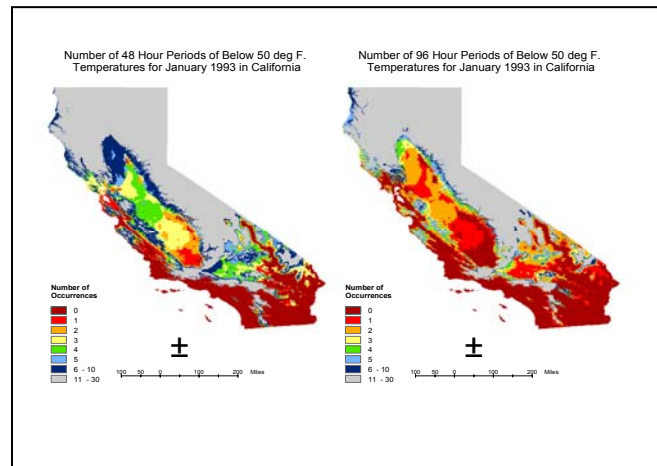


Figure 3. Extended intervals of cool temperatures (< 50°F) January 1993 illustrating microclimatic differences in the San Joaquin Valley

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FUNDING AGENCIES

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increase our present understanding of the overwintering requirements of GWSS with a focus on critical environmental and host species factors that may limit population distribution in the Central Valley of California.

OBJECTIVES

1. Identify the critical environmental constraints that influence the spatial population dynamics and overwintering success of GWSS in California's Central Valley.
2. Characterize the impact of host plant species succession on the overwintering survivorship of GWSS populations that constrain the insect's ability to become established and persist throughout the San Joaquin Valley.

RESULTS

Objective 1

Experiments designed to define the temperature-dependent feeding biology of GWSS are underway at the GWSS Experimental Laboratory on the campus of California State University Fresno (CSUF). Colonies of adult GWSS are maintained at this newly established USDA-ARS research facility in cooperation with research personnel from CSUF, the University of California (Riverside, Berkeley), and the California Department of Food and Agriculture. Plans are to characterize adult GWSS feeding and survival in climate-controlled growth chambers to determine the temperature threshold for adult feeding activity under different combinations of host type and temperature regimes. Adult insects from the rearing colonies, as well as field collected insects in reproductive diapause, will be caged on selected plant species at varying temperatures for different exposure periods in environmental chambers. At the completion of the exposure period(s), the three infested treatments of each plant species will be removed from the chamber and adult GWSS performance and survivorship monitored through the remainder of the adult insect life on the respective test plants in individual screen cages.

In preliminary trials designed to indirectly measure feeding rates, water sensitive paper placed under caged adult GWSS on cowpea collected varying levels of excreta at temperatures of 15.6, 10.0, and 4.6°C (Figure 1). Water sensitive paper strips (2" X 3"), which collect excreted honeydew, are placed adjacent to the plant stem and immediately below a 2" diameter cylindrical Lexan® cage in which adult GWSS are confined on a test plant. In future experiments, the paper will be notched and fit to the plant stem and will be manually replaced on a 4 hour interval over 24 hour intervals. Over the 24 h observations, 12 honeydew clocks will be used for each variety at each of 3 start times corresponding to 0600, 1400, and 2200 h to determine any influence of time of day (Padgham and Woodhead 1988). The amount of excreta collected upon the exposed surface(s) of water-sensitive paper will be compared among different, replicated temperature and exposure regimes to better refine the environmental conditions in which GWSS feeding is restricted or discontinued.

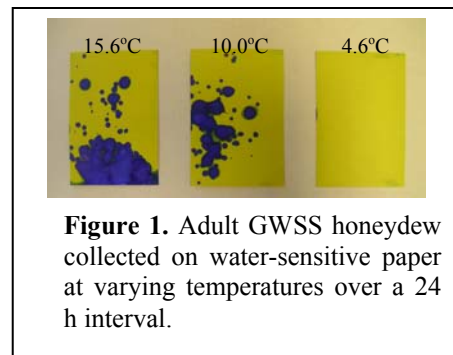


Figure 1. Adult GWSS honeydew collected on water-sensitive paper at varying temperatures over a 24 h interval.

A third set of laboratory experiments are underway using an electro-penetration feeding (EPG) monitoring apparatus to perform waveform analysis at different temperatures. Ten day old adult female GWSS are used in these EPG experiments and are initially placed in separate acclimation cages for 2 hours at the appropriate temperature upon which they will be tested. Preliminary results illustrate differences in the frequency and duration of probing events (green-shaded boxes) of adult GWSS held at temperatures of 15.6, 10.0, and 4.6°C for 12 hour testing intervals on cowpea test plants (Figure 2). Waveform excerpts were taken approximately 225 seconds after the recording began and compressed 2000 times to represent 6.5 hours of recording. These preliminary results indicate that temperature grossly affects GWSS probing behavior between 4.4-15.5°C. In planned experiments, a total of 5 tethered insects will be simultaneously monitored as experimental replicates at temperatures of 12.2, 10.0, 8.9, and 6.7 °C for exposure intervals of 6, 12, and 24 hour periods. Time course examination of waveforms will reveal the frequency and duration of insect feeding behavior and will help to accurately define the temperature threshold at which ingestion and other waveforms are halted (Serrano et al. 2000).

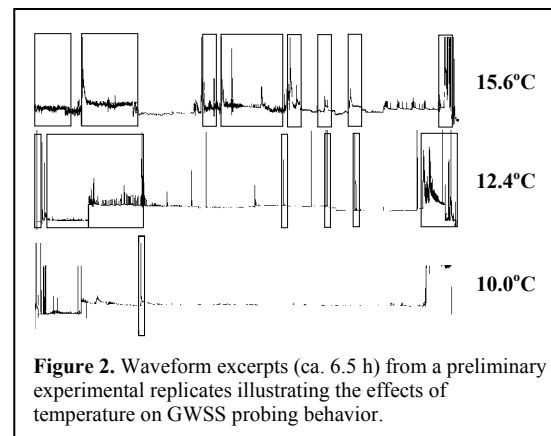


Figure 2. Waveform excerpts (ca. 6.5 h) from a preliminary experimental replicates illustrating the effects of temperature on GWSS probing behavior.

Objective 2

Seasonal population dynamics of GWSS will be monitored on selected host plants placed in different micro-climatic areas of the San Joaquin Valley: 1) the citrus-growing, foothill region of Tulare County; and 2) a GWSS-infested region of the valley floor just west of Porterville in Tulare County. In these experiments, we will examine GWSS survivorship in caged experiments on a selected host plant species. In each cage, fifty second generation GWSS adults, nearing reproductive diapause in the fall season, will be collected from natural infestations and released onto caged plants in late summer. Insects

SPATIAL POPULATION DYNAMICS AND OVERWINTERING BIOLOGY OF THE GLASSY-WINGED SHARPSHOOTER IN CALIFORNIA'S SAN JOAQUIN VALLEY

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Reporting Period: The results reported here are from work conducted from June 2004 through September 2004.

ABSTRACT

The purpose of this project is to define specific environmental constraints that influence glassy-winged sharpshooter (GWSS) population dynamics and overwintering success. We are beginning experiments to determine the temperature-dependent feeding biology of GWSS in temperature-controlled chambers. Experiments are underway in the recently established GWSS Experimental Laboratory on the campus of California State University, Fresno. Adult GWSS feeding and survival under different combinations of host plant type and temperature regimes will be monitored to determine the temperature thresholds for adult feeding activity. Complementary experiments measuring honeydew excretion rates have begun to determine the amounts of excreta collected upon exposed surface(s) of water-sensitive paper and will be compared among different temperature and exposure regimes. Electro-penetration feeding monitoring assays are underway at different temperatures on individually tethered and feeding GWSS adults. Time course examinations of waveforms reveal the frequency and duration of insect feeding behavior under varying environmental conditions. The seasonal population dynamics of GWSS will be monitored on selected host plants placed in different micro-climatic areas of the San Joaquin Valley. Results from these experiments will be coupled with climatological data to help to spatially define where GWSS can be expected to persist in the agricultural landscape and identify where continued management efforts should be directed to limit introductions into currently non-infested areas.

INTRODUCTION

The bacteria *Xylella fastidiosa* (*Xf*) causes economically important diseases of several agronomic, horticultural, and landscape ornamental crops (Pearson and Goheen 1988). The bacterium is transmitted by xylem feeding sharpshooters (Cicadellidae) and spittlebugs (Cercopidae) (Adlerz and Hopkins 1979, Purcell and Frazier 1988). In California, Pierce's disease incidence has been exacerbated following the introduction, establishment and continued spread of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, which is an effective vector of *Xf*. GWSS was first detected in southern California in the early 1990's and populations have since become established in many locations throughout southern portions of the state. First detected in Kern County in 1998, GWSS is now present in the San Joaquin Valley. However, the rapid population expansion first observed in southern California appears to be constrained to discrete regions within agricultural areas of the San Joaquin Valley and incipient, localized populations in urban areas of Fresno, Sacramento, Chico, and San Jose. The continued spread of GWSS into other California localities will almost certainly threaten the economic viability of grapes and other crop species susceptible to infection by various *Xf* strains.

Climate appears to play a significant role in the geographic distribution of diseases caused by *Xf* strains in California and throughout the southeastern U.S. (Purcell 1977, 1980, 1997). Similarly, populations of GWSS in the southeastern US appear to be constrained by climatic factors that limit the pest's establishment and persistence (Pollard and Kaloostian 1961, Hoddle 2004). Presently, limited information exists on the overwintering biology and ecology of GWSS in the San Joaquin Valley of California. An emerging hypothesis is that GWSS may be limited by certain temperature thresholds at, or below, which feeding may be discontinued. In turn, we are designing experiments to carefully determine the thresholds below which feeding discontinues. Additionally, we will determine the critical duration of time spent in this non-feeding state, which may result in increased mortality. The results of the outlined experiments will advance our ability to define the specific environmental constraints that influence GWSS population dynamics and overwintering success. This information will by

currently employed morphological characters. To do this we intend to combine three separate approaches to determine the species identity of different *G. morrilli* populations: First, we'll reassess key morphological features used to characterize *G. morrilli* with scanning electron microscopy to determine if subtle morphological differences exist between *G. morrilli* populations which could possibly indicate species differences. Such differences - should they exist - may not be easily observed with light microscopy. Second, we'll conduct mating compatibility studies to determine if different populations of *G. morrilli* are reproductively isolated, or if mating occurs, whether offspring are viable thereby defining species groups on the basis of successful interbreeding. Third, we'll determine if molecular differences exist between different *G. morrilli* populations by comparing mitochondrial and ribosomal DNA sequences. Molecular dissimilarities of key regions could potentially indicate the existence of different species, and at the same time allow their identification. Results from these three areas (morphology, behavior, and molecular avenues) of investigation will be evaluated together to determine whether *G. morrilli* as it is currently viewed is a valid species or whether it is an aggregate of morphologically indistinguishable cryptic species.

RESULTS

This project has not commenced. The reason for this is that the recruitment of the post-doc has taken some time. We expect the post-doc to be on-line in early December 2004. We will be formally requesting a no-cost extension for this project.

FUNDING AGENCIES

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

IS THE GLASSY-WINGED SHARPSHOOTER PARASITOID *GONATOCERUS MORRILLI* ONE SPECIES OR A COMPLEX OF CLOSELY RELATED SIBLING SPECIES?

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Reporting period: The results reported here are from work conducted from July 2004 to October 2004.

INTRODUCTION

This is a new proposal that was officially funded in July 2004. This project objective is to determine the status of different *Gonatocerus morrilli* populations. We intend to use three approaches to determine the species identity of different *G. morrilli* populations: (1) Reassessment of key morphological features using scanning electron microscopy to determine if subtle morphological differences exist between *G. morrilli* populations which could possibly indicate species differences (Triapitsyn to conduct this work). (2) Conduct mating compatibility studies to determine if different populations of *G. morrilli* are reproductively isolated, or if mating occurs, whether offspring are viable thereby defining species groups on the basis of successful interbreeding (Hoddle). (3) To determine if molecular differences exist between *G. morrilli* populations collected from different regions by comparing mitochondrial and ribosomal DNA sequences. Molecular dissimilarities of key regions could potentially indicate the existence of different species (Stouthamer). Results from these three areas (morphology, behavior, and molecular) of investigation will be evaluated together to determine whether *G. morrilli* as it is currently viewed is a valid species or whether it is an aggregate of morphologically similar cryptic species.

A classical biological control program is currently underway for glassy-winged sharpshooter (GWSS), which is an exotic pest in California. The native range of GWSS is the southeastern United States and northeastern Mexico (Triapitsyn & Phillips, 2000). GWSS is thought to have invaded California around 1990 as egg masses that were accidentally imported on ornamental plants from Florida. Species of GWSS egg parasitoids not present in California are currently being prospected for in the native range of GWSS. Promising candidate natural enemy species that attack eggs are being imported and released in California for GWSS control (Triapitsyn et al., 1998; Triapitsyn & Hoddle, 2001). Interestingly, one species of egg parasitoid associated naturally with GWSS in California, *Gonatocerus morrilli* (Howard) (Hymenoptera: Mymaridae), is also widely distributed in the home range of GWSS, but at the time of its initial discovery in California, *G. morrilli* had not been intentionally released here and was thought to be native to California. A potential host for *G. morrilli* in California prior to the arrival of GWSS could have been the native *Homalodisca liturata* (Ball) which has had unidentified *Gonatocerus* spp. reared from its egg masses collected in the San Diego area (Powers, 1973). The presence of *G. morrilli* in Riverside in 1980-1984 has been documented (Huber 1988). *Gonatocerus morrilli* is now the second most important natural enemy of GWSS egg masses in California (Al-Wahaibi, 2004).

The success and failure of a number of biological control projects against insect pests and weeds has hinged on the correct taxonomic identification of the target and its natural enemies (Gordh and Beardsley, 1999). Incorrect understanding of the taxonomy and subsequent interrelationships between the target and its natural enemy guild are serious impediments to an efficacious biological control program. For example, *Trichogramma minutum* and *T. platneri* are important commercially available biological control agents that are morphologically indistinguishable but reproductively incompatible (Nagarkatti, 1975). Experimental work and subsequent modeling with these two species of *Trichogramma* has indicated that because pre-mating isolation mechanisms are absent (e.g., pre-mating courtship behaviors that prevent coupling of males and females from different species) severe negative effects on biological control can occur. Negative effects manifest themselves because females that mate with males from different species fail to produce female offspring. This occurs because *Trichogramma* like *Gonatocerus* are haploid-diploid parasitic Hymenoptera. In this haplo-diploid system, fertilized eggs produce female offspring and unfertilized eggs produce male offspring. In situations where incompatible interspecies matings are occurring both species fail to produce females and the potential population growth of both parasitoid species is reduced to levels below the growth rate expected for either species in the absence of the other (Stouthamer et al., 2000).

If different populations of morphologically similar *G. morrilli* from Florida, Louisiana, Texas, and Mexico are indeed valid species that lack pre-mating isolation mechanisms, then the current biological control program against GWSS in California that is attempting to establish these new agents may reduce the current level of control achieved by the precinctive populations of *G. morrilli* in California. This could occur because of male-biased offspring production resulting from incompatible matings across species. The rationale for introducing new strains or races of *G. morrilli* into California is based on the idea that different biotypes of this parasitoid may exist and fill niches not currently occupied by the strain of *G. morrilli* already present in California.

In this grant we propose to determine if geographically distinct populations of *G. morrilli* are part of one continuous interbreeding population or if populations of *G. morrilli* are separate species that can't be easily separated on the basis of

Host specificity testing: No-choice tests were conducted with *G. ashmeadi* and STSS eggs. Single, one day old, mated, fed *G. ashmeadi* were exposed to STSS (n = 40 egg masses) and control (GWSS, n = 7 egg masses) eggs on chrysanthemum leaves in individual 100 x 15 mm Petri dishes. Each wasp was supplied one egg mass less than 48 hours of age and allowed 24 hr to parasitize the eggs before removal from the dish. The number of eggs per egg mass ranged from 2-14 (\bar{x} = 5.65) for STSS and 2-19 (\bar{x} = 5.89) for GWSS. Percent parasitism of egg masses ranged from 0-100% for both STSS (\bar{x} = 84.58%) and GWSS (\bar{x} = 71.43%) and was not found to be significantly different (Figure 5, Student's t-test, alpha = 0.05, P = 0.37702).

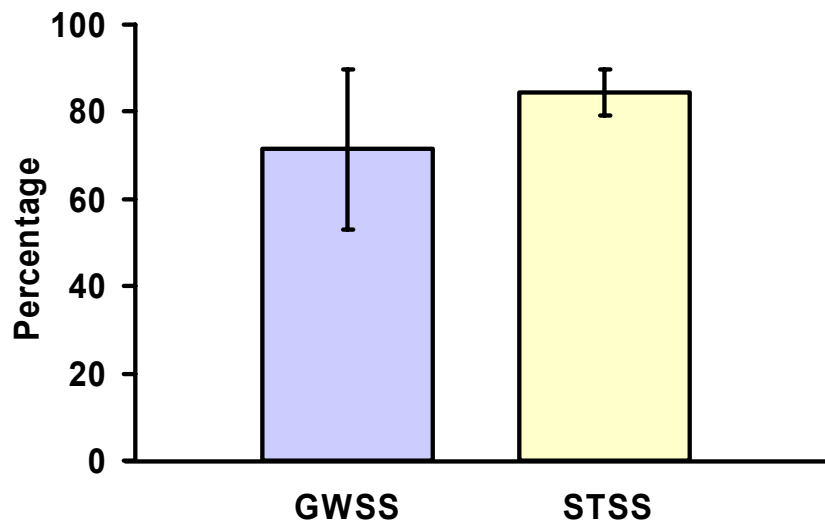


Figure 5: Percent parasitism of STSS and GWSS eggs by *G. ashmeadi* in Petri dish no-choice studies.

CONCLUSIONS

Clearly we now know BGSS oviposition preference on wild grape is for new growth, consisting primarily of the terminal 25 cm of succulent stems and tendrils that occur along the entire length of the grape cane. Additionally we have confirmed two new natural enemy host associations for the BGSS, *G. latipennis* and *Polynema* sp. While these studies were conducted on wild grape, the information acquired may have implications in developing a more complete IPM program involving this native pest species and its associated natural enemies. Overall, the new knowledge of BGSS oviposition preference provides essential information for conducting future non-target effect studies involving the exotic GWSS egg-parasitoids which we have started to investigate. Peak BGSS adult activity measured through trap catches occurred from mid-June to early August while peak emergence of nymphs and parasitoids was spread over a four week period from 24 July to 20 August 2004. Another peak of adult activity may be expected in October once the nymphs have matured into adults. No-choice tests with *G. ashmeadi* and the STSS yielded no significant differences in percent parasitism as compared with GWSS control. It is likely there will be non-target impacts by *G. ashmeadi* in STSS habitats where this parasitoid is able to successfully infiltrate and compete with other resident natural enemies such as *Ufens* and *Zagella* sp. (both Trichogrammatidae)

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FUNDING AGENCIES

Funding for this project was provided by the University of California Agriculture and Natural Resources.

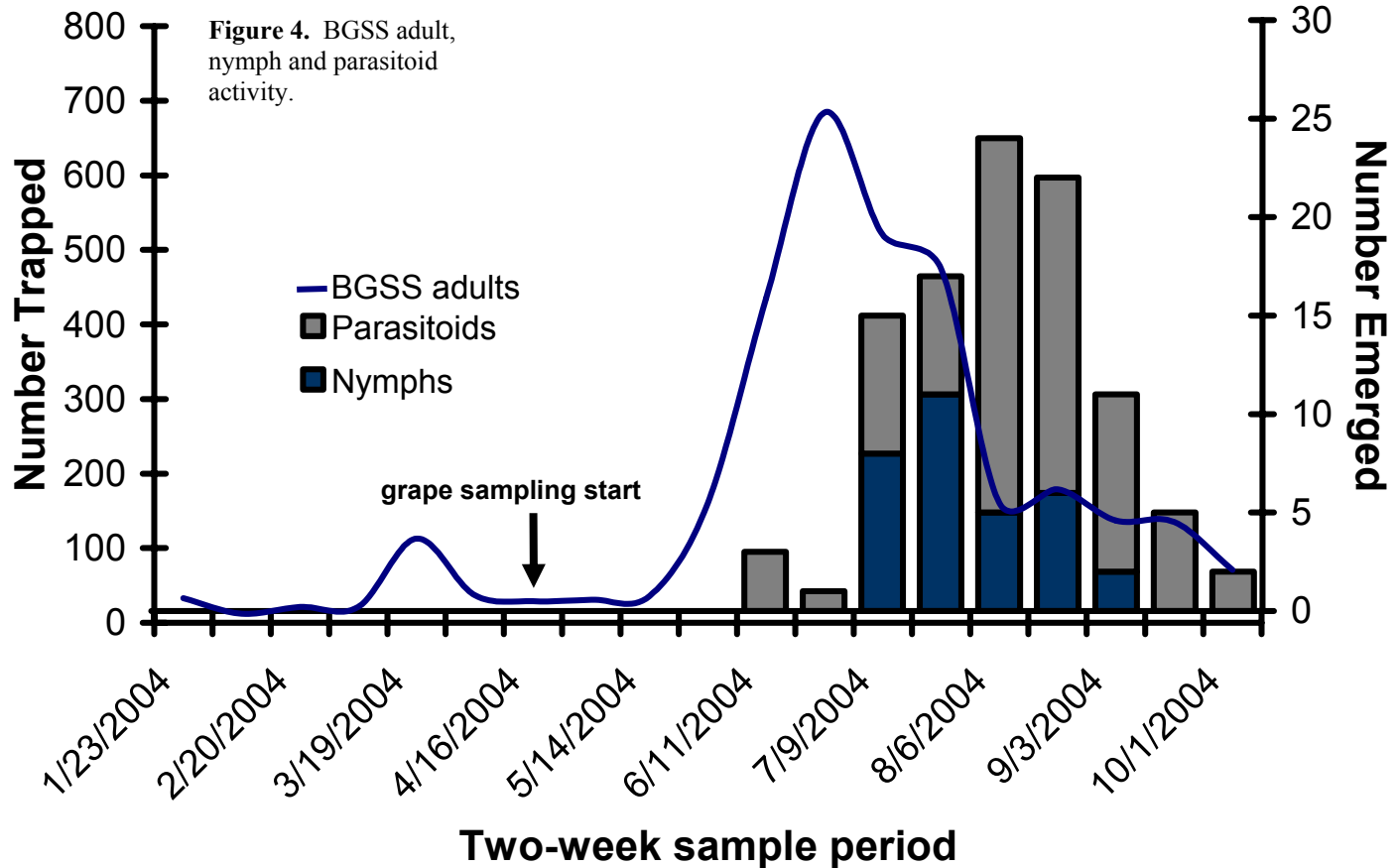
Ten entire grape canes were sampled on 14 August 2003 to account for any possible oviposition substrate not sampled in the previous survey. These canes were cut into thirds (apical, middle and basal), then placed into 10 cm of water in a Mason jar which left approximately 25 cm of cane exposed for emergence of nymphs and parasitoids. Canes and mason jars were then placed into three separate cages, according to their stem position. Cane sections were examined daily for emergence. In total, two BGSS nymphs and 16 *Polynema* sp. emerged from the canes. As there were so few insects emerged from these cane sections, the stems, leaves, petioles and tendrils were examined under the microscope for recent emergence holes from both BGSS nymph and parasitoids. A total of 65 emergence holes were counted. The majority of emergence holes were on the apical stems (n = 37) and on tendrils (n = 6, 13, 7, for apical, middle and basal portions, respectively) occurring along the length of the entire canes. Only two emergence holes were counted from leaf petioles and none were counted from middle and basal stems and leaves.

Sentinel Plant Study

To confirm the host association of the emerged parasitoids with the BGSS, three sweet-basil, a chrysanthemum and two wild grape plants were exposed to BGSS lab colonies for 3 days to allow for oviposition. Plants were removed from the colonies and transported to the oviposition survey site to allow for parasitization of BGSS eggs. After three days, the plants were brought back from field, cleaned of any insects and placed into separate cages. Plants were observed daily for any emerging insects. A combined total of 197 BGSS and *Polynema* sp. emerged from the five sentinel plants. Of these, 55 were BGSS nymphs and 142 were *Polynema* sp. (54 males, 88 females). Parasitism rates of BGSS eggs by *Polynema* sp. ranged from 33% on the mum to 78% and 86% on wild grape and basil, respectively.

BGSS and Parasitoid Activity

A total of 12 yellow sticky card traps (11 x 15 cm), were placed at the 2003 oviposition survey site to monitor BGSS adult and parasitoid flight activity. Traps were set up on 9 January 2004 and collected at bi-weekly intervals. Peak trap catch of BGSS adults occurred over the two week period of 11 June to 25 June 2004. Additionally, as soon as wild grape had sprouted and was available for collection, starting on 16 April 2004, twelve 30 cm cane sections were collected at the same bi-weekly sampling intervals. Tendrils were severed from the cane and placed into individual Petri dishes while stems were placed into dual 50 dram vials (25 cm of cane above water to allow for emergence). Plant material was checked daily for emergences of nymphs and parasitoids. Peak emergence of BGSS nymphs and parasitoids was spread over a four week period from 24 July to 20 August 2004. Data compilation is still in progress, however some of the results are shown below in Figure 4.



RESULTS:

Oviposition Survey

Wild grape plant material collected on 5 August 2003 consisted of: 50 canes (terminal 25 cm of cane), 50 tendrils, 100 large, 100 medium, and 100 small leaves with petioles. The tendrils and small leaves with petioles were selected from the terminal 25 cm sections of the canes. Each of the 50 canes was cut into thirds: upper, middle and lower. No insects emerged from large or medium leaves and their petioles and are thus excluded from further discussion. A total of 49 insects (26 *G. atropunctata*, 18 *Polynema* sp. and five *G. latipennis* parasitoids, Figures. 1 and 2) emerged from plant material collected. The highest percentage of BGSS nymph emergence (18%) occurred in the apical-most portion of the stem, with less emerging from tendrils (14%), and middle (10%) and lower (2%) stems, respectively. A very small percentage of *G. atropunctata* nymphs emerged from small leaves and their petioles. For the parasitoids the highest percent emergence occurred from the tendrils (38%). Collectively, the tendrils and stems yielded the greatest emergence (Figure 3).



G. latipennis



Polynema sp.

Figures. 1 and 2. Parasitoids of the BGSS.

BGSS Nymph and Parasitoid

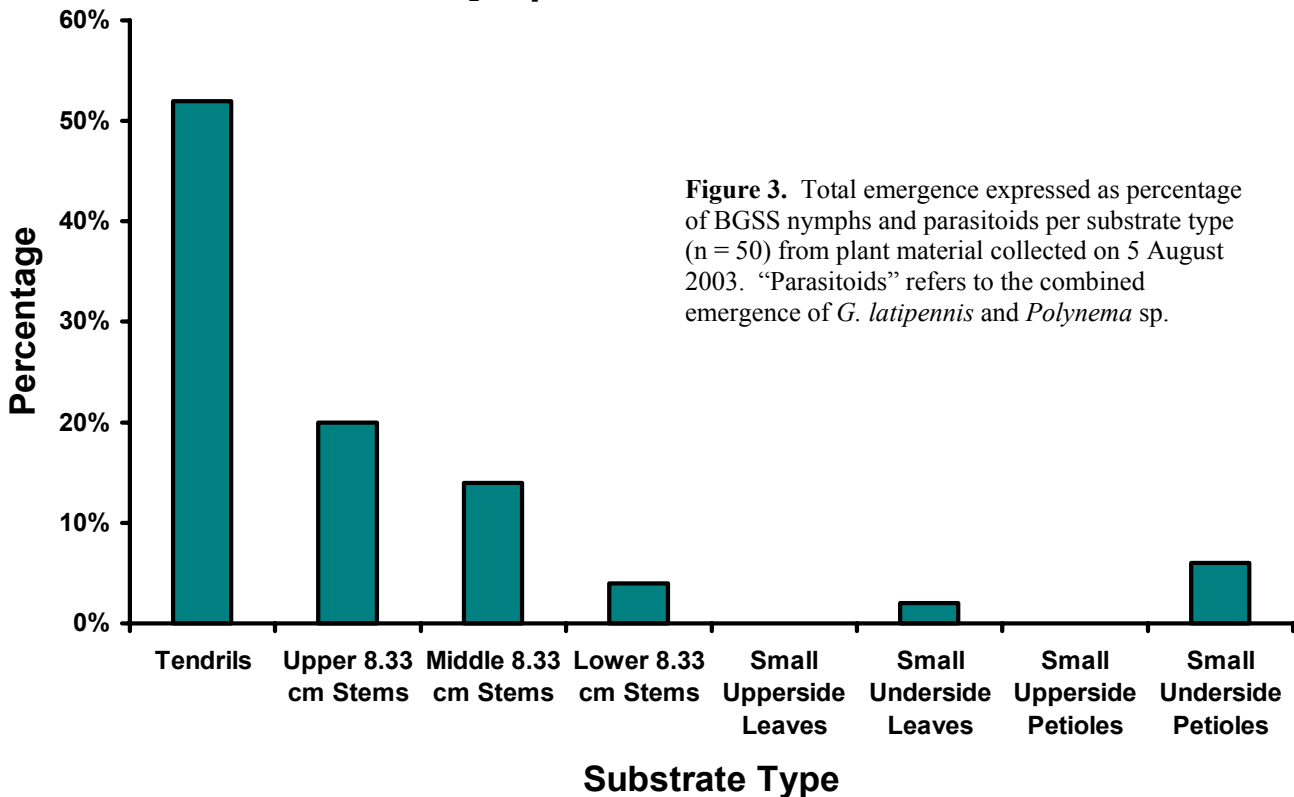


Figure 3. Total emergence expressed as percentage of BGSS nymphs and parasitoids per substrate type (n = 50) from plant material collected on 5 August 2003. “Parasitoids” refers to the combined emergence of *G. latipennis* and *Polynema* sp.

**IDENTIFICATION OF THE NATIVE PARASITOID FAUNA ASSOCIATED WITH
GRAPHOCEPHALA ATROPUNCTATA AND HOST SPECIFICITY TESTING OF
GONATOCERUS ASHMEADI ON *HOMALODISCA LITURATA***

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Reporting Period: The results reported here are from work conducted from May 2003 to October 2004

ABSTRACT

To determine the oviposition preference of female blue-green sharpshooters (BGSS), *Graphocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae), a survey was conducted on southern California wild grape, *Vitis californica* Benth (Vitaceae) growing near Temecula, California in August 2003 where populations of BGSS were known to occur. Female BGSS oviposited into new growth, primarily the succulent tendrils and stems. The under sides of small leaves and petioles were also used for oviposition, but to a lesser extent. Mature stems, large and medium sized leaves and petioles were not utilized for oviposition. Two parasitoids, *Gonatocerus latipennis* Girault and a *Polynema* sp. (Hymenoptera: Mymaridae) were reared from BGSS eggs. Literature reviews revealed a deficiency of known natural enemies for *G. atropunctata*. A sentinel plant study was conducted to further confirm the parasitization of BGSS eggs by these parasitoids. Collectively the *Polynema* sp. and *Gonatocerus latipennis* constitute the first documented parasitic natural enemies of BGSS eggs. A further examination, commencing in January 2004, of the activity of BGSS and its parasitoids in southern California is currently underway. Blue-green sharpshooter adult activity reached its peak in July while bi-weekly samples of wild grape canes and tendrils revealed peak emergence of blue-green nymphs and parasitoids occurred from mid-July to mid-August. No-choice tests with *Gonatocerus ashmeadi* Girault, a parasitoid of the gallsy-winged sharpshooter, *Homalodisca coagulata*, and BGSS eggs as part of a non-target impact assessment have yielded few results thus far. However, no-choice tests with *G. ashmeadi* and the native smoke-tree sharpshooter (STSS), *Homalodisca liturata* Ball, yielded no significant differences in percent parasitism of eggs when compared to the GWSS control.

INTRODUCTION

The native BGSS has been a threat to California grape growers for nearly a century due to its excellent transmission efficiency (Hill and Purcell 1995) of the bacterium that causes Pierce's Disease, a severe malady of commercially grown grapes. While much research has been devoted to epidemiologically related issues concerning this insect, little has been done to examine some of the most fundamental life history traits of this native pest, specifically oviposition preference (Severin 1949) and the native Californian parasitoids attacking the eggs of this pest. Further, we intend to investigate possible non-target effects of the exotic egg parasitoids that have been released to control another hemipteran pest, the GWSS, on BGSS and other native California sharpshooters and to identify the native parasitoid fauna associated with these native sharpshooter species. To address these issues, we need to know the oviposition preferences of native sharpshooters associated with particular host plants and their respective natural enemy fauna attacking oviposited eggs. The studies outlined below have determined the oviposition preferences of BGSS on wild grape, have documented its associated egg parasitoids, and provide data on host specificity of *G. ashmeadi*, a parasitoid being used as part of the classical biological control program against GWSS on the targets congener, the native STSS.

OBJECTIVES

1. Classify the native egg parasitoid fauna in California associated with sharpshooters native to California, primarily the smoke-tree sharpshooter (STSS): *Homalodisca liturata* Ball (Hemiptera: Clypeorrhyncha: Cicadellidae: Cicadellinae: Proconiini), blue-green sharpshooter (BGSS): *Graphocephala atropunctata* (Signoret), red-headed sharpshooter (RHSS): *Xyphon fulgida* (Nottingham), and green sharpshooter (GSS): *Draeculocephala minerva* Ball (the latter three, all Hemiptera: Clypeorrhyncha: Cicadellidae: Cicadellinae: Cicadellini).
2. Assess the possible non-target impacts of *Gonatocerus ashmeadi*, *G. trigutattus*, and *G. fasciatus*, parasitoids being used for the classical biological control of GWSS, on the above mentioned native sharpshooters.

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FUNDING AGENCIES

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Table 3. A hypothetical example of results yielded from a multitude of IgG-specific gut content ELISAs conducted on an individual predator (e.g., *Zelus renardii*). The number of positives yielded in all the assays indicates the number of prey consumed by this single predator.

Predator	Targeted GWSS	Protein marker designated in Table 1	Protein-Specific ELISA	ELISA result ¹
<i>Z. renardii</i>	1	Rabbit IgG	Anti-Rabbit IgG	-
	2	Guinea pig IgG	Anti-Guinea pig IgG	-
	3	Equine IgG	Anti-Equine IgG	-
	4	Mouse IgG	Anti-Mouse IgG	-
	5	Dog IgG	Anti-Dog IgG	-
	6	Pig IgG	Anti-Pig IgG	+
	7	Bovine IgG	Anti-Bovine IgG	-
	8	Cat IgG	Anti-Cat IgG	+
	9	Rat IgG	Anti-Rat IgG	-
	10	Sheep IgG	Sheep IgG	-

¹This individual predator scored positive in the anti-pig and anti-cat ELISAs; therefore it consumed 2 marked GWSSs.

CONCLUSIONS

Although it is widely accepted that predators play a role in pest regulation, we still have an inadequate understanding of, and ability to predict their impact in cropping systems. Frequently parasitoids are given major credit for suppressing pest populations; however, the impact that predators have on suppressing GWSS populations goes unrealized due to the difficulties of assessing arthropod predation as discussed above. The prey marking technique described here circumvents many of the shortcomings of the current methods used to study predation. The preliminary studies described here prove that prey marking can be a powerful method for the immunological detection of predation and can be used to study various aspects of predator feeding behavior. Over the next 2 years we plan to quantify predation rates on GWSS. Ultimately, this information can be used to improve the efficacy of conservation and inundative biological control of GWSS. This research is designed to determine which predators are exerting the greatest biological control on GWSS eggs, nymphs and adults. This information can then be used to develop a comprehensive biological control program that better conserves the populations of those predators exerting the greatest control on the various GWSS life stages.

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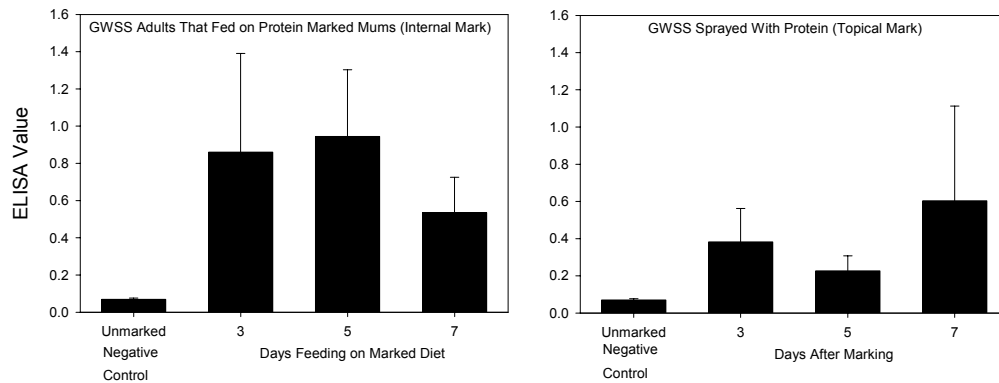


Figure 2. The efficacy of the internal (left graph) and external marking procedure (right graph) (n=8 to 16 per treatment). All of the GWSSs assayed 3, 5, and 7 days after marking yielded positive ELISA responses for the presence of rabbit IgG. All of the unmarked GWSSs yielded negative ELISA responses.

Results indicate that the protein marking procedure works for at least 7 days after marking GWSS. The next phase of our research (in progress) will be to mark individual GWSSs using the methods described above. Specifically, 10 individual GWSSs will be marked, each with a unique protein (see Table 1). The 10 GWSSs will then be placed in a field cage containing various predator species. The predator assemblage examined will represent those predators commonly found in areas inhabited by GWSS (JRH, pers. obs.). A partial list of the predator assemblage that will be examined and their probable feeding behaviors is given in Table 2. After 6 h in the cage, every remaining predator will be collected and analyzed by 10 different protein-specific ELISAs. A hypothetical example of the data we will generate over the next year is given in Table 3.

Table 1. A listing of the proteins that will be used to mark 10 individual GWSS.

Individual GWSS	Protein marker
1	Rabbit IgG
2	Guinea pig IgG
3	Equine IgG
4	Mouse IgG
5	Dog IgG
6	Pig IgG
7	Bovine IgG
8	Cat IgG
9	Rat IgG
10	Sheep IgG

Table 2. A listing of the arthropod assemblage to be examined.

Species	Stage ¹	Classification ²	Likely GWSS prey ³
<i>H. convergens</i>	Adult/immature	Carnivore	Egg
<i>Zelus renardii</i>	Adult/immature	Carnivore	Nymph/Adult
<i>Geocoris punctipes</i>	Adult	Omnivore	Egg/early instar nymph
Spiders	Adult/immature	Carnivore	Nymph/Adult
Salticidae			
Clubionidae			
Agelenidae			
Araneidae			
Earwig	Adult/immature	Omnivore	Egg, nymph, adult
<i>Chrysoperla carnea</i>	Immature	Carnivore	Egg
Preying mantis	Adult/immature	Carnivore	Nymph, adult
Syrphid fly	Immature	Carnivore	Egg
<i>Coccinella septempunctata</i>	Adult/immature	Carnivore	Egg

¹The predator life stage that will be examined.

²The primary feeding habit of each species.

³The most likely GWSS life stage that will be attacked.

the gut contents of every predator in the assemblage by a myriad of protein-specific ELISAs, and (5) all of the proteins and their complimentary antibodies are commercially available at an affordable price.

OBJECTIVES

We are in the preliminary phase of a research project dedicated to quantifying predation rates on GWSS nymphs and adults and qualifying predation on eggs. There are enough protein/antibody complexes commercially available that each GWSS in a field cage can be marked with a specific protein. We will mark individuals (e.g. adults and nymphs) and release them for 6 hours into a cage containing an assemblage of predators. The experiment will contain a day and night treatment. Observed mortality for each GWSS life stage will be determined by simply counting the number of GWSSs remaining in each cage. Each predator will then be examined by a multitude of protein-specific ELISAs to determine which predators ate GWSS nymphs and adults and how many each predator consumed. Then, each predator will be examined by a GWSS egg-specific ELISA to determine the frequency of predation on GWSS eggs (see Fournier et al. in this volume). Specifically, this study will: (1) quantify predation on GWSS nymphs and adults, (2) qualify predation on GWSS eggs, and (3) determine the circadian feeding activity of predators. Results obtained from this research will enhance our basic understanding of predator-prey interactions and aid in evaluating the efficacy of generalist predators for a conservation biological control program or an inundative biological control program.

RESULTS

We (JRH) conducted feasibility studies to determine if protein markers can be substituted for pest-specific MABs for the immunological detection of prey in predator guts. In a series of lab studies, we fed a wide variety of predators (e.g., chewing and piercing/sucking type predators) both large and small prey marked with rabbit immunoglobulin G (IgG). In turn, the gut contents of each predator was analyzed by a rabbit IgG-specific ELISA. The results showed that, regardless of the predator species and the size of prey consumed, the rabbit IgG ELISA could easily detect the mark in the predator's stomach for at least 6 hours after feeding (Figure 1).

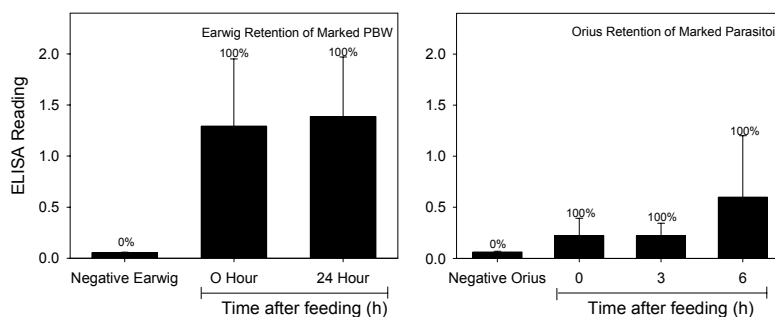


Figure 1. Mean (\pm SD) ELISA readings for the retention of rabbit IgG in the gut of two types of predators that consumed either a single 2nd instar pink bollworm larva or an adult parasitoid (*Eretmocerus emiratus*) marked with 5.0 mg/mL of rabbit IgG. The numbers above the error bars are the percentage of individuals positive for rabbit IgG. The negative predators consumed unmarked prey. Note: these data were chosen for display because they represent the extreme case scenarios (e.g., a large chewing predator eating a relatively large marked prey and a small piercing/sucking predator eating a very small marked prey). Similar studies are being conducted on GWSS.

The next study was designed to determine if we could mark adult GWSSs. In a pilot study, we marked (internally and externally) adult GWSS with rabbit IgG protein using the techniques described below.

Internal Marking

GWSSs were provided a chrysanthemum (mum) that was previously marked with a topical spray of a 5.0 mg/mL rabbit IgG solution. Individuals were allowed to feed on a protein-marked mum for 48 h. The GWSSs were removed from the protein-marked mum and placed on unmarked mums for 3, 5, or 7 days after marking and then analyzed for the presences of rabbit IgG by the anti-rabbit IgG ELISA described by Hagler (1997a). The efficacy of the marking procedure is given in Figure 2.

External Marking

We applied an external mark to individual GWSSs by spraying them with 1.0 ml of a 0.5 mg/mL rabbit IgG solution using a medical nebulizer (Hagler 1997b). The GWSS were air-dried for 1 h and then placed on mums for 3, 5, or 7 days after marking and then analyzed for the presence of rabbit IgG by ELISA. The efficacy of the marking procedure is given in Figure 2.

A NOVEL IMMUNOLOGICAL APPROACH FOR QUANTIFYING PREDATION RATES ON GLASSY-WINGED SHARPSHOOTER

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Reporting Period: The results reported here are from work conducted from August 15, 2004 to October 12, 2004.

ABSTRACT

A glassy-winged sharpshooter (GWSS) protein marking system is being developed for use as a diagnostic tool for predator gut content analysis. We determined that GWSS can be marked with 100% efficiency for at least 7 days after feeding on protein-marked plant material or spraying with a topical protein solution. Moreover, feeding trials have shown that protein marked insects can be detected by a protein-specific ELISA in the guts of predators that consumed them. Field studies are being initiated that will quantify the predation rates of an assemblage of predators on GWSS using a multitude of protein-specific ELISAs.

INTRODUCTION

Very little information exists on predaceous natural enemies of GWSS. While predaceous arthropods are important regulators of arthropod populations (Luff, 1983; Sabelis, 1992; Symondson et al., 2002); identifying the feeding choices and amount of prey consumed by generalist predators is very difficult. Predators and GWSS are small, elusive, cryptic (Hagler et al., 1991), and the predators may feed exclusively at night (Pfannenstiel & Yeorgan, 2002). Hence, visual field observations of predation are extraordinarily difficult to obtain. Moreover, predators do not leave evidence of attack. Perhaps the most frequently used experimental approach for evaluating natural enemies in the field are through studies conducted in field cages (Luck et al., 1988). Such studies require manipulation of either the natural enemy or the targeted prey population(s) within the cage (e.g., the removal or introduction of the organism of interest). Mortality of the pest can be estimated based on the presence or absence of the pest (Smith & De Bach, 1942; Leigh & Gonzalez, 1976; Luck et al., 1988; Lang, 2003). Such studies have documented the qualitative impact of manipulated predator assemblages on many types of pests, but they do not provide quantitative information on predation rates or evidence of which predator in the assemblage is exerting the greatest biological control. Often the only direct evidence of arthropod predation can be found in the stomach contents of predators. Currently, the state-of-the-art predator stomach content assays include enzyme-linked immunosorbent assays (ELISA) for the detection of pest-specific proteins (Hagler, 1998) and PCR assays for the detection of pest-specific DNA (Agustí et al., 1999; Symondson, 2002; Greenstone & Shufran, 2003).

ELISAs have been widely used to identify key predators of certain pests, including GWSS (Ragsdale et al., 1981; Sunderland et al., 1987, Hagler et al., 1992, 1993, 1994; Hagler & Naranjo, 1994ab; Bacher et al., 1999; Fournier et al., in prep). The simplicity and low cost of conducting an ELISA lends itself to the efficient screening of hundreds of field-collected predators per day. However, polyclonal antibody-based ELISAs often lack species specificity and monoclonal antibody-based ELISAs are too technically difficult, costly, and time consuming to develop for wide scale appeal (Greenstone, 1996). Moreover, pest-specific ELISAs share the same limitation as the other predator evaluation methods; the quantification of predation rates is impossible (see Hagler & Naranjo, 1996; Naranjo & Hagler, 1998 for reviews). PCR assays using pest-specific DNA probes might be less expensive to develop (Greenstone & Shufran, 2003), but PCR assays are also not quantifiable and they are more costly, technical, tedious, and time consuming to conduct than ELISAs (pers. obs.).

Due to the reasons discussed above, quantifying predation rates is extremely difficult. These difficulties have resulted in a dearth of information on the quantitative impact that generalist predators have on suppressing pest populations. The many shortcomings of each method of predator assessment described above were the impetus for us to develop a technique to quantify predator activity. The technique combines our previous research using pest-specific MAb-based ELISAs to detect predation (Hagler et al., 1991, 1993, 1994, 2003) with protein marking ELISAs we developed to study arthropod dispersal (Hagler & Miller, 2002; Hagler, 1997a, b; Hagler & Naranjo, 2004; Hagler & Jackson, 1998; Hagler et al., 2002). Here we describe a technique for marking individual GWSSs, each with a unique protein. In turn, the gut contents of each predator in the assemblage can be examined by a multitude of protein-specific ELISAs to determine how many GWSS were consumed and which predator species consumed them. The advantages of immunomarking prey over prey-specific ELISAs are: (1) prey-specific antibodies (or PCR probes) do not need to be developed, (2) the protein-specific sandwich ELISAs are more sensitive than the indirect prey-specific ELISAs (Hagler et al., 1997), (3) a wide variety of highly specific protein/antibody complexes are available, (4) the specificity of each antibody to its target protein facilitates the marking and examination of

protocols generate sufficient polymorphisms within *Xf* to enable grouping of strains according to host associations. SNP analyses represent one of the most recent technologies used for comparative studies of closely related bacteria. Based on published genomic information, strain specific primers recently will be used to investigate the pathotype profile using the 16S rDNA intergenic region. Results from our current season's research indicate that this multiplex PCR protocol can differentiate genomic populations which might co-exist in infectious vectors (Fig. 1). Here again, attempts will also be made to quantify *Xf* in selected insect vectors to identify the population dynamics of *Xf* within a vector population.

CONCLUSIONS

The results obtained from the second year of this project remains consistent with our first year observations and has generated significant new information regarding the seasonal host utilization patterns, dispersal, and overwintering biology of GWSS in the central SJV of California. This information will improve our understanding of the epidemiology of Pierce's disease which will also be useful in understanding the epidemiology of other economically important diseases caused by *Xf* for which GWSS may become an important vector. This objective directly addresses gaps in our present understanding that must be filled in order to develop comprehensive PD and GWSS management strategies. This research has expanded on previous work by documenting important aspects of the population biology of GWSS in the agricultural landscape of the central San Joaquin Valley of California. An improved knowledge of the genetic diversity of strains that comprise the population of *Xf* detected from potentially infectious GWSS will further help in devising effective strategies for managing Pierce's Disease, as well as other important diseases caused by this bacterium.

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FUNDING AGENCIES

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

pathogen, when they move into vineyards, and when they spread the pathogen to grapes is critical to understanding and managing the spread of PD in this area.

OBJECTIVES

1. To identify and characterize the seasonal abundance of the primary vectors of *Xf* and seasonal patterns of insect dispersal.
2. Compare the genetic structure of *Xf* strains isolated from GWSS collected from perennial, cultivated and non-cultivated plant species.

RESULTS

Objective 1

Examination of the seasonal host utilization patterns and dispersal biology of the glassy-winged sharpshooter, *Homalodisca coagulata* (GWSS) within and among a variety of perennial crop plant species has been monitored through the winter (2003-04) and following spring and summer seasons of 2004. Experimental sites are located in GWSS-infested areas of Tulare County, California. The results of these studies continue to provide valuable insight into the relative importance of different crop types as predominant overwintering habitats, ovipositional substrates, and preferred feeding hosts for GWSS. Patterns of crop utilization were monitored within perennial crop species including grape, citrus (navel and lemon), stonefruit (sweet cherry, peach, and plum), olive, and avocado at each of three locations for each crop type. Additionally, non-crop weed vegetation was monitored throughout the season at three experimental sites along with riparian vegetation. Host utilization was assessed monthly at each of three locations for each crop type based on sweep/beat-net sampling for adult and immature GWSS and visual inspections for GWSS egg masses. Results from our second year again indicate that host plant species influences GWSS population biology. Similar to our findings in 2003, the largest mean number of adult GWSS were collected from citrus (navel and lemon) and pomegranate whereas mean nymphal population densities were lower than the previous season. More nymphs were present in navel orange and pomegranate with fewer nymphs collected in olive, avocado, cherry, plum, and peach. Non-crop plant species upon which adult and nymphal GWSS were collected included red-root pigweed, prickly lettuce, annual sowthistle, little mallow, lambsquarters, field bindweed, blue morning glory, curly dock, evening primrose, johnsongrass, and ground cherry. The greatest mean number of GWSS egg masses were collected from both citrus and pomegranate.

Seasonal dispersal of adult GWSS was again monitored within and among the previously indicated perennial crop plant species. Traps were suspended 2 m above the ground between tree canopies along 4 linear transects at each of 3 experimental locations for each crop sampled. Beginning November 2003, a total of 11,677 adult GWSS, 29 green sharpshooters (GSS, *Draeculacephala minerva*), and 351 spittlebugs (Cercopidae) were captured on yellow sticky cards. Temporal patterns of GWSS capture were similar in citrus and pomegranate throughout the 2004 sampling season representing dispersal of both overwintered and 1st generation adult GWSS. Seasonal patterns of GWSS capture in olive, avocado, and plum was dissimilar to that of either citrus or pomegranate similar to the patterns observed in 2003. Beginning November 2003, we have begun to closely monitor the overwintering host utilization patterns of adult GWSS among the variety of perennial crop and non-crop weed species previously listed. Overwintering adult GWSS have been sampled monthly (Nov – Feb, 2003) in perennial tree crops by beating/shaking all scaffolds over two, 80 ft² white, PVC tarps that flank both sides of the tree stem and in non-crop weed species using sweep net collections described previously. Adult GWSS have been collected overwintering on citrus (lemon and navel), pomegranate, peach, plum, and avocado averaging 0.2, 0.4, 0.9, 0.02, 0.05, and 0.5 adult GWSS/tree, respectively, over the four month sample interval. Mean populations of adult GWSS swept from non-crop annual vegetation have averaged 1.1, 2.4, 0.9, and 0.3 adult GWSS/50-sweep sample over the four month sample interval, respectively. To examine the seasonal population biology of GWSS utilizing non-crop host species, GWSS, native sharpshooters, and all spittlebugs have been sampled monthly from the ground cover and surrounding vegetation at each of the 3 experimental locations with high populations of GWSS present in 2003.

At each location, sharpshooter and spittlebug adults and nymphs associated with the ground cover and surrounding non-crop vegetation are sampled using a standard sweep net (100 sweeps at each of 10 sites per location for ground cover).

Objective 2

The presence of *Xf* in a subsample of vectors captured among the different perennial crops and on non-crop species has begun using PCR. Genomic DNA is first isolated and initially screened against RST 31/33 universal primers to detect all *Xf* strains. The diversity of the chosen *Xf* isolates will be assessed using RAPD-based protocols and single nucleotide polymorphisms (SNPs) from genome loci of taxonomic importance deduced from the available genome sequences. Previous studies have demonstrated that these

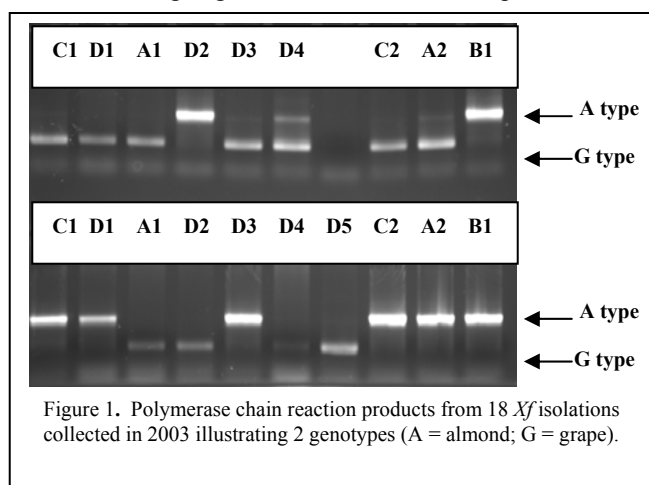


Figure 1. Polymerase chain reaction products from 18 *Xf* isolations collected in 2003 illustrating 2 genotypes (A = almond; G = grape).

EPIDEMIOLOGY OF PIERCE'S DISEASE IN THE CENTRAL SAN JOAQUIN VALLEY OF CALIFORNIA: FACTORS AFFECTING PATHOGEN DISTRIBUTION AND MOVEMENT

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Reporting Period: The results reported here are from work conducted November 2003 to September 2004.

ABSTRACT

The primary objective of this research was to characterize the seasonal abundance, dispersal, and overwintering biology of the glassy-winged sharpshooter (GWSS), a primary vector of *Xylella fastidiosa* (*Xf*). Moreover, to identify where the vector(s) acquire the pathogen, to determine when vectors move into vineyards and transmit the pathogen to grapes, and to genetically characterize the populations of *Xf* isolated from GWSS collected in different perennial cultivated and non-cultivated plant species. Based on results of seasonal plant utilization by GWSS in our study through the winter of 2003-04 and into the subsequent growing season, we conclude that host plant species can significantly influence GWSS population biology. GWSS adult, nymph, and egg mass densities varied among perennial, cultivated crop plant species and non-cultivated weed species examined in this study. Perennial crop species examined included sweet cherry, navel, lemon, olive, avocado, peach, plum, pomegranate, pistachio, and grape. Adult GWSS dispersed into and fed upon a wide range of these crop species with the largest dispersing populations observed in citrus (lemon and navel) and pomegranate, similar to our findings in 2003. Adult GWSS were also regularly collected from and observed feeding upon a wide range of non-crop weed species within and surrounding experimental orchard crops. Nymph populations were not equally represented across all perennial tree crops with increased populations collected from citrus, pomegranate, and also non-crop annual weed species. Overwintering adult GWSS were consistently collected in relatively low population densities on citrus, pomegranate, avocado, plum, peach, and non-crop annual weed species. Patterns of adult GWSS capture among the distances sampled along linear transects extending into perennial crops were dissimilar among perennial crops. The presence of *Xf* in a subsample of vectors collected from different perennial crops and on non-crop species is underway using a multiplex PCR protocol to differentiate genomic populations.

INTRODUCTION

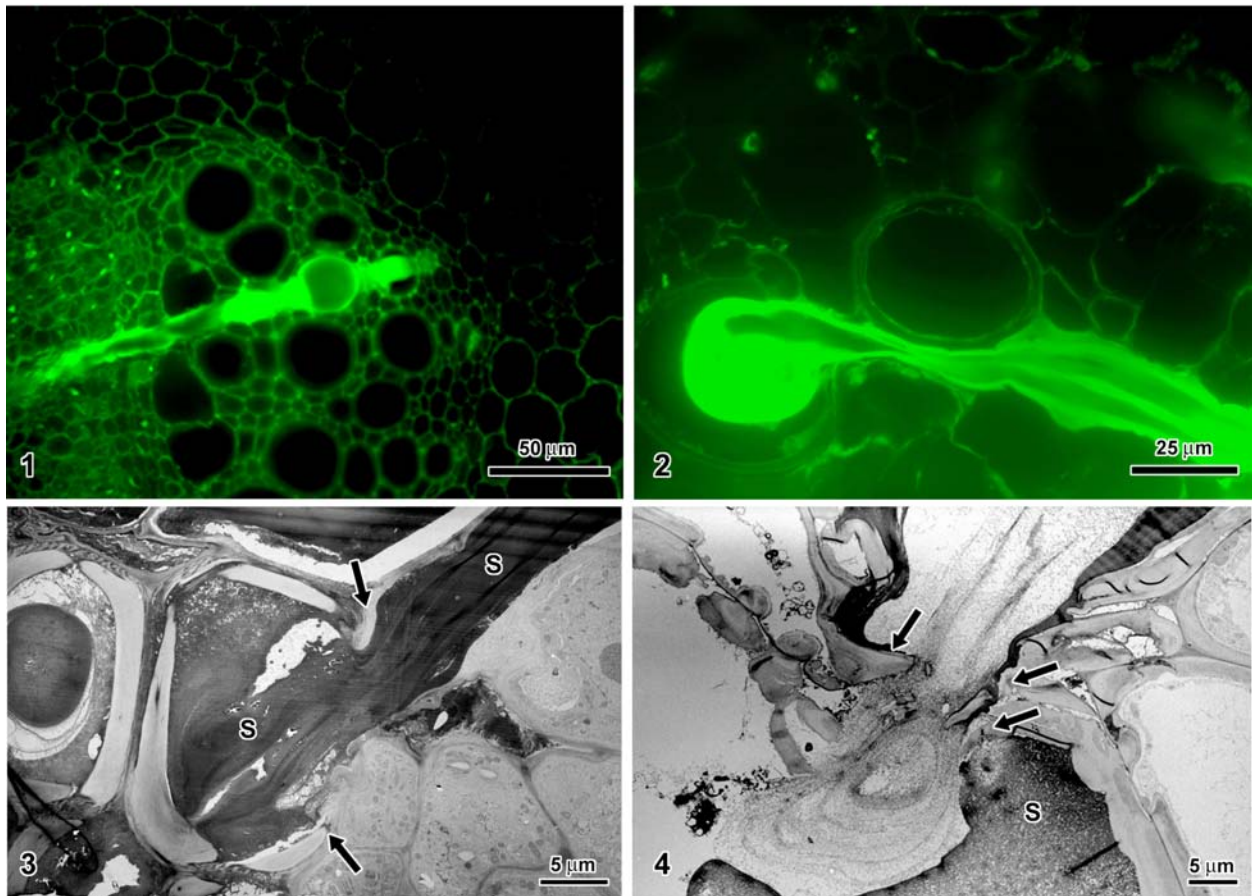
The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, was introduced into Southern California in the late 1980's and later identified in 1994 (Blua et al. 1999). The insect regularly occurs in most of Southern California and has become established along eastern portions of the San Joaquin Valley of central California. Large populations of the GWSS are becoming widely distributed and will reportedly feed and oviposit on a wide range of perennial crop and ornamental plant species as well as numerous non-crop wild plant species (Adlerz and Hopkins 1979, Daane and Johnson 2003). This sharpshooter has continued to expand its range in the state and is expected to affect the overall increase in plant diseases caused by *Xylella fastidiosa* (*Xf*) (Purcell and Saunders 1999a). Strains of *Xf* have a complex pathogenic relationship with a diverse host range including members of both monocots and dicots (Chen et al. 2000). Analyses of the genetic diversity of *Xf* have begun to elucidate differences between many of the strains (Chen et al. 1995, Hendson et al. 2001, Pooler and Hartung 1995). Knowledge of the genetic diversity of strains that comprise the population of *Xf* in the central San Joaquin Valley (SJV) of CA, especially as it relates to insect vectors, will help in devising effective strategies for managing Pierce's disease (PD), as well as other diseases caused by this bacterium.

Xylella fastidiosa is transmitted by xylem feeding sharpshooters (Cicadellidae) and spittlebugs (Cercopidae) (Hill and Purcell 1997, Purcell and Frazier 1985). In California, there are at least 20 species capable of transmitting the pathogen, although only four species are considered to be epidemiologically important in grapes (Pearson and Goheen 1988). Based on the population dynamics of native sharpshooter species in coastal California vineyards, much of the spread of *Xf*, especially early in the season when it is most damaging to grapevines, are by adults that move into the vineyard from outside host sources (Purcell and Saunders 1999b). Knowledge of which vector species transmit *Xf* in the central SJV, where they acquire the

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FUNDING AGENCIES

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



Figures 1, 2. Confocal scanning light micrographs. Figure 1. Several vessel elements damaged by a single GWSS stylet probe.

Figure 2. Salivary sheath material occluding a fragmented vessel element

Figures 3, 4. Transmission electron micrographs showing fragmented vessel element walls (arrows) and salivary sheath occlusions (s).

In our greenhouse and laboratory studies, host plants fed on by sharpshooters for several days to weeks begin to show symptoms similar to those of plants infected with the bacterium *X. fastidiosa*. These symptoms occur in our host plants even though the sharpshooters we are studying are free of *Xylella*. Previous reports indicated that the symptoms of Pierce's disease may occur very shortly after inoculation with *X. fastidiosa*, long before there is a significant increase in the population of the bacteria to a level believed necessary to produce symptoms (Labavitch *et al.* 2002). Many plant species infected by strains of *X. fastidiosa* show no symptoms of Pierce's disease (Purcell and Saunders 1999). Our research is ongoing to determine the correlation of mechanical damage and occlusion of vessel elements to the onset of symptoms in non-infected host plants

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ULTRASTRUCTURAL CONTRIBUTIONS TO THE STUDY OF THE GLASSY-WINGED SHARPSHOOTER AND PIERCE'S DISEASE

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ABSTRACT

A variety of microscopic techniques including light microscopy, confocal scanning light microscopy, transmission electron microscopy, and scanning electron microscopy are helping to elucidate the structure and function of the mouthparts and the salivary sheath of the glassy-winged sharpshooter, a vector of Pierce's disease.

OBJECTIVES

1. Describe the morphology and ultrastructure of the glassy-winged sharpshooter mouthparts.
2. Describe stylet penetration and the function of each stylet pair during feeding.
3. Ascertain the path of mouthparts from the epidermal layer to the vascular tissue of the host plant, and to ascertain if the sharpshooter has fed in parenchymatous or phloem tissue en route to xylem tissue.
4. Determine the ultrastructure of the salivary sheath and its association with all plant tissues encountered from the epidermal layer to the xylem tissue.

RESULTS AND CONCLUSIONS

The glassy-winged sharpshooter (GWSS) has a significant economic impact as the vector for the transmission of *Xylella fastidiosa*, which causes Pierce's disease in grapes, leaf scorch in oleander and almonds, and variegated chlorosis in citrus. Different strains of the bacterium also cause diseases of avocados, peaches, plums, apricot, cherries, and many other trees and ornamentals (Purcell and Saunders 1999, Purcell et al. 1999). The GWSS feeds primarily on the xylem fluid of more than 100 different host plants from more than 35 plant families.

In response to the tremendous economic importance of this insect, a variety of research avenues are under investigation to develop control or management strategies. One important research area that has not received adequate attention is the interaction between the GWSS and the host plants. Until very recently we knew very little regarding the structure of the GWSS mouthparts, and simply assumed that they were similar to those of other leafhoppers. During the last two years, we have provided extensive ultrastructural descriptions of the GWSS mouthparts, including several new sensory structures associated with the sharpshooter stylets and labium (Leopold et al. 2003, Freeman et al. 2002, 2003).

Many unbranched salivary sheaths and branches of very complex sheaths, formed by nymph and adult sharpshooters, do not always extend directly from the host-plant epidermis to the xylem tissue. GWSS stylets may penetrate only as far as the vessel element wall or they may actually fragment the lignified wall and enter the cell lumen (Figures 1-4). Several vessel elements in a vascular bundle or secondary xylem may be damaged during a single sharpshooter probe (Figure 1). Fragmented vessel elements (Figures 2-4) would change the dynamics of water translocation. Penetrated vessel elements are only infrequently surrounded by salivary sheath material, which raises questions as to the function of the sheath in reducing or preventing cavitation. Penetrated vessel elements can, however, become partially or completely occluded with GWSS salivary sheath material (Figures 1-3), a situation that would also disrupt water translocation even in the absence of *X. fastidiosa*.

The glassy-winged sharpshooter ingests large volumes of xylem fluid during feeding, most of which is quickly excreted. We have noted that both nymph and adult sharpshooters produce exudates during probes that do not reach the xylem, suggesting that they may be feeding in host cells located between the epidermal layer and the xylem. The transfer of *Xylella* to parenchyma cells outside of the xylem (Backus et al. 2003) might be another indicator that sharpshooters are feeding in non-xylem tissues. With a high assimilation efficiency of carbon (Brodbeck et al. 1993, 1995, 1996), there may be a nutritive advantage for even limited feeding in parenchymatous tissues. We now have preliminary data showing that first, second, and third-instar nymphs successfully feed on sunflower stems where the xylem is located too distant from the epidermis to be reached by the length of their stylets. We note that less than 50% of first and second instars have salivary sheaths terminating in the xylem even when the xylem is within the reach of their stylets. Third and fourth instars are only slightly more successful.

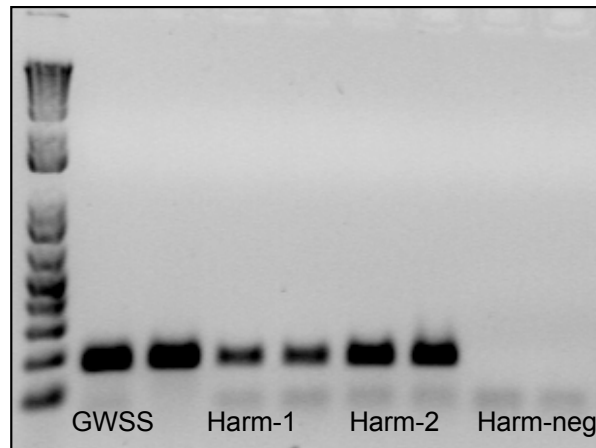


Figure 1. PCR assays were performed using GWSS-specific COII primers on *Harmonia axyridis*. This 2% agarose gel shows that GWSS DNA fragment (178bp) was amplified from the following samples (duplicates): positive control (GWSS), predators fed six GWSS eggs (Harm-1, Harm-2). No amplification occurred for the *H. axyridis* individual that did not consumed any GWSS eggs (Harm-neg).

CONCLUSIONS

We showed that molecular gut content assays can be used to detect GWSS remains in the guts of predators. Once optimization tests are complete we will assay extensive numbers of field-collected predators. We will be able to distinguish specimens that preyed upon immature and adult life stages of the GWSS via the PCR assay and those that consumed eggs via the ELISA assay. An understanding of the key natural enemies of GWSS will contribute to an areawide IPM approach for GWSS control. Once key predators are identified they can be better exploited for conservation and augmentative biological control programs.

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FUNDING AGENCIES

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ELISA for the presence of GWSS egg antigen (methods described in Hagler et al. 2002). Data indicate that the number of ELISA positive reactions decreased over time (Table 1). All negative controls yielded negative ELISA absorbance values. Significant differences between the mean absorbance of values of the lacewings fed GWSS eggs and their negative control counterparts was found in all post-feeding time intervals, except for time=24 and 36 h.

Table 1. ELISA results testing for the presence of GWSS egg antigen in the guts of *Chrysoperla carnea* (3rd instar larva).

Treatments ^a	Negative Control			Lacewing fed with GWSS eggs		
	Absorbance at 405 nm, mean ± SD	Critical value ^b	% positive reactions (N) ^c	Absorbance at 405 nm, mean ± SD	% positive reactions (N)	Significance ^d
0h	0.089±0.003	0.098	0 (15)	0.526±0.488	95 (19)	***
6h	0.072±0.006	0.090	0 (22)	0.176±0.142	62 (21)	***
9h	0.076±0.004	0.088	0 (19)	0.197±0.167	76 (21)	**
12h	0.074±0.007	0.095	0 (21)	0.147±0.149	43 (23)	*
24h	0.077±0.008	0.101	0 (14)	0.170±0.180	36 (22)	N.S.
36h	0.073±0.005	0.088	0 (22)	0.072±0.011	0 (22)	N.S.

^a post-GWSS egg consumption intervals (hour).

^b Mean + 3SD of the negative controls (Sutula et al. 1986).

^c Based on the critical value of the negative control predators. N=total no. of individuals assayed for each treatment.

^d Significant differences (*t* test) between negative control predators and their counterparts fed GWSS eggs: ***, *P* < 0.001; **, *P* < 0.01; *, *P* < 0.05; N.S., not significant.

ELISA Response to Multicolored Asian Lady Beetle that Consumed GWSS Eggs

Adult beetles were placed in individual Petri dishes and starved for 36 h. Each adult was fed six GWSS eggs (within a 60-min time frame) and isolated from food for 0 or 6h and then frozen (-80°C). Negative controls were individuals that did not eat any GWSS eggs. We analyzed the dissected gut of each individual by indirect ELISA for the presence of GWSS egg antigen. All negative controls yielded negative ELISA absorbance values. We found that 65% of the individuals that ate GWSS eggs scored positive at time=0 h, and 8% at time=6h. A significant difference between the mean absorbance values of the beetles fed GWSS eggs and their negative control counterparts only occurred for the time=0h treatment.

Predator Gut Content Analysis Using PCR Assays

We are currently optimizing a PCR assay to detect GWSS DNA in the guts of various species of predators. Several pairs of primers were designed to amplify GWSS-specific fragments from: (1) randomly amplified polymorphic DNA (RAPD) based on sequence characterized amplified regions (SCAR); and (2) the mitochondrial cytochrome oxidase subunit I (COI) and subunit II (COII) genes (de León & Jones 2004). The size of amplified fragments of GWSS DNA varies from 166 to 302 bp. Adult *H. axyridis* fed six GWSS eggs were immediately frozen (-80°C) after eating. Negative controls were beetles that did not eat any GWSS eggs. Each individual was homogenized in a lysis buffer solution, DNA was extracted using a DNeasy kit (Qiagen Inc., Valencia CA) and subjected to PCR using GWSS-specific COII primers. GWSS DNA was successfully amplified from *H. axyridis* extracts (Figure 1). Further tests are underway comparing the efficacy of different primer sets and determining the half-life detection interval of GWSS DNA in the guts of several predator species (*C. carnea*, *Z. renardii*, *S. diadema*, and several species of spiders).

IDENTIFYING KEY PREDATORS OF THE VARIOUS GLASSY-WINGED SHARPSHOOTER LIFESTAGES

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Reporting Period: The results reported here are from work conducted from November 1, 2003 to October 1, 2004.

ABSTRACT

Glassy-winged sharpshooter (GWSS) egg-specific monoclonal antibody (MAb) and GWSS-specific genetic markers have been developed for use as diagnostic tools for predator gut content analysis. Feeding trials were conducted to determine how long a MAb-based ELISA can detect GWSS remains in the guts of *Chrysoperla carnea* and *Harmonia axyridis*. We found that *C. carnea* can yield positive ELISA reaction for the presence of GWSS egg antigen for up to 24 hours after eating an egg. Further results showed that the detection period of GWSS egg antigen in *H. axyridis* is less than 6 hours. Using mitochondrial COII primers specific to GWSS, we obtained successful amplification of GWSS DNA fragments from *H. axyridis* that consumed six GWSS eggs. Optimization tests are underway to increase the efficacy of GWSS-specific genetic primers to detect pest DNA in predator guts. Feeding trials with additional predators (*Zelus renardii*, *Sinea diadema*, and several spider species) are currently being performed.

INTRODUCTION

Effective control of GWSS will require an areawide integrated pest management approach (AW-IPM). A major component of AW-IPM is the exploitation of the pest's natural enemies, which, when utilized to their greatest potential, can increase the effectiveness of other control tactics. Unfortunately, very little information exists on GWSS's predaceous natural enemies. Evidence of predation of GWSS eggs and adults has been observed in the field (JH pers. obs.); however, the composition of the predator complex, and the relative impact of each predator on GWSS mortality is unknown. A major obstacle is the difficulty of studying predators in their natural environment. Unlike parasitoids, predators rarely leave evidence of attack. Laboratory experiments can be used to evaluate the suitability of particular prey and the rates of predation. However, lab studies seldom translate to field situations. Direct field observations are sometimes used to identify predators of key pests, but the small size and cryptic nature of predators and GWSS make direct observations difficult and laborious. Predator gut content analysis represents a valid approach to investigate predation. Currently, the state-of-the-art predator stomach content assays include enzyme-linked immunosorbant assays (ELISA) for the detection of prey-specific proteins (Hagler 1998; Hagler & Naranjo 1994ab) and polymerase chain reaction (PCR) assays for the detection of prey-specific DNA (Symondson 2002). To this end, we have developed GWSS egg-specific MAbs (Hagler et al. 2002; Fournier et al. submitted) and GWSS-specific primers (de León & Jones 2004). Both assays provide an avenue to qualitatively assess the impact of predator species on GWSS populations.

OBJECTIVES

Our main objective is to identify the composition of the GWSS predator complex using pest-specific ELISA and PCR assays. However, several optimization studies are needed (e.g. detectability half-life) before these assays can be used to examine field-collected predators. Here we report results of laboratory tests on detection periods of GWSS egg antigen in the guts of two generalist predators, the green lacewing, *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) and the multicolored Asian lady beetle, *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) using a GWSS egg-specific ELISA. We also present preliminary results on predator gut content analysis using PCR.

RESULTS:

ELISA Response to Lacewing that Consumed GWSS Eggs

Predators were placed individually in Petri dishes and starved for 36 h. Lacewings were then fed one or two GWSS eggs (within a 30-min time frame) and isolated from food for 0, 6, 9, 12, 24, or 36h at 25°C, photoperiod of 16:8h (L:D), and then frozen (-80°C). Negative controls were individuals that did not eat any GWSS eggs. Each lacewing was analyzed by indirect

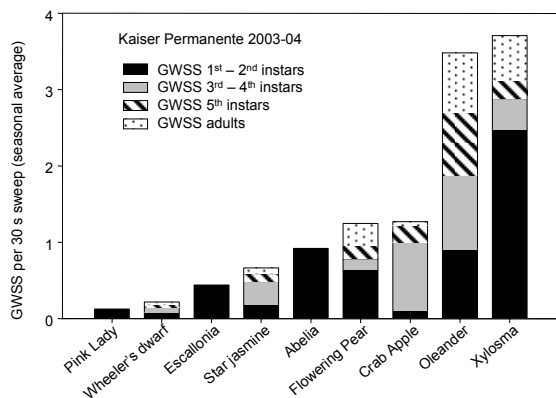


Figure 1. The seasonal average for host plant preference GWSS adults and nymphs was clearly towards oleander and Xylosma at this sampling site. Data of the seasonal average are skewed by the large spring GWSS population density.

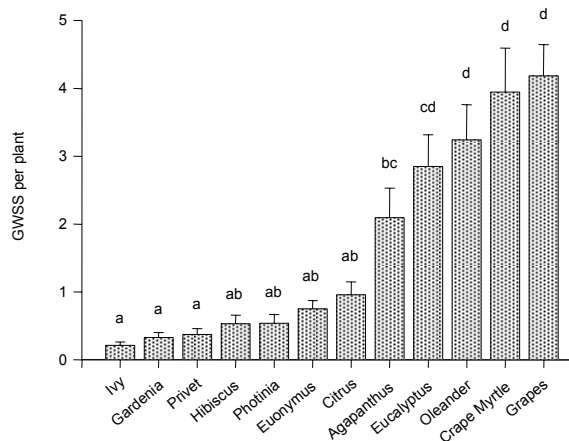


Figure 2. Average densities (\pm SEM) of GWSS (nymphs and adults) were significantly different among perennial host plants, Tukey's HSD at $P < 0.05$. Data are seasonal averages, and biased towards host species preferred in June and July, when GWSS densities were the highest.

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FUNDING AGENCIES

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

collected GWSS material to the experimental site – basically, many of the GWSS nymphs died or left the tested host plant almost immediately after being transfer. We are currently improving inoculation techniques.

Objective 2 – Natural Enemies

During the surveys of GWSS population dynamics in non-agricultural regions, described previously, we collected information on GWSS natural enemies, using sampling techniques such as GWSS egg mass collections (>100 leaves per perennial plant species per collection) and potential GWSS predator collections (beat and sweep samples). As in all studies, we recorded host plant species and seasonal period. We found *Gonatocerus ashmeadi* and *G. triguttatus* (Triapitsyn et al. 1998) comprised about 95 and 4%, respectively, of collected parasitoids. As has been suggested, these parasitoids kill >90% of the summer GWSS population. Parasitoid numbers drop during the winter, when most GWSS are in the adult stage – although large nymphs were present as well. No egg masses or recently hatched nymphs were found from November through February. The first fresh egg masses were collected in April (2003) and March (2004), and we found parasitized eggs within as soon as April (2004). Our results suggest that egg parasitoids are the primary biological control factor. Combined with the winter / spring area wide insecticide control programs (which dramatically reduce the over-wintered population on citrus, the primary GWSS host plant during this period, and lower the overall GWSS population levels in the SJV) the egg parasitoids reduce the GWSS population in the urban regions to such an extent that GWSS can be difficult to find in large numbers in late summer samples.

Predators may play a small role controlling GWSS nymphs. Spiders were the most common predator found, and there was a significantly positive relationship between the number of spiders found and the number of GWSS egg masses ($P < 0.001$, $r^2 = 0.28$). Still, there has not yet been any concrete evidence that links these generalist predators with the regulation or suppression of GWSS. During the GWSS urban surveys, predators were collected, identified to family or genus, and stored at -80°C. These specimens have been shipped to the Western Cotton Research Laboratory, where the predator gut content is being assayed with immunologically-based assays that employ pest-specific monoclonal antibodies (MAbs) for the presence of GWSS egg protein using the ELISA by Drs. Hagler, Fournier and Leon (Hagler et al. 2003). These studies will provide direct evidence of predation by generalist predators.

Objective 3 - Xylella

How important are glassy-winged sharpshooter populations in the urban regions as vectors of *Xf* in nearby agricultural areas? First, GWSS population densities have been relatively low in the SJV urban centers, as previously described. Second, GWSS has a relatively low *Xf* transmission efficiency. Together, the low density and poor transmission efficiency would suggest few GWSS would have *Xf* in their mouthparts and play any role in the movement of the pathogen. We tested adult GWSS collected from ornamental plants in Bakersfield and, to our surprise, found *Xf* in GWSS (mouthparts) collected from oleander, *Xylosma*, and Chinese elm. The positive results do not necessarily mean that the GWSS acquired the *Xf* from the plants that they were collected on as the adults move between host plants often.

How important are GWSS nymphs in the movement of *Xf* among ornamental plants and to vineyards? Nymphs shed the lining of their gut with each molt before adulthood, loosing any *Xf* living there and therefore provide a better indication of acquisition. The initial screening of GWSS nymphs used a “presence” or “absence” of groups of nymphs collected and therefore data are presented as such, rather than a percentage. In the initial collections, *Xf* was found only in GWSS nymphs collected from oleander (in the Bakersfield region). It is also important to note that all GWSS samples testing positive for *Xf* were analyzed for bacterial strain differences and analyses showed that the bacteria present are not of the PD type, but could be oleander, almond, oak, peach or plum. Most likely the *Xf* is oleander strain, which does not pose an immediate threat to nearby vineyards because this strain does not cause PD in grapes

CONCLUSIONS

We have described GWSS population density and age structure on ornamental plants common in residential landscaping in the SJV. We have further described natural enemy presence. This research can be added to information collected in Riverside and Ventura counties to help predict GWSS movement and develop control programs. The research has broader implications for use of ornamental landscape and riparian plants within agricultural settings (e.g., landscaping around farm buildings and homes). Plants which act as preferred hosts for both vector and pathogen can be target for control. By testing GWSS for the presence of *Xf*, researchers will identify potential sources of the pathogen, thereby preventing potential epidemic spread of Pierce’s disease causing *Xf* throughout a reservoir of ornamental host plants. To see a list of host plants, for both *Xf* and GWSS) go to: <http://nature.berkeley.edu/xylella>.

OBJECTIVES

1. Determine glassy-winged sharpshooter biology and ecology throughout the season, particularly its age structure on and utilization of the different host plants that represent common breeding or dispersion refuges for glassy-winged sharpshooter in the San Joaquin Valley.
2. Determine the contribution of resident natural enemies on glassy-winged sharpshooter mortality and whether natural enemy abundance or species composition varies significantly on different GWSS host plants or ecosystems in the San Joaquin Valley.
3. Determine the presence of *Xylella fastidiosa* in glassy-winged sharpshooter collected from different host plant species and in selected ecosystems in the San Joaquin Valley.

RESULTS

Objective 1 - Survey.

GWSS numbers, age structure and natural enemies were surveyed in residential areas in Bakersfield, California. In the 2003-2004 season, six residential sites were sampled. Each site was selected for its combination of different GWSS and *Xf* host plants; most of the sampled sites had 3-8 individual plants of each plant species, with 3 or more GWSS host plant species in close proximity. Host plants surveyed included: carob, rose, star jasmine, Chinese elm, flowering pear, apple, escallonia, pink lady, ivy, nectarine, photinia, citrus, gardenia, privet, euonymous, hibiscus, agapanthus (lily of the Nile), grape, crape myrtle, eucalyptus, mock orange, oleander, *Xylosma* and Wheeler's dwarf. Each month, samples were taken for GWSS and natural enemies. We also recorded plant condition. From April 2003 to October 2004, we made >3000 plant samples (sample plant × sample date).

A thorough analysis of this data set will be made at the end of the residential survey (April 2005) when we project to have >5000 samples, each with information on host plant species, condition and phenology; GWSS density and age structure; and potential natural enemies present. An initial analysis show strong host plant preferences GWSS adults and nymphs, especially towards oleander, crape myrtle and *Xylosma* during the spring and summer months (Figure 1). Host plant preference for adult and nymph feeding sites was not always the same as those preferred for egg deposition – especially with respect to oleander, as reported by other researchers.

The seasonal population dynamics showed a strong spring GWSS population on all hosts followed by a summer decline, which is largely attributed to egg parasitism of the summer brood. We believe that the winter period is critical for GWSS population dynamics as this period represents the low point in the population density. Oleander and privet may be the most important overwintering hosts in the urban regions. In contrast, host plants as crape myrtle and crabapple are dormant throughout winter and, according to our samples, play no role in the GWSS overwintering. However, they are excellent hosts for oviposition and nymphal development during late spring and summer time. For some host, GWSS are confined to specific sections. For example, the flowering pear trees brake dormancy early in the year and start blooming by the first week of February. GWSS adults have been found on the twig tips in the middle of the winter in these trees. It is unknown whether they survive the entire winter in this plant or the early physiological activity of the flowering pear attracts the GWSS. We also found GWSS overwintering exclusively on the “suckers” of the following tree species: eucalyptus, carob tree, Chinese elm, and olive.

Objective 1 – Manipulative Experiments

To categorize GWSS age structure, ecology, and resident natural enemies (particularly predators) on different host plants common in urban areas, potted (6.6 L) plants were used to provide a replicated array of similarly-conditioned (e.g., age, size, irrigation) GWSS host plant species. These preference studies were conducted in an unsprayed, GWSS infested citrus orchard, and two unsprayed residential areas in Bakersfield, California. Perennial species included ivy, photinia, citrus, gardenia, privet, euonymous, hibiscus, agapanthus (lily of the Nile), grapevine, crape myrtle, eucalyptus, and oleander. Annual (or weed) species included prickly lettuce, little mallow, annual sowthistle, coast fiddleneck, common groundsel, London rocket, fox tail brome, lambsquarters, blue grass, and shepperd purse. Both perennial and annual species were set in a randomized block design. Results show GWSS seasonal-long densities were influenced by host plant species, with a significant difference (ANOVA, $P < 0.001$) among host plants, for both perennial and annual categories (Daane et al. 2003, 2004a). Results are provided for perennial host plants in the citrus orchard (Figure 2), which shows a 20-fold difference in the number of GWSS on ivy, the least preferred host planted tested, and grape, the most preferred. We found a relatively similar pattern in the 2002/03 and 2003/04 seasons. Interestingly, GWSS egg mass density was not related to adult or nymphal densities ($P = 0.25$, $r^2 = 0.03$; $P = 0.35$, $r^2 = 0.01$, respectively). As with the urban survey, we conclude that GWSS adults have oviposition preferences that may be different from the nymphal feeding preference. We believe this difference is a result of both GWSS adults and nymphs switching among host plants, and to a disparate level of predator and parasitoid activity.

In a second experiment, we manipulated combinations of GWSS host plant species in cages. Four plant species have been planted in different combinations (e.g., citrus only, citrus and oleander, oleander only, oleander, citrus and crape myrtle), with a total of 7 plant species (4 replicates). Initial progress was slowed by the difficulty we encountered in transferring field-

BIOLOGY AND ECOLOGY OF THE GLASSY-WINGED SHARPSHOOTER IN THE SAN JOAQUIN VALLEY

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Reporting Period: The results reported here are from work conducted from November 1, 2002 to October 1, 2004.

ABSTRACT

We followed glassy-winged sharpshooter (GWSS) preference and age structure on ornamental host plants in Bakersfield, California. Results of an urban survey showed GWSS host utilization varied greatly. This was especially true during the growing season when the mobile GWSS nymphs and adults would frequently shift amongst abutted host plants. While host plant utilization was dynamic, yet there were clear seasonal patterns. In late-fall through mid-winter, GWSS were most commonly found on privet, oleander, and citrus. In late-winter through spring, the preferred hosts were *Xylosma*, photinia, and flowering pear. In summer, host utilization was most dynamic and often dependent on host condition (such as irrigation). Nevertheless, GWSS adult and nymph summer and early-fall populations were consistently found on *Xylosma*, photinia, oleander, star jasmine, and Crape myrtle. Controlled experiments with potted host plants found similar results and highlight differences in GWSS feeding and oviposition preferences. Throughout all studies, we sampled the numbers of predators and parasitoids. Emerged parasitoids show *Gonatocerus ashmeadi* and *G. triguttatus* were reared from egg masses collected on most host plants, and accounted for a large percentage of summer GWSS mortality. Predators were present, especially spiders, and often observed feeding on GWSS. However, our data has not yet found any one predator species to be consistently associated with GWSS or with a reduction in GWSS densities. Collected predators are being analyzed using immunologically-based assays that employ pest-specific monoclonal antibodies (MAbs) to help identify the key predators of GWSS. During the urban surveys, we collected plant material (e.g., potential vector host plants) and potential insect vectors to determine the incidence of *X. fastidiosa*. This material was processed in the laboratory using "immunocapture DNA extraction" to determine the presence of *X. fastidiosa*. Results show that GWSS collected in urban regions often (>10%) carry *Xylella fastidiosa*, however, it is not the strain that cause PD.

INTRODUCTION

The primary focus of this research is the description of glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, GWSS preference, egg deposition, age structure, population dynamics and levels of natural regulation on different host plants in the urban / agricultural interface in the San Joaquin Valley (SJV). Currently, such a description of GWSS biology and ecology in the SJV is lacking. The developed information from this research will help understand GWSS seasonal movement and infestation foci. Of primary concern to regional control programs is whether or not untreated urban GWSS populations serve as an inoculum source for either the insect vector or the bacterial pathogen, *Xylella fastidiosa* (*Xf*).

To develop a more complete description of host plant influence on GWSS age structure and natural enemy impact, we conducted both urban surveys and manipulative experiments. Specifically, we sought to determine the potential of common plant species used in residential landscaping to either reduce or increase GWSS densities. We further screened common plants and GWSS collected for the presence of *Xylella fastidiosa*. When completed, information on the abundance, host plant use, and seasonal dispersal patterns of GWSS and natural enemies in urban better enable researchers to determine GWSS movement and host plant succession in the SJV, and the data may be useful for modification of surrounding vegetation, such as trap crops, to suppress GWSS movement into a vineyard.

will also provide insights into the potential efficacies of anti-PD plant modifications.

OBJECTIVES

1. Develop an artificial diet delivery system for rearing the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*.
2. Formulate and evaluate an artificial diet for the development and reproduction of GWSS.
3. Investigate the utilization of proteinaceous components in the food stream of GWSS in order to refine and improve the artificial diet using physiological and proteomic/genomic approaches.

RESULTS AND CONCLUSIONS

This project has just been funded. Preparation of quarantine facilities is complete and the identification of insect cultures to be used in our studies is underway. The process to hire an additional researcher has been initiated. Preliminary experiments, in collaboration with Jones and Setamou at ARS in Weslaco, have demonstrated continuous feeding by adult GWSS for over 30 days on artificial diets presented through a specialized feeding tube. Additionally, differences in survival have been noted as a result of changes in amino acid concentration and composition within the diet.

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FUNDING AGENCIES

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DEVELOPMENT OF AN ARTIFICIAL DIET FOR THE GLASSY-WINGED SHARPSHOOTER

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Reporting Period: Funding for the study was initiated in October, 2004 and the project is in the start-up phase at the time of this reporting.

ABSTRACT

The intent of this project is to develop an artificial rearing system for the glassy-winged sharpshooter (*Homalodisca coagulata*) (GWSS), the primary vector of Pierce's Disease (*Xylella fastidiosa*) (PD). In order to accomplish this, a diet delivery system will first be developed and then used to test artificial diets. Diet formulations will be based, in part, on previous studies performed by Cohen (2002) using GWSS, as well as on artificial diets developed for other Hemiptera (Mitsuhashi, 1979; Coudron *et al.*, 2002) and on the xylem chemistry of GWSS host plants (Andersen, *et al.*, 1992). Diets will be evaluated based on their effects on life history analyses, reproductive rate and intrinsic rate of increase of GWSS. Another aspect of our project involves investigating nitrogen source(s) for GWSS, as that may represent a nutrient limitation for xylem feeders. Two potential sources for nitrogen, i.e. proteins or peptides, will be studied by determining the fate of dietary proteins/peptides (Brandt, *et al.*, 2004) and the ability of salivary and midgut proteolytic enzymes to digest proteins/peptides (Wright, *et al.*, 2004). In this way, we will identify the role(s) proteins and peptides play in GWSS nutrition and their potential uses in artificial diet formulations.

INTRODUCTION

The formulation of an artificial diet for GWSS will greatly enhance the ability of researchers to rear this insect. Presently, the rearing of GWSS is labor-intensive and costly because of its dependence on the propagation of appropriate host plants, with researchers often needing to propagate several species of plants to enable them to rear GWSS under optimal conditions. The development of an artificial diet would likely be more cost effective and portable, increasing the availability of high quality insects for Pierce's disease researchers and decreasing the costs and time-constraints associated with maintaining the insect in culture. The increased accessibility of GWSS to researchers can lead to more rapid developments in novel control measures for this major vector of PD, with these new measures being directly applied by growers. Furthermore, the coupling of an artificial diet with a suitable delivery system can lead to an improved understanding of the relationship between GWSS nutrition and other PD-related issues (including GWSS' varying abilities to acquire/maintain/transmit infectious *Xf* under different circumstances, e.g., via artificial membranes vs. plants, Redak *et al.*, 2004). In addition, the diet delivery system alone would have other potential uses such as in studying the interactions between GWSS, *Xf*, and the host plant, as well as in testing potential anti-GWSS and anti-*Xf* control agents. This could be accomplished by incorporating into the feeding system: 1) selected host plant-associated compounds; 2) media containing the causative agent of PD (*Xylella fastidiosa*, *Xf*) (although some studies have suggested that *Xf* acquired via an artificial membrane by GWSS may not be infectious, Redak *et al.*, 2004); 3) control agents including anti-GWSS or -*Xf* compounds (such as proteins to be engineered into host plants to control either GWSS or *Xf*; Dandekar *et al.*, 2003; Lin, 2003; Meredith and Dandekar, 2003; Reisch *et al.*, 2003) or anti-GWSS microbials (Kaya, 2003; Mizell & Boucias, 2003). In summary, the development of an artificial diet and a corresponding delivery system for GWSS could lead to insights that can be used to generate improved methods for controlling GWSS and, therefore, Pierce's disease.

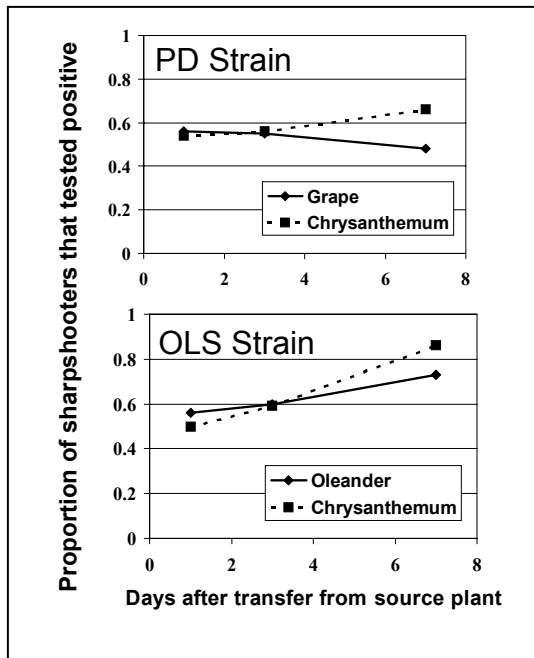
An important part of our project also involves gaining a better understanding of the digestive physiology of GWSS. This will be investigated by focusing on the role proteins and peptides play in GWSS nutrition, as these or similar compounds have been isolated from some xylem fluids (Cohen, 2002; Jain and Basha, 2003; Rep *et al.*, 2003). We will accomplish this by determining the extent to which GWSS can digest proteins and peptides, as well as elucidating the fate of specific ingested proteins in GWSS. This information will be directly used in the generation of an optimal artificial diet for GWSS. Furthermore, GWSS' ability to degrade proteins/peptides will also shed light on the degree to which GWSS can disable defensive proteins/peptides in plants, which is important when dealing with salivary enzymes that are secreted into plant tissues and could alter anti-*Xf* defense components (e.g., either naturally occurring or genetically engineered proteins/peptides; Lin, 2003; Meredith and Dandekar, 2003; Reisch *et al.*, 2003). This knowledge could be used when modifying target plants such as grapevines to improve their resistance against Pierce's disease (PD). Therefore, our investigation into nutritional requirements will not only aid us in the development of a suitable artificial diet for GWSS, but

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FUNDING AGENCIES

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



Results from retention experiments using the OLS strain acquired from oleander showed no significant difference in the mean proportion of insects testing positive when insects were subsequently fed for on either a host (oleander), or a non-host (chrysanthemum) of the OLS strain for 1, 3, or 7 days after acquisition. Similarly experiments using the PD strain acquired from grapevine also found no significant difference in the mean proportion of insects testing positive at 1, 3 or 7 d after acquisition regardless of whether the insects were subsequently fed on either a host (grapevine) or a non-host (chrysanthemum) of the PD strain. Thus, both PD and OLS strains of *X. fastidiosa* remained detectable in GWSS, even when the insects fed on a non-host of the strain for 7 d.

Objectives 2 and 3

To test if feeding substrate can influence the ability of insects to acquire and transmit a particular strain of *X. fastidiosa*, we plan to use a pathogen delivery system to allow us to either maintain or manipulate the feeding substrate as desired. The method described by Bextine and Miller (2002) was originally used for an *Alcaligenes* sp. of bacteria. This technique was modified to provide an environment suitable to survival of *Xylella fastidiosa* and the test plants used. We are using sections of chrysanthemum stem about 10 cm long that are connected

by tubing to a syringe with a suspension of *X. fastidiosa* in PBS. The distal end of the stem is also cut and left open. The syringe is depressed until liquid is extruded from the distal end of the cut stem. Then GWSS are allowed to feed on these stems.

To demonstrate that live *X. fastidiosa* cells could survive movement through a cut stem, *X. fastidiosa* was suspended in a PBS buffer, and the syringe was depressed until liquid was extruded from the distal end of the cut stem. Droplets forming on the distal end were collected and analyzed using PCR to determine if *X. fastidiosa* cells were present. In all cases, *Xylella* was detected within the first 10 drops extruded. Thus, in these experiments, material was injected into stems until at least 10 drops of material was extruded from the distal cut end to ensure that the bacteria have been moved the entire distance of the stem.

In transmission experiments, adult insects were fed for 24 hours on either infected plants, or media-grown bacteria delivered through the cut stem system as described above. Adults were then individually moved to uninfected test plants and allowed to feed for 4 d. When GWSS adults were fed on PD-infected grapevines, 12/26 (46%) transmitted the pathogen to healthy grapevine test plants. In contrast, when insects were fed on media-grown PD bacteria through the cut stem method, no individuals (0/48) transmitted the pathogen to test pants. Similar results were found with OLS-infected plants (9/37, or 24% of individuals transmitted) compared to media-grown OLS delivered through cut stems (0/22 transmitted). Thus, insects did not transmit PD or OLS strains when media-grown bacteria were delivered through the cut-stem system. Purcell et al. (personal communication) found similar results when leafhoppers were fed *X. fastidiosa* through parafilm sachets.

Additional studies are being conducted to determine why insects are unable to transmit the pathogen from the cut stem delivery system. For example, a recent study demonstrated that *X. fastidiosa* cultures will produce different levels of “biofilm formation” when grown on different types of media (Leite et al. 2004). We will test if growing our strains on different media may help induce transmissibility by insects. In addition, we will conduct further studies to determine the pathway of the bacteria through the system. For example, by testing the honeydew of insects feeding on the cut stem system, we can determine if the bacteria are successfully passing through the insect. In the interim, work on the remaining objectives will continue using insects fed on PD and OLS infected plants.

CONCLUSIONS

In retention experiments (Objective 1) for both the PD and OLS strains, we found the proportion of insects retaining the pathogen was the same, regardless of whether insects subsequently fed on a host or a non-host of that strain. This indicates that retention of a particular strain of the pathogen by an individual insect is not dependant on host-specific xylem content of the plant on which it is feeding. In transmission experiments (Objectives 2 and 3) insects successfully transmitted the PD and OLS when they acquired the pathogen from infected grapevine and oleander plants respectively, but did not transmit either the PD or OLS strains when the media-grown bacteria were delivered through the cut-stem system. This could be the result of biological characteristics of media-grown bacteria that contribute to non-transmissibility by insects, or failure of the cut stem system to properly deliver bacteria to the insect. Further experiments are being conducted to determine the basis for lack of transmission of media-grown bacteria by GWSS.

EFFECTS OF FEEDING SUBSTRATE ON RETENTION AND TRANSMISSION OF *XYLELLA FASTIDIOSA* STRAINS BY THE GLASSY-WINGED SHARPSHOOTER

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Reporting Period: The results reported here are from work conducted from October 2003 to September 2004.

ABSTRACT

In this project we are testing the effects of feeding substrate on the acquisition and retention of *Xylella fastidiosa* by the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*. We are using two strains of *X. fastidiosa* that are present in California: a Pierce's disease (PD) strain that infects grape, and an oleander leaf scorch (OLS) strain that infects oleander. A series of experiments were conducted to compare the retention of PD or OLS strains after acquisition, when insects were subsequently maintained on a plant species that was either a host or non-host of that particular strain. In these studies, we found no significant difference in the mean proportion of insects testing positive for the PD or OLS strains, regardless of whether the insects were subsequently fed on either a host or a non-host of the PD or OLS strain. Thus, retention of a particular strain of the pathogen by an individual insect does not appear to be dependant on the xylem content of the plant host on which it is feeding. In a second study transmission efficiency of adult GWSS fed for 24 h on *X. fastidiosa*-infected plants was compared to those fed for 24 h on *X. fastidiosa* from pure media-grown cultures delivered through a cut stem system. In these experiments insects transmitted PD and OLS strains when they acquired the bacteria from a plant, but did not transmit either strain when media-grown bacteria were delivered through the cut-stem system.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) is capable of acquiring and transmitting several different strains of *X. fastidiosa* from a variety of host plants. In this project we are testing the effects of feeding substrate on the acquisition, retention and transmission of *X. fastidiosa* by GWSS. Two strains of the pathogen present in California are being used in these experiments: a Pierce's disease (PD) strain that infects grapevine, and an oleander leaf scorch (OLS) strain that infects oleander. These two strains have different host ranges; the PD strain does not infect oleander, and the OLS strain does not infect grape.

OBJECTIVES

1. Compare retention times of *X. fastidiosa* when infected glassy-winged sharpshooter (GWSS) are subsequently fed on plants that are either hosts or non-hosts of the strain they carry.
2. Compare acquisition and transmission efficiency of insects fed on infected plants to those fed on media-grown cultures delivered through cut stems.
3. Compare retention times of two strains of *X. fastidiosa* in GWSS when simultaneously acquired through cut stems, then subsequently fed on either (a) a non-host of both strains, (b) on a host of only one strain, or (c) alternating hosts of each strain.
4. Test the effects of antibacterial materials on acquisition and transmission of *X. fastidiosa* by GWSS.
5. Test the effects of variation in substrate pH and free ion availability on the acquisition and transmission of *X. fastidiosa* by GWSS.

RESULTS

Objective 1

We began by comparing the relative proportion of insects that tested positive after acquisition of a given strain of *X. fastidiosa*, when they were subsequently maintained on a plant species that was either a host or non-host of that strain. Grape plants (*Vitis* spp.) infected with a Pierce's disease (PD) strain of *Xylella fastidiosa*, and oleander plants (*Nerium oleander*) infected with an oleander leaf scorch (OLS) strain were used as sources of inoculum. The strain of *X. fastidiosa* infecting plants was confirmed by PCR. Groups of GWSS adults were caged on either an OLS infected oleander plant, or a PD infected grapevine for 2 days. Insects were then moved to an uninfected plant of the same species as the source plant (oleander or grape), or to a non-host of the strain (chrysanthemum). Samples of insects were collected at 1, 3, and 7 days after transfer to uninfected hosts and frozen. Insects were subsequently tested for the presence of *X. fastidiosa* using PCR.

CONCLUSIONS

These findings will help solve the PD/GWSS problem by:

- Identifying the mechanism of *Xf* inoculation and using EPG to observe it real-time as it occurs,
- Identifying one determinant of inoculation efficiency, i.e. the role(s) of inoculation behavior vs. bacterial presence and/or detachment in the foregut,
- Developing protocols for further tests of transmission biology and efficiency, especially with respect to acquisition.
- Developing a Stylet Penetration Index for testing among host and non-host species or cultivars, diets, etc. for performance of transmission behaviors, ultimately leading to improved host plant resistance.

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Appendix Table A. Current definitions of the AC EPG waveform phases, families and types of GWSS on grape.

Waveform Phase	Waveform Family	Waveform Type	Waveform Characteristics	Proposed Biological Meanings	
				Plant Tissue/Cell	Insect Activity
Pathway	A	A1	Highest amplitude, hump-like waveform at beginning of probe; usually with spike at the top	Parenchyma or mesophyll	Major salivary sheath formation; deep extension/retraction of stylets; some watery salivation
		A2	Medium amplitude, variable slope; irregular, high frequency with occasional trenches and/or potential drops	Parenchyma or mesophyll	Lengthening and/or hardening of salivary sheath; cell membrane breakage; some watery salivation
	B	B1	Short, single- or multi-peak "spikelet bursts" (20-28 Hz) separated by flutter, wave-like sections	Parenchyma or xylem or pith	Stylet tip fluttering; possible internal muscle/valve movement; involved in inoculation
		B2	Extremely regular, stereotypical pattern of peaks (6 Hz), with distinct phrases	Parenchyma or xylem or pith	Stylet sawing through salivary sheath or tough wood; sheath branching; sheath salivation
Ingestion	C	C (to be subdivided)	Very regular, low rep. rate (3 Hz) with distinct phrases	Parenchyma or xylem or pith	Trial (short) or sustained (long) ingestion (watery excretory droplets correlated)
Interruption	N	N (to be subdivided)	Irregular, appearing A-like at times, but interrupting continuous C; ave. dur. 16 sec.	Parenchyma or xylem or pith	Sheath or watery salivation in ingestion cell; sheath extension

FUNDING AGENCIES

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correlated with all other findings to determine how often the inoculation behavior, when performed by bacteria-laden insects, actually results in expulsion of *Xf*. Present findings [3] still implicate waveforms B1, C and N, especially during long probes. All data analysis will be completed and a manuscript submitted in early 2005 [6].

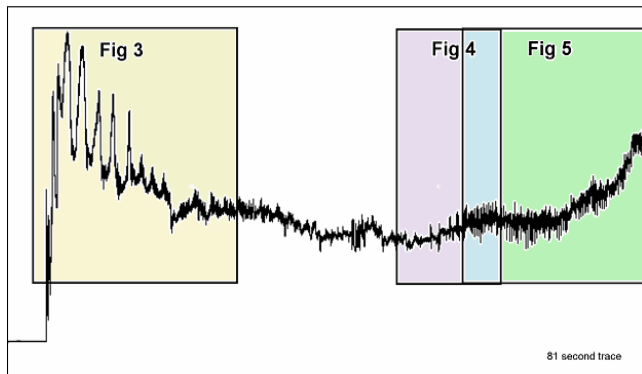


Figure 1. Waveform of GWSS probe in artificial diet compressed 35 times. Box labels indicate where Figures 3-5 were taken from this trace.

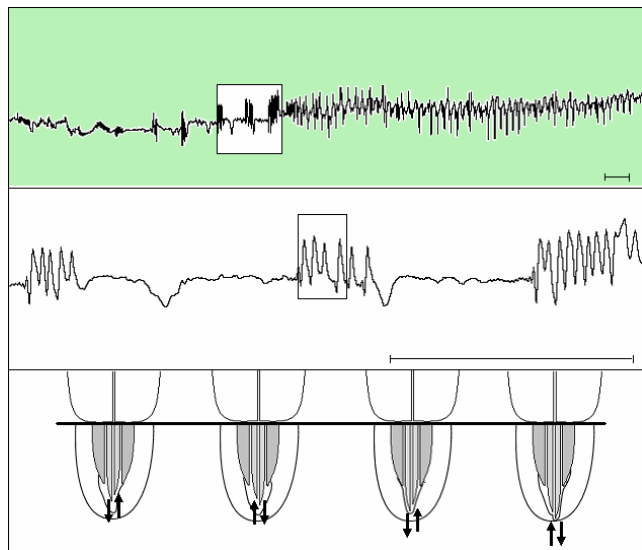


Figure 3. Correlation of B1 waveforms with GWSS stylet activities in artificial diet. Top panel is a waveform trace with B1 compressed 5 times. The middle panel is an uncompressed B1 waveform trace that corresponds to the boxed waveform portion in the top panel. The boxed waveform portion of the middle panel is a B1 spikelet burst and correlates with the stylet activities in the bottom panel. Time marks in the lower right hand corner of the top and middle panel equal one second.

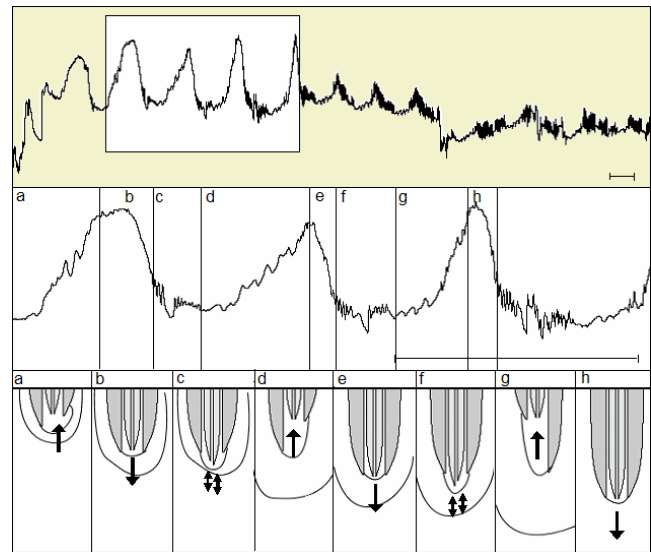


Figure 2. A1 waveforms were correlated with GWSS stylet activities in artificial diet. Top panel trace contains an A1 waveform compressed 5 times. The middle panel is an uncompressed A1 waveform trace that corresponds to the boxed waveform trace in the top panel. Subdivisions, a-h, in middle panel are correlated with stylet activities in the bottom panel with the same subdivision letters. Time marks in the lower right hand corner of the top and middle panel equal one second.

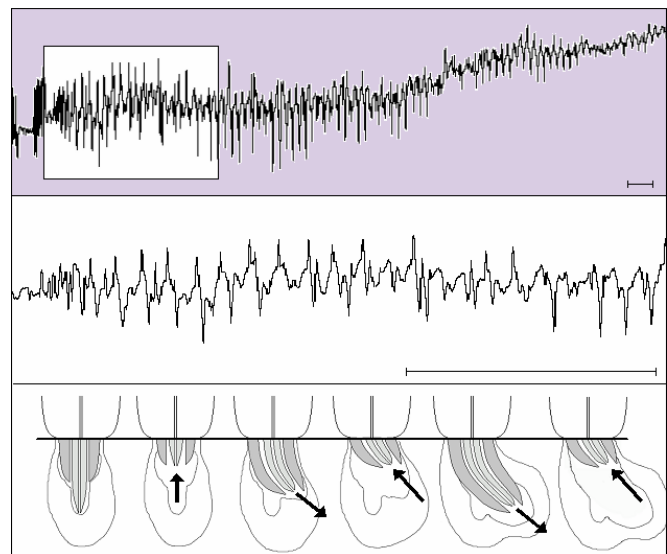


Figure 4. Correlation of B2 waveform with GWSS stylet activities in artificial diet. Top panel is a B2 waveform trace compressed 5 times. The middle panel is an uncompressed B2 waveform trace that corresponds to the boxed portion of the waveform in the top panel. The bottom panel are the stylet activities that were observed at the onset of the B2 waveform and through out the waveform. Time marks in the lower right hand corner of the top and middle panel equal one second.

September 2004. The new USDA-ARS/CSU-Fresno Insect Maintenance and Research facility went into full operation in October 2004. Also during this time we established colonies in the greenhouse in Parlier of the following species: smoke tree sharpshooter, *H. liturata* (STSS), as well as (with Groves) red-headed sharpshooter, *Xyphon fulgida* (RHSS), green sharpshooter, *Draeculacephala minerva* (GSS) and three-cornered alfalfa hopper, *Spissistilus festinus* (3CAH) (collected locally). Preliminary studies of the feeding behavior and EPG waveforms of all of these species are underway.

In addition to major infrastructure improvements in the first 6 months, we also analyzed past data, and Joost performed extensive preliminary tests to develop new protocols in electromyography and real-time imaging of sharpshooter muscles controlling feeding. We also wrote papers, and reviewed and wrote grant proposals. Among these were revisions of the Almeida & Backus paper on blue-green sharpshooter waveforms, now in print [1] and a newly funded UC PD proposal to continue research on mechanisms of *Xf* transmission and details of ingestion behavior. Once we had moved back into the lab and set up, progress resumed on existing objectives during the last four months of the reporting period (July – October 2004).

Objective 1 - Waveform Correlations

Experiment 1: AC-DC Correlation Monitor

Significant progress was made this year in the continuing development of this technology. Bennett built two new prototype monitors, the last of which included design suggestions developed by Backus in consultation with W. F. Tjallingii, Wageningen Agricultural University, The Netherlands. These prototypes for the first time succeeded in achieving waveform fidelity with the original, separate AC and DC waveforms, a goal sought for the last two years of work developing these instruments [2].

Experiment 2: Salivary Sheath-Cell Type Correlation

Backus analyzed histological images produced last year by Habibi from recordings made by Yan (see methods and preliminary findings in [2, 3]). Preliminary findings and waveform appearances are the same as those pictured in the 2002 and 2003 progress reports [2, 3], but waveform names are as in [3]. Results show that early pathway activities, especially A1, occur in the shallow epidermal/parenchyma tissues, A2 and continuous B1 usually occur in the parenchyma peripheral to the vascular bundle (although the sample size of tissues collected for B1 is very small). B2 usually occurs in the parenchyma or phloem, and is often associated with a large deposit of sheath saliva sometimes at a branching point in the sheath. The number of B2 events is also correlated with the number of sheath branches. Short, early C and N events can occur variably, in parenchyma, phloem or xylem; however, longer later C and N events are almost always in mature xylem cells. It is still uncertain whether B1 or C may represent the first penetration of a xylem cell. Correlations were completed and a manuscript is *in prep* for submission in late November [4]. Appendix Table A further summarizes the plant tissue/cell correlations known at the end of the reporting period (late Sept. 2003).

Experiment 3: Stylet Activities Correlation

Joost analyzed the videomicrography data collected by Yan of the stylet activities in artificial diet (see methods and preliminary findings in [2, 3], as well as a schematic of the equipment in the Backus et al. 2004 poster). Stylets could clearly be seen performing stereotypical behaviors during three waveform types frequently seen on grape, i.e. A1, A2 and B1. Results are summarized in Figures 1 – 4 below, Table A and in the Backus et al. 2004 poster. They reveal for the first time that A1 represents the primary formation of the salivary sheath (Figures 1, 2), B1 represents stylet tip fluttering (Figures 1, 3), and B2 represents stylet sawing through the hardened sheath (and, we speculate, perhaps also through tough plant material) (Figures 1, 4). It is particularly interesting that the B1 spikelet burst is dispersed intermittently throughout other pathway waveforms, e.g. between peaks of A1 (Figure 2), as well as in continuous durations by itself (Figure 3). This dispersion, plus last year's Experiment 4 finding [3] that B1 was the only pathway waveform associated with *Xf* inoculation, suggest that the spikelet bursts might represent precibarial valve movement, an important component of a hypothesized inoculation behavior [4]. A manuscript describing these results is *in prep* for submission in late November [5].

Objective 2 - Inoculation Behavior:

Experiment 4: EPG Waveforms Associated with Inoculation

Habibi completed sectioning and photomicrography of the remaining grape tissues probed by EPG-recorded GWSS, i.e. those during the short probe treatment (see the 2003 progress report [3] for methods and preliminary findings). Results from each of the three bacterial detection methods used (Table 1) continue to support that immunocytochemistry may be the most sensitive detection method; 56% of probes showed positive detection of *Xf* near the salivary sheath, while 45% were positive with PCR, and only 10% with culturing. These findings continue to support the interpretations discussed in the 2003 progress report [3]. Unlike PCR,

Table 1: Number of EPG-GWSS-probed grape samples that was positive for *Xf* near the probe out of the total number tested, for each of the three bacterial detection methods.

Probing Treatment	PCR	Culture	Immunocyt.
3 short probes	5/10	0/10	3/8
1 long probe	4/10	1/8	6/8

immunocytochemistry results suggest that detectable bacteria are inoculated more often during long than short probes (Table 1). However, it will be important to determine how many insects were actually inoculative before we can state that conclusively. We have begun to dissect the fixed, dried heads of the recorded sharpshooters for scanning electron microscopy, to determine how many of them contained *Xf* and in exactly which areas in the precibarium/cibarium. This information will be

SHARPSHOOTER FEEDING BEHAVIOR IN RELATION TO TRANSMISSION OF THE PIERCE'S DISEASE BACTERIUM

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Reporting Period: The results reported here are from work conducted from November 1, 2003 to September 30, 2004.

ABSTRACT

Progress this year consisted of completing past projects as well as building infrastructure for future research. Backus's new lab in Parlier was renovated, upgraded and equipped with state-of-the-art facilities for electrical penetration graph (EPG) monitoring of insect feeding and histology of plant and insect tissues. Extensive colonies of glassy-winged, smoke tree, green, and red-headed sharpshooters were established in Fresno and Parlier (with R. Groves, ARS Parlier). New personnel were hired; data was intensively analyzed and grant proposals written. Much effort was also expended in developing new protocols and preliminary findings for feeding waveform correlations with bacterial expulsion and muscle contraction, as well as AC and DC waveforms for several species in colony. Stylet activities and salivary sheath-cell type correlations for the major GWSS waveforms were completed (Objective 1), as was all of the plant histology for the GWSS inoculation test (Objective 2). Results to date support a modified version of last year's hypothesis for the mechanism of *Xf* inoculation to grape. *Xf* bacteria may exit the stylets during brief stylet activities represented by the B1 spikelet burst, B1-like portions of N and/or C, probably within seconds of the first puncture of any penetrated cell, both along the pathway to and within xylem. Proper placement of the bacteria appears to be crucial; placement in xylem leads to growth of the bacteria sufficient for detection by less sensitive methods such as culturing. Otherwise, when more sensitive detection methods such as immunocytochemistry of the tissues immediately surrounding the salivary sheath are used, they can detect *Xf* in non-xylem tissues. Three papers from this research are in preparation for submission in late 2004 – early 2005. This work will help solve the PD/GWSS problem by identifying the mechanism of *Xf* inoculation and crucial aspects of inoculation efficiency, and eventually aid host plant resistance through the development of the Stylet Penetration Index.

INTRODUCTION

Almost nothing was known, until this work, about the stylet penetration behaviors of the glassy-winged sharpshooter (GWSS), and how they interact with populations of *Xylella fastidiosa* (*Xf*) to facilitate transmission to grapevine. This project is combining the three most successful methods of studying leafhopper feeding (i.e. histology of fed-upon plant tissues, videotaping of feeding on transparent diets, and electrical penetration graph [EPG] monitoring) to identify most details of feeding.

OBJECTIVES

1. Identify and quantify all feeding behaviors of GWSS on grapevine, and correlate them with location of mouthparts (stylets) in the plant and presence/ population size of *Xf* in the foregut.
2. Identify the role of specific stylet activities in *Xf* transmission, including both the mechanisms of acquisition and inoculation, and their efficiency. This project's emphasis is on inoculation.
3. Begin to develop a simple, rapid method to assess feeding, or detect the likelihood of *X. fastidiosa* transmission (an "inoculation-behavior detection method"), for future studies.

RESULTS

During the first six months of this reporting period (Nov. 2003 – April 2004), Backus's new lab at USDA-ARS in Parlier was closed due to extensive renovation construction underway. Notwithstanding this delay, we made significant progress on several sharpshooter research fronts during this time. We hired new personnel (a post-doc and a second technician), purchased many supplies and pieces of equipment (including a new confocal microscope), and trained in the use of the equipment. Also, we received CDFA importation permits and permission for a GWSS maintenance colony to be established in Fresno Co., at a site on the campus of CSU-Fresno. A trailer was rented, retrofitted for quarantine infrastructure, and inspected by officers of the Fresno Co. Agricultural Commissioner's office. Insect maintenance and research rooms were built and outfitted with lighted shelves, cages, growth chambers, and research equipment. Also, a contract was arranged by Groves and Civerolo with Morgan to supply greenhouse-reared GWSS on a monthly basis. Acquisition of insects began in

moving an estimated $28\pm 3\%$ (mean \pm SE) of the observation period in both treatments. Spiders in the bean treatment caught and fed on 0.22 ± 0.07 GWSS per day, whereas those in the mixed-plant treatment fed on 0.33 ± 0.09 GWSS. All GWSS were sexed after observation, and data were examined for possible behavioral differences. However, there were no differences between the sexes in terms of their behavior (MANOVA with sex and plant-spider treatment as the factors; $F=1.29$, $df=5,276$, $p=0.27$).

CONCLUSIONS

The availability of multiple plant species increased GWSS interplant movement, and feeding times were reduced in these cages, suggesting GWSS 1) can detect the presence of other host species in the vicinity, probably through olfaction, and 2) that diet-mixing helps GWSS obtain needed nutrients more rapidly. However, the increased movement between plants also may correspond to an increased in acquisition and spread of the bacterium that causes Pierce's Disease. The effects of potentially toxic plants, such as tree tobacco, are not currently understood on GWSS interplant movement. Further data analysis should help clarify the insects' response. Spiders did not affect GWSS feeding and intra- and inter-plant behavior in the observations described here. Thus, these (and possibly other arthropod) predators should not affect the GWSS' acquisition and spread of Pierce's Disease.

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v.8) for differences due to the plant availability (beans-only or mixed plants), spiders (presence or absence), and whether the GWSS were field-collected as adults or lab-reared. Adults that had been reared from birth only on bean plants in laboratory colonies were used in 27 cages, and GWSS that had been captured in the wild as adults were used in 9 cages. One behavior was omitted from the analysis to allow independence of the observations (see Cisneros and Rosenheim 1998).

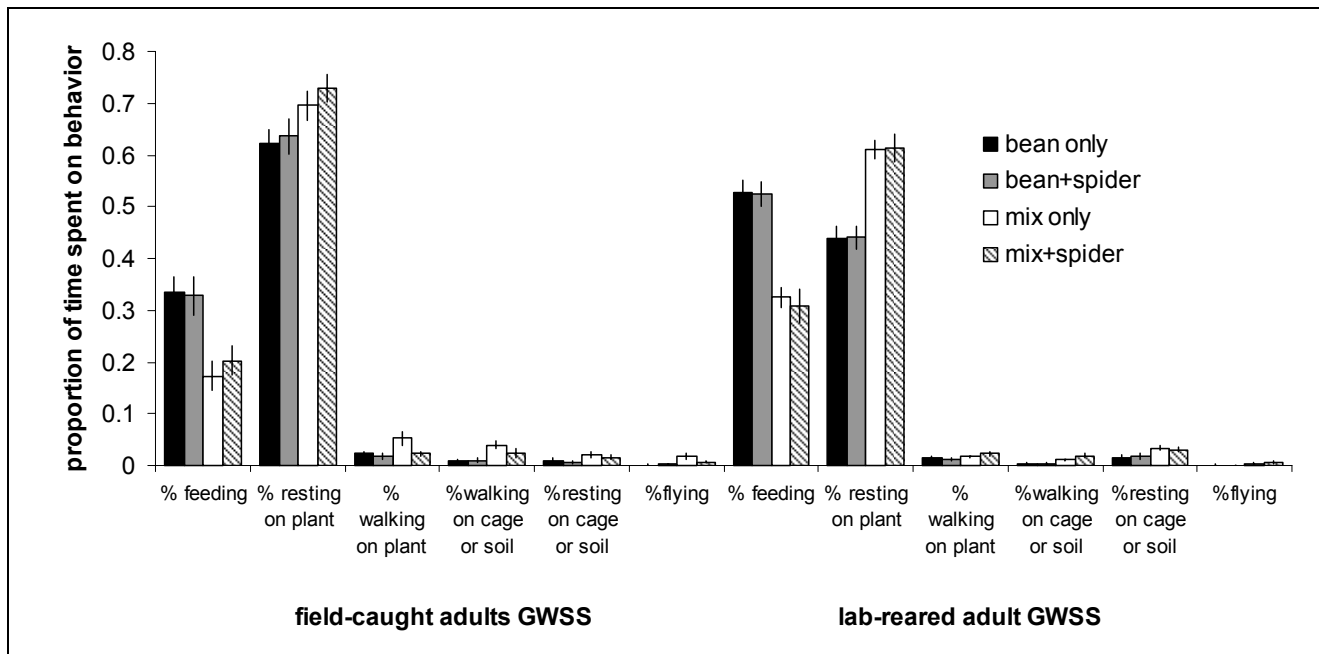


Figure 1. Behaviors performed by caged GWSS adults observed during daylight hours. The average time spent by individuals in each cage on each behavior is shown; error bars indicate standard error.

GWSS spent nearly all of their time either feeding or resting on plants (Figure 1). About 2-5% of the time was devoted to walking on a plant, 1-5% to walking on the cage or soil, 2-5% to resting on the cage or soil, and 0-2% to flying. Plant treatment (bean-only or mixed species) affected all behaviors ($F=13.87$, $df=5,132$, $P<0.0001$). Individuals on beans spent more time feeding and less time resting than insects did on plants in mixed-species cages. Field-caught insects varied significantly from laboratory-reared individuals in their behaviors ($F=16.20$, $df=5, 132$, $P<0.0001$), feeding less and resting more than laboratory insects. However, both groups of insects showed similar time budgets. Both spent less time feeding on beans than on mixed plants. However, lab-reared insects spent less time resting than feeding on beans, and field-reared insects rested more than feeding on beans. This interaction between plant treatment and insect origin (field-caught vs. lab-reared) was significant ($F=2.58$, $df=5,132$, $P=0.029$). Both plant treatment and insect origin significantly affected all insect behaviors at the $p=0.01$ level or greater.

Interplant movement, either by walking or by flying, was higher in the mixed-species cages. GWSS also spent more time resting on the cage or on the soil in the mixed-plant treatment cages, although such a small amount of time was spent in this behavior that it was probably not biologically significant. However, the increase in movement between plants in the mixed cages, although small, is significant in that such behavior increases the GWSS' opportunities to acquire and transmit Pierce's disease.

The three plant species were selected because one provided a host on which GWSS can complete multiple generations (bean), one was an alternate host favored in the field (sunflower), and the final plant contains potentially toxic nicotine in the xylem (tree tobacco), and so may be preferentially avoided. All three plant species were used as host for feeding, but the amount of time spent feeding on each species has not yet been calculated. Both the time spent feeding, and the frequency of leaving each species of plant, will indicate the GWSS' preference for the 3 species.

The presence of spiders did not affect GWSS behaviors ($F=1.08$, $df=5, 132$, $P=0.376$). There were no interactions between spiders and plant species or origin of GWSS. Spiders used in the experiments were field-collected, and the species changed as the season progressed. Predation activity also varied within species, perhaps due to hunger levels of each individual. The presence of spiders did not affect GWSS, but wide variation in spider activity level might hide predation effects. We therefore examined spider activity levels (% of observations in which the spider moved), based on intra- and inter-plant movements, to correlate predation pressure to GWSS movement and feeding behavior. GWSS did not show a behavioral response to spider activity levels (spider activity not correlated to GWSS time spent feeding, moving on the same plant, resting on the plant, moving on the soil or cage, flying) in either plant treatment, nor was the number of GWSS eaten related to spider activity (all non-significant in direct regressions). The spiders were equally active in the two plant treatments,

PLANT AND PREDATOR EFFECTS ON INTERPLANT MOVEMENT BY THE GLASSY-WINGED SHARPSHOOTER

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Reporting period: The results reported here are from work conducted from May 2004 through September 2004.

ABSTRACT

Adult GWSS in caged habitats were monitored hourly to determine the effects of plant species availability and predator presence on intra- and inter-plant movement, as these factors are directly related to the acquisition and spread of Pierce's Disease. GWSS were placed in caged habitats with either a monoculture of beans or polyculture of bean, sunflower, and tree tobacco, and either with or without spiders, in a 2x2 factorial design. Origin of the GWSS (field-caught or laboratory-reared) was also included as a third factor in the multi-factor MANOVA to determine the importance of each treatment on GWSS feeding, resting, and intra- and inter-plant movement. Approximately 85-90% of the day was spent feeding or resting on plants. Only 0.5-1.5% of the observations recorded flying GWSS, and another 1-2% found GWSS walking between plants. More insects moved between plants in the mixed-plant cages than in the bean-only cages, suggesting the GWSS are able to detect the presence of other species of plants in the vicinity. This increase in interplant movement would probably correspond to an increase in Pierce's disease transmission. Field-collected insects spent less time feeding and more time resting on plants than did laboratory-reared insects. Both sets of insects spent more time feeding in bean-only cages than in mixed-plant cages. Beans may not have provided optimal nutrients, and GWSS may have moved to other plants to supplement nutrient intake. GWSS fed on sunflower and tobacco readily, although preferences have not yet been calculated. No predator-mediated spread of Pierce's Disease is expected to occur, as the presence, activity levels, and predation by spiders had no effect on GWSS behavior. Further analysis of feeding times and movement between plant species may clarify the relative importance of toxin dilution (nicotine from tree tobacco) and nutrient balancing from bean and sunflower plants.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) *Homalodisca coagulata* Say, is primarily of economic importance because it vectors the Pierce's disease-causing bacterium, *Xylella fastidiosa* (Blua et al. 1999). The insect feeds on hundreds of species of plants (Adlerz 1980; Hoddle et al. 2003), many of which harbor asymptomatic populations of *X. fastidiosa* (Purcell and Hopkins 1996). Every time a GWSS moves to a new plant to feed, the chances of acquiring and transmitting Pierce's Disease increase. Therefore, the factors causing GWSS to move between plants are directly related to the spread of Pierce's disease.

Generalist herbivores such as the GWSS may move to new plants to balance nutrients, to avoid intra- or inter-specific competition, to dilute plant defensive toxins, or to avoid predation. GWSS feeds primarily, if not exclusively, on the xylem, where nutrients are very dilute (Andersen et al. 2003). The nutritional requirements of GWSS have been determined (Andersen et al. 1992; Brodbeck et al. 1996), and only cowpea and soybean have been found to reliably sustain GWSS throughout a complete generation (D.J.W. Morgan, pers. comm.; Brodbeck et al. 1999). However, why GWSS move between plants, especially when a nutritionally adequate host such as bean is available, is unknown. Interspecific competition is rarely a concern for GWSS, as few other organisms feed on the xylem on the host plants on which GWSS can feed. Intraspecific competition may occur, as GWSS move off plants when present in very high densities (Armer, pers. obs.), but these densities will not occur frequently when biological control is in place. Plant defensive compounds are not common in the xylem (Raven 1983), but alkaloids and quinones are present in certain plant families and may be more prevalent than scientists have previously expected. For example, solanaceous plants carry defensive compounds from synthesis sites in the roots to the leaves via the xylem. Tree tobacco is one such solanaceous plant, which contains nicotine in the xylem. Finally, predators may affect herbivore behavior, as some herbivores can detect and respond to the presence of predators by halting feeding or altering host plant selection (Schmitz et al. 1997; Schmitz and Suttle, 2001). Alternately, an herbivore that moves frequently between plants to optimize feeding may be more apparent to visual predators.

OBJECTIVE

Determine the effect of plant species variety and predators on GWSS interplant movement.

RESULTS

Caged habitats of 0.56m² contained 6 plants in soil. Plants and predators were set up in a 2x2 factorial design, with either a monoculture (all bean plants) or polyculture (2 bean, 2 sunflower, and 2 tree tobacco plants) and with or without spiders. Sixteen adult GWSS were placed in each cage and their location and behavior were monitored every hour throughout as daylight was available, for 10-14 hours. The behaviors are shown on the x-axis of Figure 1. The percent of adult GWSS in a cage performing each activity was averaged over all hours observed. The data were compared by a 3-factor MANOVA (SAS



Section 2: Vector Biology and Ecology



Figure 1. Representative clusters from two promising *Xf* resistance source subgroups. BO2SG and BO3SG are the resistant female parents. Cabernet Sauvignon and Pinot noir are shown for size/shape comparisons. Crosses to BO2SG are in the top row while crosses to BO3SG are in the bottom row. The other clusters are from first generation crosses. Analytical details can be found in Table 2.

Figure 2. Juice extracted from selected clusters of *Xf*-resistant crosses shown in Figure 1 and detailed in Table 2. Note the high quantity of red color and the variation in hue from some of the crosses. This variation allows for tailoring varieties to meet particular enological needs. Juice from Cabernet Sauvignon and Pinot noir are on the left in the first two vials respectively.



a = (1=low, 4= high); b = (1=green, 4= brown)

Table 3. UC Davis field plantings of wine crosses made in 2003. F2-7 and F2-35 are respectively a black and a white female seedling of the cross Cabernet Sauvignon x Carignane. B34-82 is a USDA cross.

Cross	Resistance Source	Seedlings Planted
F2-7 x F8909-08	<i>V. arizonica</i>	10
F2-35 x F8909-08	<i>V. arizonica</i>	38
F2-35 x BD5-117	SEUS complex	164
F2-7 x BD5-117	SEUS complex	149
BD5-117 x B34-82	SEUS complex	141
	Total	502

Table 4. Wine grape crosses made at UCD in 2004.

Female Parent	Male Parent	Resistance Source	# Seeds
BO2SG	Cabernet Sauvignon	<i>V. smalliana</i>	376
BO2SG	Carignane	<i>V. smalliana</i>	196
BO2SG	Sauvignon blanc	<i>V. smalliana</i>	404
BO3SG	Chambourcin	<i>V. smalliana-simpsonii</i>	412
BO3SG	Petite Sirah	<i>V. smalliana-simpsonii</i>	419
BO3SG	Cabernet Sauvignon	<i>V. smalliana-simpsonii</i>	371
BO3SG	Carignane	<i>V. smalliana-simpsonii</i>	350
BO3SG	Sauvignon blanc	<i>V. smalliana-simpsonii</i>	223
F2-7 (CabS x Carig.)	BD5-117	SEUS complex	1131
F2-7	Midsouth	<i>V. champinii</i>	522
F2-7	F8909-08	<i>V. arizonica - candicans</i>	4,500
F2-7	F8909-17	<i>V. arizonica - candicans</i>	300
F2-35 (CabS x Carig.)	B55-1	<i>M. rotundifolia</i>	18
F2-35	B43-17	<i>V. arizonica-candicans</i>	323
F2-35	B43-36	<i>V. arizonica</i>	141
F2-35	B43-56	<i>V. arizonica</i>	56
F2-35	BD5-117	SEUS complex	783
F2-35	Midsouth	<i>V. champinii</i>	522
NC-11J	UCD0124-01	<i>M. rotundifolia</i> -SEUS complex	175
Midsouth	Midsouth	<i>V. champinii</i>	500
NC6-15	Sauvignon blanc	<i>M. rotundifolia</i>	50
Total			11,772

Table 5. Ratios of *Xf*-resistant: susceptible (R:S) progeny in populations from various resistance sources by *V. vinifera* parents based on a greenhouse screen. Resistance is defined as a mean value less than 100,000 cfu/ml (colony forming *units per ml*).

Resistant Parent	Resistance Source	Number Resistant	Number Tested	Percent Resistant	Approx: R/S ratio
Midsouth	<i>V. champinii</i>	9	17	53%	1:1
BO2SG	<i>V. smalliana</i>	11	23	48%	1:1
Cha3-48	<i>V. champinii</i>	8	26	31%	1:2
DC1-39	Complex	9	33	27%	1:3
BO3SG	<i>V. smalliana-simpsonii</i>	1	6	17%	1:5
F901	<i>V. shuttleworthii</i>	1	7	14%	1:6
AW c52-94	<i>V. simpsoni</i>	2	15	13%	1:6
Z 71-50-1	Complex	2	25	8%	1/11
AT0023-019	<i>V. arizonica</i> (La Paz)	2	29	7%	1/11
F902	<i>V. shuttleworthii</i>	0	16	0%	-
Roucanneuf	Complex	0	22	0%	-
Villard blanc	Complex	0	6	0%	-
JS23-416	Susceptible	0	19	0%	-
Total			244		

Table 2A. Analytical evaluation of representative progeny from three different sources of *Xf* resistance.

Genotype	Species or Cross	Cluster Wt. (g)	Brix	pH	TA (g/L)	Berry Wt. (g)	Est. Yield (gal/ton)
BO2SG	<i>V. smalliana</i>	45	24.5	3.28	19.7	0.3	129
BO3SG	<i>V. smalliana-simpsonii</i>	66	25.0	3.53	12.1	0.3	90
Cab Sauv	<i>V. vinifera</i>	269	23.0	3.52	6.8	1.0	160
Pinot noir	<i>V. vinifera</i>	299	25.5	3.72	6.1	1.2	182
J13-09	BO2SG x Melissa	184	24.2	3.16	12.1	1.3	160
J13-13	BO2SG x Melissa	62	25.5	3.22	9.8	1.4	162
J14-09	BO2SG x C1020	90	25.2	3.36	9.1	1.2	176
J14-12	BO2SG x C1020	125	27.0	3.46	8.3	1.0	167
J14-16	BO2SG x C1020	120	26.0	3.38	9.8	1.4	170
J17-3	BO3SG x C67-129	100	25.0	3.32	7.1	1.3	150
J17-06	BO3SG x C67-129	102	25.8	3.53	6.4	1.4	149
J17-08	BO3SG x C67-129	117	26.5	3.43	7.7	1.0	135
J17-14	BO3SG x C67-129	200	27.0	3.68	5.9	0.9	148
J17-24	BO3SG x C67-129	224	26.0	3.62	6.7	1.1	137
J17-25	BO3SG x C67-129	70	27.0	3.65	5.9	1.0	146
J17-36	BO3SG x Melissa	110	26.5	3.76	4.5	0.9	154
J17-39	BO3SG x Melissa	70	25.0	3.33	7.4	0.8	176
J17-50	BO3SG x Melissa	185	24.0	3.32	6.8	1.2	165
J18-18	BO3SG x Melissa	195	23.0	3.14	9.8	1.1	143
J18-24	BO3SG x Melissa	60	26.5	3.54	5.5	1.1	148
J18-35	BO3SG x Melissa	93	26.2	3.55	6.2	0.9	152
J18-37	BO3SG x Melissa	100	23.5	3.14	9.7	0.7	158
J18-38	BO3SG x Melissa	101	25.0	3.23	8.6	1.0	154
J27-03	Midsouth x B90-116	99	23.5	3.85	8.3	1.2	168
J27-06	Midsouth x B90-116	125	25.0	3.76	5.2	1.2	145

Table 2B. Sensory evaluation of representative progeny from three different sources of *Xf* resistance.

Genotype	Species or Cross	Skin Tannin Intensity ^a	Seed Color ^b	Juice Hue	Juice Color Intensity	Juice Flavor
BO2SG	<i>V. smalliana</i>	2	4	red	dark	fruity, peppery
BO3SG	<i>V. smalliana-simpsonii</i>	1	4	red	dark	fruity, peppery
Cab Sauv	<i>V. vinifera</i>	3	2.5	pink	light	slightly vegetal
Pinot noir	<i>V. vinifera</i>	1	4	pink	very light	fruity
J13-09	BO2SG x Melissa	2	4	red	medium +	tart, red fruit
J13-13	BO2SG x Melissa	2.5	4	red-purple	medium +	fruity, slight hot pepper
J14-09	BO2SG x C1020	2	4	red	medium	tart, jammy, very slight hot pepper
J14-12	BO2SG x C1020	2	4	pink	light	slightly jammy, broad fruity
J14-16	BO2SG x C1020	2	4	green		green pepper, hot pepper
J17-3	BO3SG x C67-129	1.5	4	red-purple	medium +	slightly fruity, hot pepper
J17-06	BO3SG x C67-129	2	3.5	pink-red	medium	hay, hot pepper
J17-08	BO3SG x C67-129	1.5	4	pink-orange	light +	vinifera-like, acidic, hot pepper
J17-14	BO3SG x C67-129	2	4	red	medium	slightly jammy, fruity
J17-24	BO3SG x C67-129	4	4	red	medium +	fruity, hot pepper
J17-25	BO3SG x C67-129	1.5	4	red	medium	very slightly vegetal-herbal
J17-36	BO3SG x Melissa	2	4	pink	medium -	slight hay, hot pepper
J17-39	BO3SG x Melissa	2	4	red	medium +	tart, raspberry, very slight hot pepper
J17-50	BO3SG x Melissa	2	4	pink-red	medium	simple fruit, berry
J18-18	BO3SG x Melissa	3	4	pink-red	medium -	slight hay, canned
J18-24	BO3SG x Melissa	2	4	red	medium	slight hay, fruity
J18-35	BO3SG x Melissa	2	3.5	pink-red	medium -	hay, hot pepper
J18-37	BO3SG x Melissa	2	4	pink-brown	light	tart berry, slightly buttery
J18-38	BO3SG x Melissa	1	4	red	medium -	berry, slight hot pepper
J27-03	Midsouth x B90-116	1	4	purple	dark	current, vegetal
J27-06	Midsouth x B90-116	1	4	red	medium-	strawberry, herbal

Progeny from crosses of field resistant parents, like JS23-416 – judged resistant in Florida (Herb Barrett, personal communication) yet has been susceptible in our greenhouse tests, to *V. vinifera* do not seem to be resistant (<100,000 fu/ml). However, they do produce a broad and relatively even distribution of progeny from 170,000 to almost 6,500,000 cfu/ml. Although we would not consider those at the low end of this scale to be resistant, they have as low or lower bacterial levels than do some of the field resistant genotypes from the SEUS we have tested. We have avoided these progeny and using these parents to prevent release of field resistant cultivars that may survive PD infection, but allow vine-to-vine movement in vineyards.

We are beginning testing of about 200 genotypes with results expected in March 2005. These results will be used to direct backcrossing of the most resistant genotypes to *V. vinifera* wine grapes.

Napa Field Trial

This year we planted another block in our field trial at Beringer Vineyards in Yountville. We expanded the plot by adding 6 vine replicates of 20 different genotypes from 4 different resistant sources. Based on our GH screen results, both highly resistant and highly susceptible genotypes from each resistant source were planted. These will be inoculated with *Xf* next April and ELISA tested in October 2005.

This fall we observed the most pronounced visual PD symptoms to date in the 2001 and 2003 plantings following inoculation with *Xf* early this spring. We used a mixture of 5 different Napa PD strains as inoculum. The 2001 planting consists of known field resistant selections from the SEUS, and the 2003 planting consists of 3 vine reps of some of our early crosses and a few more SEUS field resistant types. On October 8, 2004 we scored these vines for visual symptoms and took samples for ELISA testing from 291 vines in these blocks. Results will be reported in December.

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Table 1. Summary of different crosses within the subgroups and the relative number of genotypes within each group that merit further evaluation.

Resistance Source	<i>V. vinifera</i> Parent	Genotypes Evaluated	Genotypes Selected
BO2SG (<i>V. smalliana</i>)	C1020	36	10
	Princess	21	9
BO3SG (<i>V. smalliana-simpsonii</i>)	C67-129	30	7
	Princess	81	14
AW C52-94 (<i>V. simpsonii</i>)	C51-63	353	71
Midsouth	B90-116	39	4
	C67-129	46	1
	Princess	8	1
Total		614	117

Completion of these objectives is tied to the speed with which seedlings can be produced, fruited and evaluated and subsequent generations produced.

- Develop multiple lines of *Xf* resistant wine grapes using 8909 (*V. rupestris* x *V. arizonica* selections; *Xf* resistant breeder selections (DC1-39, Zehnder selections, etc); and southern grape species (*V. arizonica*, *V. champinii*, *V. shuttleworthii*, *V. simpsonii*, *M. rotundifolia*, and others).
- Continue backcross generations with 8909-08, DC1-39, and other lines to advanced *vinifera* selections and select for high quality wine grape characteristics.
- Continue to identify and characterize additional sources of *Xf* resistance with high levels of powdery mildew resistance.
- Maintain current and produce additional populations for genetic mapping efforts aimed at characterizing *Xf* resistance genes, and identifying and mapping fruit quality traits such as color, tannin content, flavor, production, etc. in *Xf* resistant backgrounds.
- Study the inheritance of *Xf* resistance from a broad range of resistance sources.

RESULTS AND CONCLUSIONS

Shift From Table Grape Breeding to Wine Types

Because the California Table Grape Commission's decision to not fund the breeding of PD resistant grapes, as of May 2004 we are now solely breeding PD resistant wine grapes. This year we evaluated 4,042 seedlings from 39 different crosses made in the last three years for use as wine grapes. From this number, four subgroups based on different resistance source were identified as particularly promising (Table 1). Promise was based on resistance to *Xf* and powdery mildew, fruit quality parameters, and viticultural characteristics such as yield and growth habit.

Evaluation of Fruit Quality

Within a cross we observed useful segregation of wine grape quality factors such as quality and quantity of color, acidity, pH, flavor, and skin and seed tannin. Table 2A and 2B present data for typical genotypes from three of the four resistance groups. These were harvested on August 26, 2004. Figure 1 displays clusters from two of the four promising *Xf* resistance subgroups listed in Table 1. Their morphology is becoming very *vinifera*-like in the first generation. Figure 2 displays juice extracted from some of the *Xf* resistant crosses in comparison with the juices from Cabernet Sauvignon and Pinot noir. There are a wide variety of colors that should allow matching enological needs with our selection process.

Planting of 2003 Crosses

Table 3 summarizes the field planting of wine crosses made in 2003. We did not germinate the 2,150 seeds of the cross of a SEUS cultivar by Syrah since our GH screening of progeny from the same SEUS female by pure *V. vinifera* indicated only 1 in 12 of the seedlings was likely to be resistant. Crosses made in Spring 2003 contained efforts directed at table and raisin grape production. This year's crosses were entirely devoted to wine grape efforts.

Wine Crosses Made in 2004

Table 4 details the wine grape crosses made during Spring 2004. We were able to tailor our choices for PD resistant parents with our previous experiences directed at table grape breeding. The assays of subsets of progeny from crosses with various parental sources found that the expression of PD resistance in progeny varies. *Vitis arizonica/candicans* selections from near Monterey, Mexico (b43-17, b43-36, and b43-56) produced 100% resistant progeny in the testing of the subset and should therefore be homozygous resistant. F8909-08 and F8909-17 were both derived from b43-17. The heritability of selections from Florida varied: BO2SG, BD5-117 and Midsouth produced 50% resistant progeny; while only 20% of the progeny of BO3SG was resistant, so progeny from it will be planted sparingly. NC-11J x UCD0124-01 represents a resistant x resistant cross from two different resistant backgrounds. B55-1 and NC6-15 are opportunities to ingress resistance from *Muscadinia rotundifolia* into wine crosses. We plan to plant between two and three thousand of the most promising seedlings from the crosses detailed above in Spring 2005.

Greenhouse Screen Results

We screened 474 genotypes with our greenhouse screen. The tested genotypes included cultivars and species from the SEUS, many Olmo *Vinifera/Rotundifolia* (VR) hybrids with potential PD resistance and for use as parents, table and wine grape crosses, and possible *Xf* resistant wine grape selections from a private breeder in North Carolina. Several promising *Xf*-resistant SEUS genotypes were identified. Six of 19 Olmo VR hybrids tested resistant. Two may be promising parents. None of the wine grape selections from North Carolina proved to be adequately resistant.

Table 5 presents the ratio of resistant to susceptible (R:S) progeny from crosses of highly susceptible *V. vinifera* parents crossed with a variety of *Xf* resistance sources. One *V. smalliana* and one *V. champinii* F1 hybrid progeny had R:S ratios of close to 1:1, suggesting that the resistance in these parents was heterozygous and controlled by a single gene. Other parents had ratios ranging from 1:3 through 1:11. Details are summarized in Table 5. We made crosses onto the *V. champinii* hybrid this year and they will be tested to see if the inheritance ratio remains 1:1, as does our F8909-17 resistance source (see Walker-Krivanek report). In other backgrounds, resistance seems to erode with continued backcrossing to *V. vinifera*, thus these stable resistance sources are very valuable and are easily adapted to marker-assisted selection.

BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

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Reporting Period: The results reported here are from work conducted from November 2003 through October 2004.

ABSTRACT

Strong and continued progress is being made in breeding Pierce's disease (PD) resistant grapes. Fruit quality has markedly improved while maintaining high levels of PD resistance. We continue to make many crosses, produce thousands of seeds, and plant about two thousand plants in the field each year. We have been increasing the number of seedlings and high fruit quality selections we test under our greenhouse screen. This screening is very severe, but material that passes the screen is reliably resistant and dramatically restricts *Xylella fastidiosa* (*Xf*) movement. We are also co-screening for powdery mildew resistance. The heritability of *Xf* resistance from a range of resistant southeast US (SEUS) cultivar and species parents is not consistent – some parents produce few resistant offspring, while others produce a large percentage – making careful parental screening very important. We have been able to expand our *Xf* screening the past few years and have tested hundreds of potential parents before we need to make breeding decisions the following year.

INTRODUCTION

Renewed and intensified PD outbreaks in historic PD zones in wine regions around the state and the introduction of GWSS into the southern San Joaquin Valley demonstrate the vulnerability of *V. vinifera* wine grape culture in California. All of California's wine grapes are susceptible to PD and no effective prevention or cure currently exists. Under severe PD pressure, culture of *V. vinifera* grapes is not possible. We are currently breeding PD-resistant wine grape cultivars for localized use in traditional PD "hot-spots" that are common in the North Coast, and it is likely that acceptable white and red wine grapes for these areas can be produced in two generations of crosses with our current *Xf* resistant selections. To further improve the utility of these *Xf* resistant cultivars, we are co-selecting for high levels of powdery mildew resistance. Unlike wine varieties for widespread use where the need for "pure *V. vinifera*" cultivars is enforced by marketing, given adequate quality (neutrality, color, season, cultural characteristics) varieties for localized use should prove useful to industry as blenders and by keeping "hot-spot" vineyard acreage in production. Our concurrent efforts to identify *Xf* resistance genes (see companion proposal – Walker and Riaz) will make it possible in the future to transform wine grapes with grape-derived resistance genes. Using grape genes to transform grapes should help overcome public reluctance about GM grapes and provide durable PD resistance.

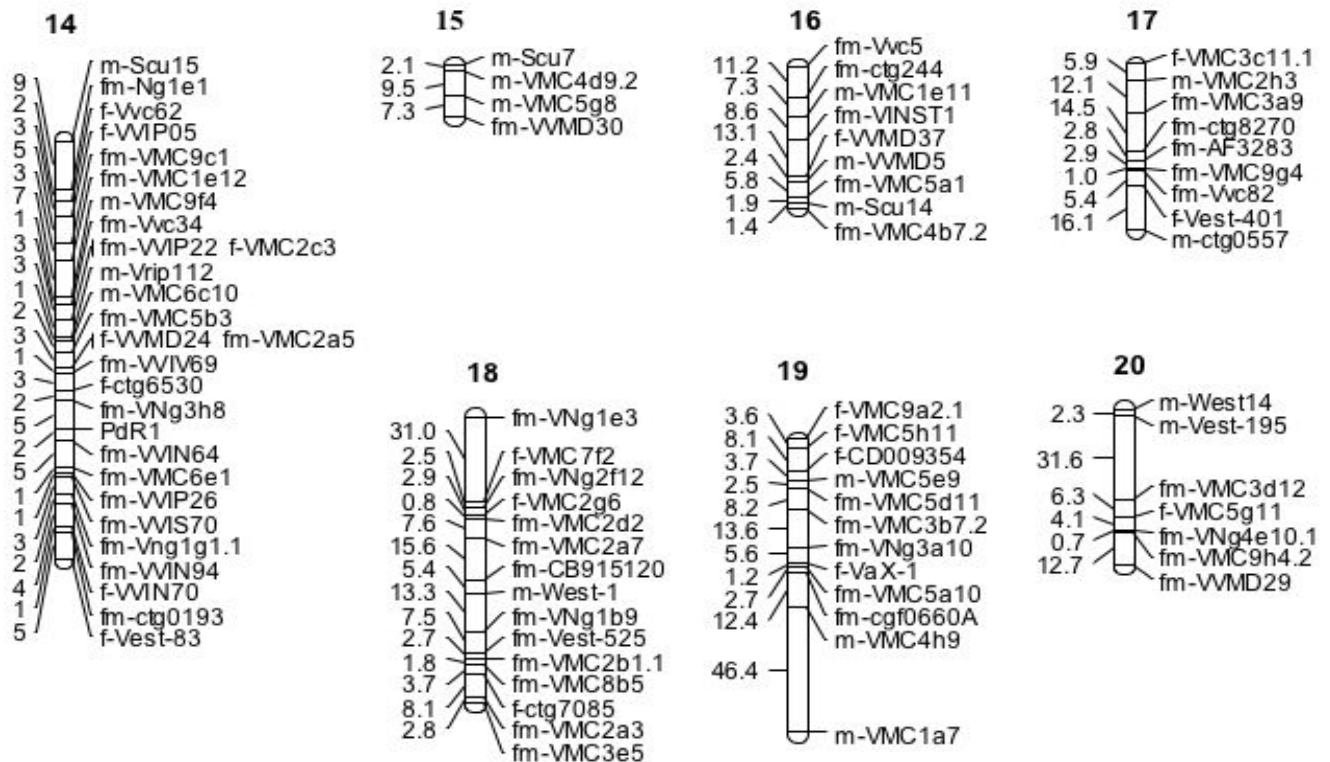
PD resistance exists in a number of *Vitis* species and in the related genus, *Muscadinia*. Resistant cultivars have been developed in public and private breeding programs across the southeastern United States (SEUS). These cultivars have high PD resistance, but relatively low fruit quality relative to *V. vinifera* grapes. In the southeastern US, they must also resist downy and powdery mildew, black rot and anthracnose, which have as great an effect on viticulture in the southeast as PD does. Most of these diseases are not found in California, allowing breeders to incorporate more high quality *V. vinifera* into their breeding efforts and enabling the production of much higher quality PD resistant cultivars in a shorter time span. We have characterized (see past reports) and employed a wide range of PD resistant germplasm from the collections at the National Clonal Germplasm Repository, Davis; selections obtained from breeders in the southeastern U.S.; from *V. rupestris* x *V. arizonica* selections that have exceptional PD resistance; and from several *V. vinifera* x *M. rotundifolia* hybrid winegrape types that have some fertility. These breeding efforts have already resulted in relatively high quality selections with excellent PD resistance.

At UC Davis we are uniquely poised to undertake this important breeding effort. We have developed rapid screening techniques for *Xf* resistance and have optimized ELISA and PCR detection of *Xf* (Buzkan et al. 2003, Buzkan et al. 2004, Krivanek et al. 2004, Krivanek and Walker 2004). We have unique and highly resistant *V. rupestris* x *V. arizonica* selections, as well as an extensive collection of southeastern grape hybrids, that offer the introduction of extremely high levels of *Xf* resistance into commercial grapes. We also have several years' worth of seedlings in the ground that need evaluation as winegrape types.

OBJECTIVES

The objectives of our PD breeding project are divided into two primary parts. The first is the breeding of *Xf* resistant wine grapes through backcross techniques using *V. vinifera* wine grapes and *Xf* resistant selections and sources characterized from our previous breeding efforts. The second is the continuing 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. These efforts support both the breeding program and the genetic mapping program.

Figure 1b. Riaz & Walker2004 SSR based genetic linkage map of 9621 (8909-15 X8909-17)



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FUNDING AGENCIES

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board. Previous mapping efforts upon which this research is based received funding from the American Vineyard Foundation, the California Grape Rootstock Improvement Commission, and the Louis P. Martini Endowed Chair in Viticulture.

resistant genotypes are in process and will be tested for AFLP polymorphisms utilizing florescent primers and visualized on a PE 3100 sequencer.

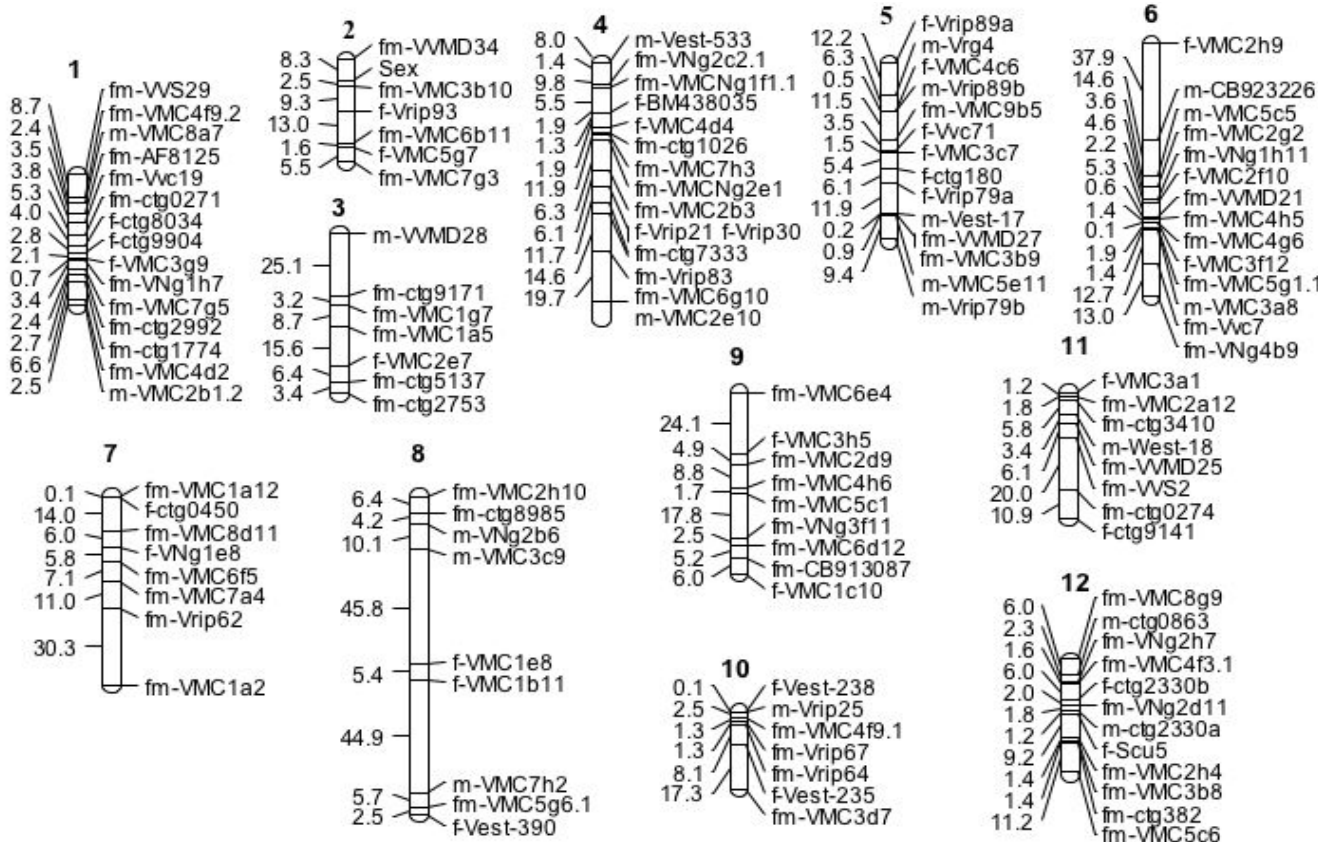
Table 1. Data on number of markers mapped for the 9621 (D8909-15 x F8909-17) mapping population.

Molecular Markers		
Genomic SSR	VMC published/unpublished	134
	VVMD	10
	VVS	2
	INRA	9
EST derived SSR	Southern Cross University, Australia	4
	INRA, France	7
	Genome Facility (U.C. Davis)	35
ESTP markers	Doug Adams/NCBI data base	16
Grand Total		217

Table 2. Details of the 9621 genetic linkage map.

Linkage groups	19
Linked markers	214
Total map length	1300 cM
Average distance between markers	5.98 cM
Largest group (PD linkage group)	30 markers 80cM (group14)
Smallest group	4 markers 18cM (group 15)

Figure 1a. Riaz & Walker2004 SSR based genetic linkage map of 9621 (8909-15 X8909-17)



Spring 2004 and a total of 4,500 seeds have been collected and placed into cold stratification. Should the initial subset of the family segregate in a 1:1 resistant to susceptible ratio as expected the expanded family of approximately 2,000 to 3,000 genotypes will be an excellent choice for fine resolution placement of the *PdRI* resistance gene. This would be the first step toward placement of resistance markers (flanking the *PdRI* locus) onto a bacterial artificial chromosome (BAC) within a genomic library in a procedure termed "chromosome landing" (Tanksley et al. 1995). Plans for construction of the library are underway.

Objective 2

The original genetic linkage map was based primarily on AFLP markers with 375 placed on the map, with an additional 32 ISSR, 25 RAPD and 9 SSR markers (Douceff et al. 2004). Our efforts expanded to more reliable SSR markers in order to construct a repeatable framework map useful for more precise placement of primary resistance genes, QTL analysis and marker-assisted selection. Among the marker classes added to the map 310 SSR markers have been tested, 155 were polymorphic in the parents and all have been added to the map; 90 EST derived SSR markers have been tested, 60 of them were polymorphic and 46 have been added to the map; 20 EST markers (provided by Doug Adams) have been tested and 16 were added to the map (Table 1). A total of 217 markers (SSR, EST-SSR and ESTP) tested on 188 genotypes have now been utilized for map construction.

The 217 SSR markers included some that have been previously published and many that were developed by Vitis Microsatellite Consortium and are as yet unpublished. All markers were tested on a small set of 8 DNA samples including both parents and run on 6 % polyacrylamide gels. DNA on the gels was visualized by silver staining with a commercial kit (Promega). We have tested and used all available informative genomic microsatellite markers for the 9621 population. Meanwhile, we also initiated collaboration efforts with the research group at INRA (Montpellier, France) to obtain primer sequences of SSR markers developed at their facility.

To develop ESTP (expressed sequence tagged polymorphism) markers, sequences of grape cDNA were obtained from Dr. Doug Adams (Department of Viticulture and Enology, UC Davis). Potential PCR primers were designed using the computer program PRIMER 0.5. Primers were selected to have similar properties to facilitate standard conditions for PCR reactions. Primers are 20 to 23 nucleotides long with GC contents of 50-60% and melting temperature ranging from 59-64°C. Amplification and polymorphism for each EST was tested on 2% agarose gels. If length base polymorphisms were not revealed, then a set of 10 different restriction enzymes (*HindIII*, *EcoRI*, *Ava II*, *BstNI*, *DraI*, *Hae III*, *HinfI*, *Msp I*, *EcoRV*, *Rsa I*) were tested to find restriction site based polymorphism among parents D89090-15 and F8909-17.

Objective 3

There are now a large number of EST derived SSR markers available, in addition to the genomic SSR markers from the Vitis Microsatellite Consortium. The EST derived SSR markers are more valuable if the cDNA sequence from which the EST was derived has a known function as determined by comparisons with homologs from other EST databases. We plan on selecting EST-SSR markers that show homology to genes which control disease resistance along with those that control other important morphological, physiological and agronomic traits. So far we have tested 90 EST-SSR markers from three different sources (Table 1) and 45 of informative markers were added to the entire core set of 9621 population. Our goal is to screen an additional 100-150 EST-SSR markers with putative known function and we are adding to the map as they are completed.

Objective 4

In order to develop the core framework map based on SSR markers, preliminary linkage analysis for each parent was carried out with MAPMAKER 2.0. Each segregating locus was paired with a "dummy" locus, resulting in a doubled data set. Linkage groups obtained from the doubled data set were then divided into two symmetrical sets of groups and one set was chosen for further detail. The "first order" and "compare" commands were used to determine the probable order of all markers in each linkage group. The integrated linkage analysis to obtain the sex-average map was performed with JOINMAP 2.0 (LOD 5.0 and recombination frequency 0.45). Using the fixed sequence command, the order of markers was determined relative to the established order obtained from the initial MAPMAKER analysis. Map units in centimorgans (cM) were derived from the Kosambi (K) mapping function. The integrated consensus map analysis was carried out with JOINMAP 3.0. The consensus linkage map was developed with 217 markers (155 SSR markers, 45 EST-SSR, 16 ESTP markers and the Pierce's disease resistance locus). A total of 214 markers fall in 19 linkage groups and only 3 markers were unlinked. Total map length is 1300 cM with average distance between markers of 5.9 cM. All markers were evenly distributed. The current map is depicted in Figure 1. The largest linkage group was comprised of 30 markers and smallest group consisted of 4 markers (Table 2). The locus for Pierce's disease resistance mapped to linkage group 14 with flanking markers on each side (Figure 1). Many additional markers have been added but have not been included on the map.

To saturate a narrow region around the *PdRI* locus resistance locus with molecular markers, the strategy of bulk segregant analysis (BSA) (Michelmore et al. 1991) in concert with the AFLP marker system has been initiated in cooperation with our report titled "Optimizing marker-assisted selection (MAS) for resistance to *Xylella fastidiosa* to accelerate breeding of PD resistant grapes." Work has begun within two segregating families from susceptible by resistant crosses. One family, C8909-07 by F8909-08, segregates 1:1 resistant to susceptible and a good correlation between resistance and resistance marker alleles has been established. A bulk of the DNA from the 12 most susceptible and a bulk of the DNA the 12 most

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

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Reporting Period: The results reported here are from work conducted from November 2003 to October 2004.

ABSTRACT

Development of an SSR genetic linkage map based on the 9621 family is continuing. The family segregates for PD resistance and is based on the cross of PD resistant D8909-15 x PD resistant F8909-17. We expanded the mapping population size from 116 to 188 genotypes. The current genetic linkage map consists of 217 non-AFLP markers (SSR, EST-SSR and ESTP) in 19 linkage groups. The PD resistance locus *PdRI* maps to linkage group 14 of the male parent (F8909-17), which now consists of 30 markers, 9 of which are localized within 10 cM of *PdRI*. To avoid confounding affects from resistance inherited from D8909-15 additional families derived from a susceptible by resistant cross are currently being evaluated for map based cloning of the *PdRI* locus. A family from the cross of F2-7 (a cross of two *V. vinifera* wine grapes, Cabernet Sauvignon x Carignane) x F8909-08 (a PD resistant sibling of F8909-17) has been made and is currently being screened for PD resistance via our standard greenhouse testing procedure. To saturate a narrow region around the resistance locus with molecular markers, bulk segregant analysis (BSA) in concert with the AFLP marker system has been initiated in cooperation with our report titled "Optimizing marker-assisted selection (MAS) for resistance to *Xylella fastidiosa* to accelerate breeding of PD resistant grapes."

INTRODUCTION

This project expands upon and continues a genetic mapping effort initiated with funding from the California Grape Rootstock Improvement Commission, the Fruit tree, Nut tree and Grapevine Improvement Advisory Board, the California Table Grape Commission and the American Vineyard Foundation. The project has been mapping resistance to *Xiphinema index*, the dagger nematode, and *Xylella fastidiosa* (*Xf*) in an "F2" population designated as the 9621 family (D8909-15 x F8909-17). A genetic map of 116 individuals from the 9621 population was created primarily with AFLP markers (Doucleff et al. 2004). Our efforts were expanded to informative markers, such as microsatellites or simple sequence repeats (SSR) for two main reasons. First, a genetic map based on SSR markers provides a reliable and repeatable framework for initial mapping of candidate genes and quantitative trait loci (QTLs). Secondly, SSR markers tightly linked to resistance and phenotypic traits of interest are ideal for marker-assisted selection due to their applicability across different genetic backgrounds and ease of use. The grape genetic research community formed the International Grape Genome Program (IGGP) to increase coordination and cooperation and to enhance knowledge of the grape genome. Use of the SSR marker system is common among the different research groups so that our mapping efforts can be linked to others. Integrating the 9621 genetic linkage map to other mapping populations will facilitate targeting genomic regions that harbor quantitative trait loci. Comparison to other maps will allow us to identify more markers that are linked to *Xf* resistance and optimize marker-assisted selection strategies applied to breeding programs. For fine scale mapping a narrow region around the primary resistance locus, we include procedures here. The proposal will expand to include construction and utilization of a genomic library of a resistant parental genotype for eventual cloning of the PD resistance gene.

OBJECTIVES

1. Increase the base population from 116 to 188 genotypes within the 9621 family and expand to a family based on a susceptible by resistant cross of 2,000 to 4,000 genotypes.
2. Increase the number of SSR and EST markers on the core genetic linkage map from 100 to 300 markers.
3. Screen an additional 100-150 EST derived SSR markers for which functions are known after their comparison to homologues in available EST databases.
4. Develop core framework map with an average distance of 2 to 5 cM between markers and utilize Bulk Segregant Analysis (BSA) with the AFLP marker system to saturate a 1 cM region around the *PdRI* resistance locus.

RESULTS AND CONCLUSIONS

Objective 1

The original starting material for this project was a molecular marker linkage map of the 9621 population based on 116 individuals (Doucleff et al. 2004). We expanded the core set of individuals from the 9621 to 188 genotypes to take advantage of 96-well plate based techniques and to increase resolution on the map to improve marker association with PD resistance. A second family derived from a susceptible by resistant cross of F2-7 (a *V. vinifera* wine grape, Cabernet Sauvignon x Carignane) x F8909-08 (a PD resistant sibling of F8909-17) has been made, and 40 individuals are currently being screened for PD resistance via our standard greenhouse testing procedure. An expansion of the family was made in the

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FUNDING AGENCIES

Funding for the 2004-2005 funding year was received in mid-September 2004. This proposal was not submitted to other funding agencies. However, it is linked to the Walker/Tenscher Pierce's disease resistance breeding project funded by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board (and formerly by the California Table Grape Commission and the California Raisin Advisory Board), and the Walker/Riaz mapping project. This project was initiated through funding by the American Vineyard Foundation and CDFA for the Genetics of Resistance to Pierce's disease, a project that developed a framework map for the 9621 population. Funding from the Louis P. Martini Endowed Chair in Viticulture has also supported Pierce's disease mapping and marker development projects.

resistant and susceptible genotypes within the 25000 series have shown improved fruit quality (Figure 2) and are currently being screened to confirm the correlation between the resistance markers and the PD resistance trait. We are also utilizing these populations to confirm the effectiveness and economics of the MAS relative to our greenhouse screening procedure.

Table 1. Resistance classification and marker genotypes for the individuals of the full-sib family derived from the susceptible by resistant cross of C8909-07 x F8909-08. * = Genotypes selected for Bulk Segregant Analysis procedure.

Genotype	Overall resistance level to PD	Mean natural log (cells/ml)	Mean CMI score	Mean % leaf scorch	Alleles of SSR markers flanking the PdR1 resistance
99217-21 *	Resistant	9.51	1.00	58.3	Rr / Rr
99217-40 *	Resistant	9.70	1.33	75.0	rr / Rr
99217-18 *	Resistant	9.77	2.75	95.0	Rr / Rr
99217-41 *	Resistant	10.19	4.25	76.3	Rr / Rr
99217-35 *	Resistant	10.55	1.33	100.0	rr / Rr
99217-19 *	Resistant	11.08	2.50	76.7	rr / Rr
99217-01 *	Resistant	11.52	2.25	90.0	rr / Rr
99217-23 *	Resistant	11.57	3.00	87.5	Rr / Rr
99217-34 *	Resistant	11.83	3.75	65.0	Rr / Rr
99217-46 *	Resistant	11.87	5.75	100.0	Rr / Rr
99217-27 *	Resistant	12.20	4.25	100.0	Rr / rr
99217-22 *	Resistant	12.29	4.00	100.0	Rr / Rr
99217-12 *	Resistant	12.50	4.00	95.0	Rr / Rr
99217-38	?	12.69	5.00	100.0	Rr / Rr
99217-36	?	13.09	5.00	100.0	rr / rr
99217-50	?	13.52	4.25	83.8	Rr / Rr
99217-14	Susceptible	14.06	5.50	88.8	rr / Rr
99217-07	Susceptible	14.87	5.50	100.0	rr / rr
99217-04 *	Susceptible	15.42	6.00	100.0	rr / rr
99217-33 *	Susceptible	15.59	5.75	100.0	rr / rr
99217-06 *	Susceptible	15.80	5.25	68.3	rr / rr
99217-09 *	Susceptible	15.81	5.75	100.0	rr / rr
99217-10	Susceptible	15.82	4.75	100.0	rr / rr
99217-13 *	Susceptible	15.84	5.50	100.0	rr / rr
99217-42	Susceptible	15.85	4.25	75.0	rr / Rr
99217-15 *	Susceptible	15.87	5.25	100.0	rr / rr
99217-32 *	Susceptible	15.87	5.50	100.0	rr / rr
99217-28 *	Susceptible	15.91	5.75	100.0	rr / rr
99217-05 *	Susceptible	15.91	5.75	100.0	rr / rr
99217-37 *	Susceptible	15.92	5.25	100.0	rr / rr
99217-26 *	Susceptible	15.95	5.50	100.0	rr / rr
99217-24 *	Susceptible	16.04	6.00	100.0	rr / rr

Figure 2.

Vitis arizonica PD
Resistant poor fruit
quality

Hybrid BC1-25017 with
flanking PD resistance markers
Improved fruit quality

Vitis vinifera PD
Susceptible Excellent fruit
quality



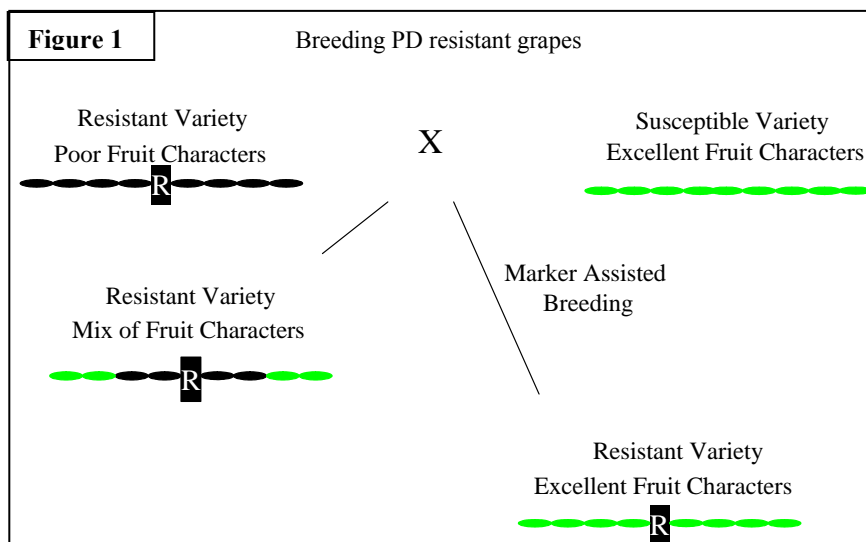
- Utilize Bulk Segregant Analysis (BSA) with the AFLP marker system to saturate with markers the region around the previously mapped *Xf* resistance locus and eventually convert confirmed candidate markers to stable SCAR primers.
- Confirm candidate marker linkage to resistance within families derived from resistant by susceptible crosses such as the '8909' x *V. vinifera* and ('8909' x *V. vinifera*) x *V. vinifera* back-cross generations.

RESULTS AND CONCLUSIONS

Sub-objective 1.

Initial mapping of the PD resistance locus *PdR1* in the male parent F8909-17 of the 9621 family localized it to chromosome 14, and identified 6-8 SSR markers on the same linkage group. Marker placement on published SSR linkage maps of *Vitis* were used to preferentially target chromosome 14, bringing the total number of SSR markers on the linkage group up to 30. Approximately 9 SSR markers are localized within a 10 cM distance of the resistance gene. These SSR markers are reliable and are the easiest of the molecular markers to incorporate within a MAS breeding program. Correlation tests of these candidate markers to PD resistance when functioning within a *V. vinifera* genetic background are underway and described in sub-objective 3. The SSR marker analysis has allowed us to confirm that marker alleles linked in coupling to PD resistance alleles of the *PdR1* locus in another PD resistant progeny of b43-17 (F8909-08) are different than the alleles linked in coupling the resistance alleles in F8909-17. It is apparent from these results that b43-17 is homozygous resistant for the *PdR1* locus, and that F8909-17

inherited its resistance allele from one chromosome 14 and F8909-08 inherited its resistance allele from the homologous chromosome 14. In either case the markers linked to resistance will function for MAS, however, different alleles linked in coupling to the resistance alleles will have to be followed through the downstream MAS process. Placement of SSR markers to chromosome 14 via the comparative mapping strategy continue as the markers become available, however, the number of SSR markers that can be targeted to a specific chromosomal region via comparative mapping is limited.



Sub-objective 2.

For high density marker saturation within a narrow window around the *PdR1* locus, a bulk segregant analysis (BSA) strategy (Michelmore et al. 1991) in concert with the AFLP marker system was chosen as the method of choice. Initial BSA was attempted within the 9621 family, however, confounding effects of the resistance loci within the D8909-15 parent made the attempt more difficult than expected. To avoid confounding affects from resistance inherited from other genetic backgrounds and focus the BSA procedure only on the *PdR1* locus, work has begun within two segregating families from susceptible by resistant crosses. The first family, 99217 (C8909-07 x F8909-08) consists of 33 genotypes, has been screened for PD resistance (Krivanek et al. submitted) and segregates 1:1 resistant to susceptible (Table 1). DNA has been extracted from these genotypes, flanking SSR markers were run and a good correlation between resistance and resistance marker alleles has been established (Table 1). A bulk of the DNA from the 12 most susceptible and a bulk of the DNA from the 12 most resistant genotypes are in process and will be tested for AFLP polymorphisms utilizing florescent primers and visualized on a PE 3100 sequencer. The second family derived from a susceptible by resistant cross is a *V. vinifera* x F8909-08 family; it consists of 40 genotypes and has been designated as 0062. Testing of this family for PD resistance is currently underway via our standard greenhouse testing procedure (Krivanek et al. in press; Krivanek and Walker in press). It is expected that the progeny in this family will segregate in a 1:1 manner, and if so, DNA extraction and BSA procedures will be undertaken as with the 99217 family. Candidate AFLP markers will be converted to stable and more reliable SCAR primers before incorporation into the MAS program.

Sub-objective 3.

Work is progressing with two distinct breeding populations for testing of candidate resistance markers and initial application of those markers to MAS. One family is a cross of the PD resistant F8909-08 to a female *V. vinifera* wine grape F2-7 (Cabernet Sauvignon x Carignane) and designated as the 0062 family. A second breeding population consists of a cross of F8909-08 to several elite *V. vinifera* table grape genotypes (the 500 series). A subset of the 500 series has been screened for PD resistance and screened for markers flanking the *PdR1* locus. Five confirmed resistant genotypes have been utilized in the development of the first backcross generations BC1 (backcrossed to additional elite *V. vinifera* genotypes). The BC1 population (25000 series) consists of approximately 200 individuals and was planted in the field in 2003. Marker analysis for flanking markers to the *PdR1* locus has been completed for the 25000 series and the marker information was utilized in selection of genotypes for the spring of 2004 crosses for the development of the BC2 generations. Subsets of candidate

OPTIMIZING MARKER-ASSISTED SELECTION FOR RESISTANCE TO *XYLELLA FASTIDIOSA* TO ACCELERATE BREEDING OF PIERCE'S DISEASE RESISTANT GRAPES OF HIGH FRUIT QUALITY

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Reporting Period: The results reported here are from work conducted from October 2003 to October 2004. Research on this project was initiated under the "Genetics of Resistance to Pierce's Disease" of the Long-term American Vineyard Foundation Pierce's Disease Project.

ABSTRACT

Efforts at identifying molecular markers linked to *Xylella fastidiosa* (*Xf*) resistance are continuing. Our primary focus is on resistance derived from b43-17, a *Vitis arizonica/candicans* type collected near Monterrey, Nuevo Leon, Mexico. The '9621' *V. rupestris* x *V. arizonica* hybrid mapping family (PD resistant D8909-15 x PD resistant F8909-17) was used to localize *PdRI*, a primary PD resistance locus within the linkage map of the male parent F8909-17 (progeny of b43-17) and identify candidate linked resistance markers. In more recent research, a comparative mapping strategy between the '9621' linkage map and other SSR maps within *Vitis* was used to identify 9 SSR markers within 10 cM of the resistance locus. Resistance from the female parent D8909-15 has not yet been localized to a genetic map. The strategy of bulk segregant analysis (BSA) in concert with the AFLP marker system has been initiated to saturate the region around the resistance locus and is expected to yield an additional 20 to 50 markers linked to the resistance trait. All candidate resistant markers have been and will continue to be applied to breeding populations derived from '8909' x *V. vinifera* and ('8909' x *V. vinifera*) x *V. vinifera* back-cross generations in order to confirm resistance marker effectiveness in *V. vinifera* backgrounds and continue with marker assisted selection for development of high quality PD resistant grapes.

INTRODUCTION

Several American *Vitis* species are native to the regions where PD is endemic, and resistance from these sources has been introgressed into many different cultivars grown in the south-eastern United States. The acceptance of the new hybrid cultivars has been limited due in part to some undesirable non-vinifera fruit quality traits. The development of high quality PD resistant cultivars will be facilitated by the use of molecular markers to achieve a more precise introgression of the resistance genes into domesticated backgrounds and avoid introgression of undesirable traits (Figure 1). Backcross introgression via molecular markers has been accomplished successfully in other crops (Young and Tanksley 1989). This type of introgression is generally termed Marker Assisted Selection (MAS), whereby indirect selection on a trait of interest (such as disease resistance) is made by screening for the presence of a DNA marker allele tightly linked to the trait. MAS for disease resistance can also be used to eliminate susceptible genotypes in a breeding population early in the selection process, which allows for evaluation of much larger effective populations. Larger effective population sizes increase the opportunity to identify genotypes with high disease resistance and good horticultural qualities (such as good flavor traits, color, berry and cluster size, etc.). Other key aspects of the MAS process include avoiding confounding environmental effects on the trait phenotype and accelerating breeding progress while saving space and time, allowing for more efficient use of resources (Paterson et al. 1991, Kelly 1995). Rapid screening time is particularly valuable when applied to perennial crops such as grape with relatively long generation times (Alleweldt 1988, Striem et al. 1994). To effectively use linked markers in MAS only requires that the markers be highly reproducible, linked in coupling phase i.e. on the same homologous chromosome, and within 5 centimorgan (cM) mapping units of the resistance locus (Kelly 1995).

Within grapevines, markers linked to powdery mildew resistance (Dalbo et al. 2001, Pauquet et al. 2001), downy mildew resistance (Luo et al. 2001) and seedlessness (Lahogue 1998) have been published. In the case of powdery mildew resistance, MAS has already been successfully utilized for screening a grape breeding population. We are successfully developing a MAS system for screening PD resistant genotypes that will greatly benefit our breeding of PD resistant wine grapes.

OBJECTIVES

Our overall objective is to identify DNA markers that are tightly linked to the primary locus or loci required for complete resistance to PD within *Vitis*. Research will focus on PD resistance as inherited from *V. arizonica* and will utilize an established *V. rupestris* x *V. arizonica* genetic map. These markers will be utilized for MAS to eliminate susceptible seedling progeny our continuing PD resistance breeding program.

Sub-objectives

1. Continue with a comparative mapping strategy between the *V. rupestris* x *V. arizonica* 9621 (D8909-15 x F8909-17) linkage map and other SSR maps within *Vitis* in order to identify additional SSR markers linked to resistance.

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

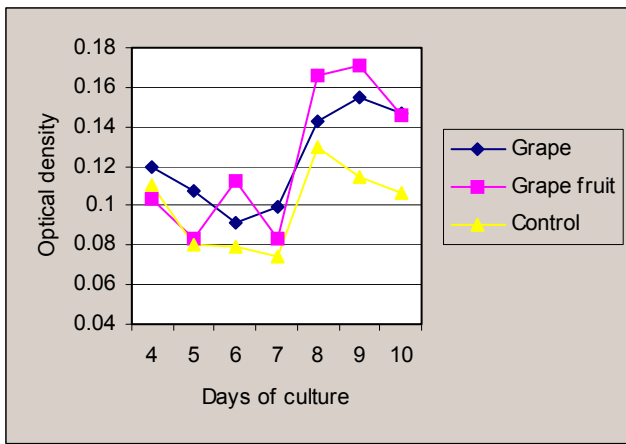


Figure 2. Effect of host plant xylem fluid on *Xf* growth.

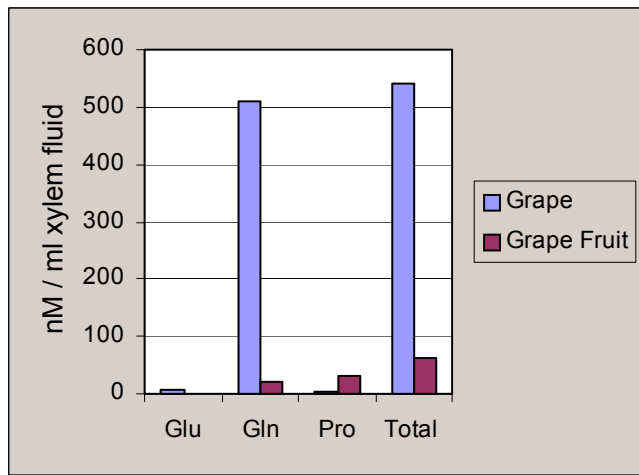


Figure 5. Some amino acid contents in grape and grape fruit xylem fluid.

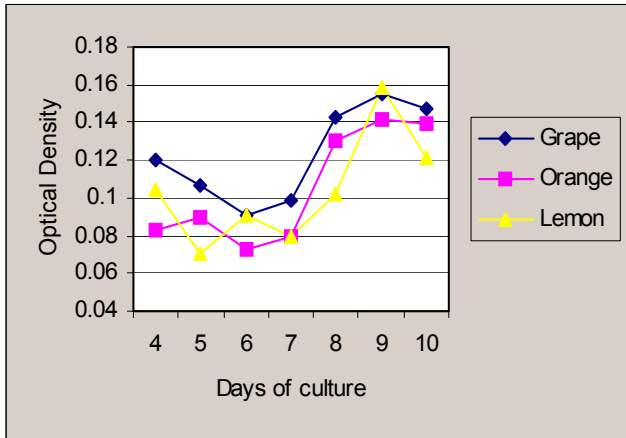


Figure 3. Effect of host plant xylem fluid on *Xf* growth.

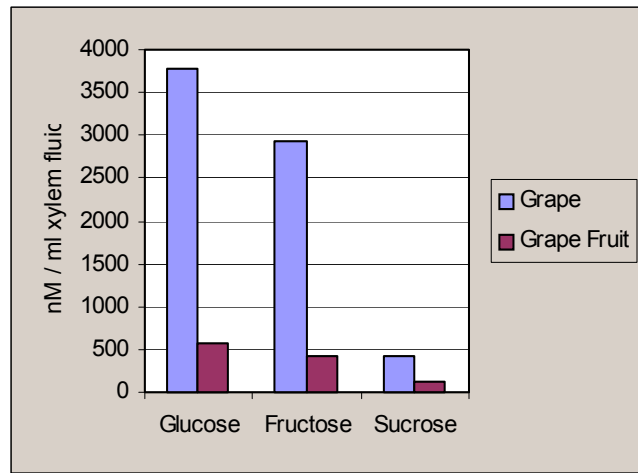


Figure 6. Sugar contents in grape and grape fruit xylem fluid.

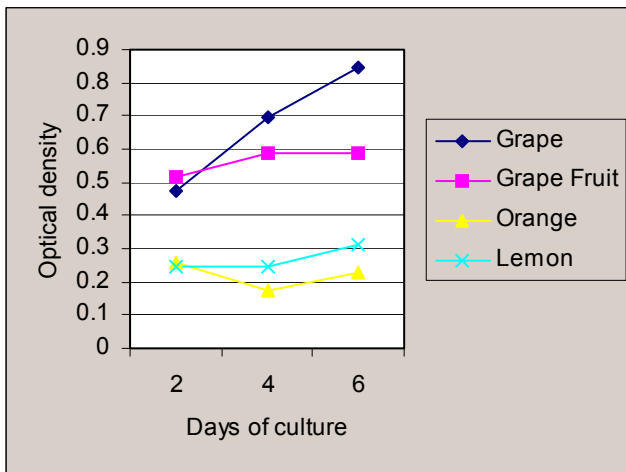


Figure 4. Effect of host plant xylem fluid on *Xf* biofilm formation.

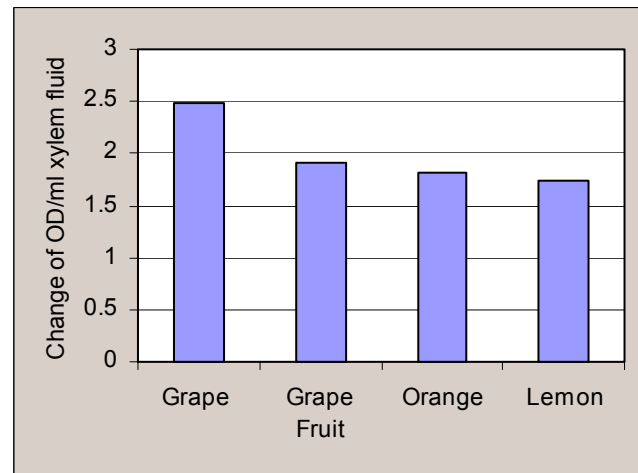


Figure 7. Peroxidase levels in host xylem fluid.

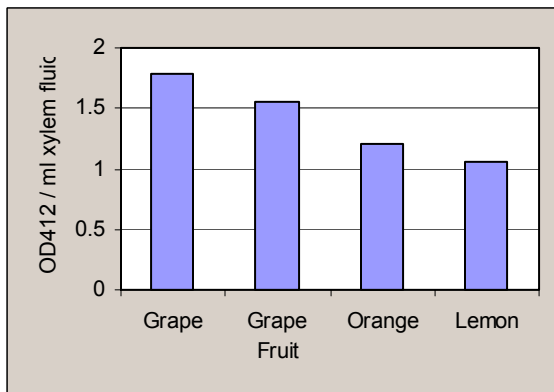


Figure 8. Total thiol contents in host xylem fluid.

and grape may differentially affect growth of *Xf*. Redox status also likely affects the tendency for *Xf* aggregation and biofilm formation. Adding reducing agents such as glutathione to artificial medium promotes *Xf* aggregation and biofilm formation (Leite et al., 2004). It was reported that thiols mediate the aggregation and adhesion of *Xf* (Leite et al., 2002). Thiol-containing compounds in xylem fluid include cysteine, methionine and glutathione. The redox status in citrus and grape xylem fluid and its role in *Xf* aggregation and biofilm formation, and host plant resistance/susceptibility to *Xf* need to be further investigated.

OBJECTIVES

1. Investigate the effect of host plant xylem fluid on *Xf* multiplication, aggregation and attachment.
2. Determine the biochemical mechanisms of host xylem fluid influence on *Xf* multiplication, aggregation and attachment.

RESULTS

Commercial citrus (lemon, orange and grapefruit) groves in proximity to vineyards were selected in the Temecula Valley, California. Three blocks of 30 citrus and 30 grape vines were used. A minimum of 15 citrus trees and 15 vines were randomly selected from each block (making a total of 15 trees or vines from each plant species) to extract xylem fluid. Terminal shoots from each plant were used for xylem extraction with a pressure bomb apparatus (Anderson et al., 1989). Upon collection, the xylem fluid was immediately placed on dry ice before final storage in a -80 °C freezer. The samples were used to test the impact of these xylem fluid on *Xf* resistance and chemical analyses of soluble carbohydrates, free amino acids, and redox status.

Effects of xylem fluid of each plant species on *Xf* attachment were evaluated on the biofilm formation. Formation of biofilm on the abiotic surfaces was assessed as described by Espinosa-Urgel et al. (2000). The analyses of *Xf* multiplication and aggregation were based on the fact that optical density (540 nm) is correlated with bacterial cell numbers and aggregation state as described by Burdman et al. (2000).

Our data indicated that, when the xylem fluid of grapefruit, orange and lemon was added to the PD Temecula strain of *Xf* in PD3 medium in glass culture tubes, there were heavy *Xf* cell aggregations to form large white clumps in suspension of the culture and the culture fluid was clear with no significant turbidity; in contrast, grape xylem fluid added to the same *Xf* culture did not cause visible clumping, but rather a visible thick biofilm was formed on the surface of glass tube and the culture was turbid (Figure 1). After homogenization of the culture, we found that the numbers of *Xf* cells in the grapefruit xylem fluid treatment were significantly higher at 6, 8 and 9 days after culture compared with those in the grape xylem fluid treatment (Figure 2). The numbers of *Xf* cells in orange or lemon xylem fluid treatments were generally lower than those in grape xylem fluid treatment (Figure 3). These data suggest that the citrus species, especially grapefruit, are suitable hosts for *Xf* growth and may serve as a great reservoir of the pathogen for GWSS acquisition. Our assay results revealed that xylem fluid of the citrus species significantly inhibited *Xf* biofilm formation compared to that of grape (Figure 4). Our attempt to investigate the biochemical mechanisms likely to be involved indicated that 96% of amino acids in grape xylem fluid was comprised of glutamine, while 47% of amino acids in grape fruit xylem fluid was proline (Figure 5). The content of total amino acids in grape xylem fluid was near 9-fold higher than that in grapefruit xylem fluid (Figure 5). Sugar contents were 1.4- to 5.5-fold higher in grape xylem fluid than those in grapefruit xylem fluid (Figure 6). Peroxidase and total thiol levels were also higher in grape xylem fluid than in citrus xylem fluid (Figures 7 and 8).

CONCLUSIONS

Xylem fluid of grapefruit, orange and lemon caused PD Temecula strain of *Xf* cells to aggregate and form large white clumps but inhibited the attachment. In contrast, grape xylem fluid did not cause visible clumping but led to heavy attachment. Grapefruit xylem fluid significantly increased multiplication of *Xf* cells compared with grape xylem fluid. Citrus species, especially grapefruit, appear to be suitable hosts for *Xf* growth and may serve as a reservoir of the pathogen for GWSS acquisition and transmission to grape vines. Further research is underway to elucidate the biochemical mechanisms.

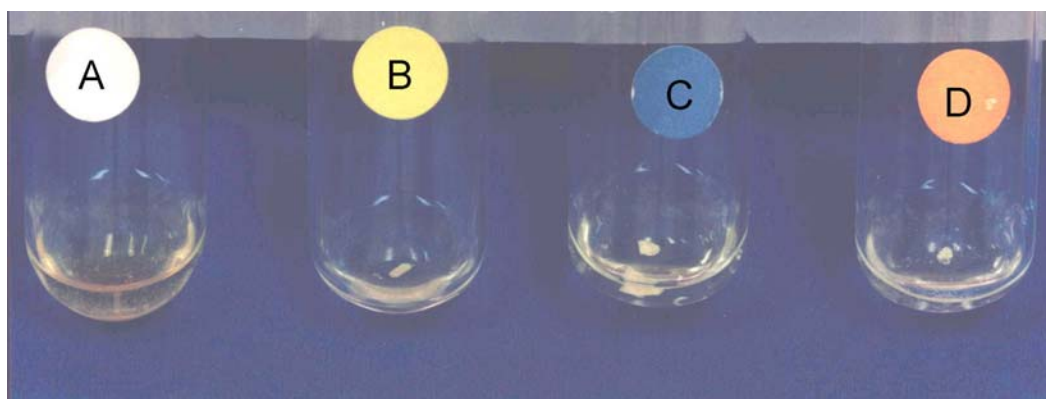


Figure 1. Effect of host plant xylem fluid on *Xf* aggregation. A, treatment with grape xylem fluid. B, treatment with grapefruit xylem fluid. C, treatment with orange xylem fluid. D, treatment with lemon xylem fluid. Note that white clumps of *Xf* aggregates are formed in the grapefruit, orange and lemon xylem fluid treatments.

IMPACT OF HOST PLANT XYLEM FLUID ON *XYLELLA FASTIDIOSA* MULTIPLICATION, AGGREGATION, AND ATTACHMENT

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Reporting Period: The results reported here are from work conducted from October 2003 to August 2004.

ABSTRACT

Research in Temecula Valley indicated that the proximity of citrus groves to vineyards has influenced the incidence and severity of Pierce's disease (PD), *Xylella fastidiosa* (*Xf*), in grapes. Although the glassy-winged sharpshooter (GWSS) feeds on and moves back and forth between Temecula citrus groves and vineyards, there are no visible *Xylella fastidiosa* (*Xf*) symptoms in the citrus. This implies that citrus trees are resistant or tolerant to the *Xf* but may be a reservoir to harbor the pathogen for GWSS acquisition while grape vines are susceptible. We investigated the mechanisms of host plant resistance/susceptibility by examining the impact of xylem fluid of grapefruit, orange, lemon and grape on *Xf* multiplication, aggregation and attachment as well as the related xylem fluid chemistry. Our laboratory experiments revealed that xylem fluid of grapefruit, orange and lemon caused an aggregation of Temecula PD cells to form large white clumps while grape xylem fluid did not cause visible clumping, but created a visible thick biofilm. The numbers of *Xf* cells in grapefruit xylem fluid treatment were significantly higher at 6, 8 and 9 days after culture compared with those in grape xylem fluid treatment. The numbers of *Xf* cells in orange or lemon xylem fluid tests were generally lower than those in grape xylem fluid treatment. Citrus xylem fluid significantly inhibited *Xf* biofilm formation compared to grape xylem fluid. The content of total amino acids in grape xylem fluid was near 9-fold higher than that in grapefruit xylem fluid. Sugar contents were 1.4- to 5.5-fold higher in grape xylem fluid than those in grapefruit xylem fluid. Peroxidase and total thiol levels were also higher in grape xylem fluid than in citrus xylem fluid. Our results indicate that the differences between citrus and grape plants in their responses to *Xylella* may be due to differences in their xylem fluid chemistry.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a xylem-limited, plant pathogenic bacterium that causes Pierce's disease (PD) in grapes (Purcell, 1981). *Xf* is mainly vectored by the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, in Southern California. Although a comprehensive list of suitable hosts for the GWSS has been identified, comprising 75 plant species in 35 families (Turner and Pollard, 1959), the major crop hosts in Temecula Valley are citrus and grapes. Previous studies in California have identified 94 plant species in more than 28 of plant families as host of *Xf* (Freitag, 1951; Raju et al., 1983; Raju et al., 1980). Most identified *Xf* hosts show no symptoms but serve as inoculum sources of *Xf* for vector acquisition. Perring et al. (2001) studied the incidence of PD in the Temecula Valley and found that proximity of citrus groves to vineyards has influenced the incidence and severity of PD in grapes. The PD infection is most severe when the grape vines are adjacent to citrus, and that the damage declines as one moves away from citrus (Perring et al., 2001). Although the GWSS feeds on and moves back and forth between citrus trees and grape vines, there is generally no *Xf* caused disease symptom in citrus in the area. This implies that citrus trees are resistant or tolerant to the *Xf*, but may be a reservoir to harbor the pathogen for GWSS acquisition and transmission while grape vines are susceptible. Little is known about the biochemical mechanisms involved in host plant resistance/susceptibility to *Xf* in the system. Additional information is required to determine if citrus can be suitable reservoirs for *Xf*. Elucidation of the biochemical mechanisms may be useful for developing host plant resistance in grapes as a sustainable component of integrated pest management program.

Xf aggregates to form biofilm inside its host plants and insect vectors. The biofilm formation is considered as a major virulence factor of PD (Marques and Ceri, 2002). Biofilm is defined as structured communities of sessile microbial aggregates enclosed in a self produced polymeric matrix and attached to a surface (Costerton et al., 1995). It was recently reported that a defined medium with some components based on susceptible grape cultivar "Chardonnay" xylem fluid chemistry better supports *Xf* growth and stimulates *Xf* aggregation and biofilm formation in vitro (Leite et al. 2004). However, the effect of citrus xylem fluid on *Xf* multiplication, aggregation and biofilm formation remains unknown.

Xf is a nutritionally fastidious bacterium (Wells et al. 1987). In defined medium certain amino acids are essential for *Xf* growth, glucose stimulates the growth while fructose and sucrose have inhibiting effect (Wells et al. 1987; Chang and Donaldson, 2000). It is not known whether differences in contents of amino acids and the sugars in the xylem fluid of citrus

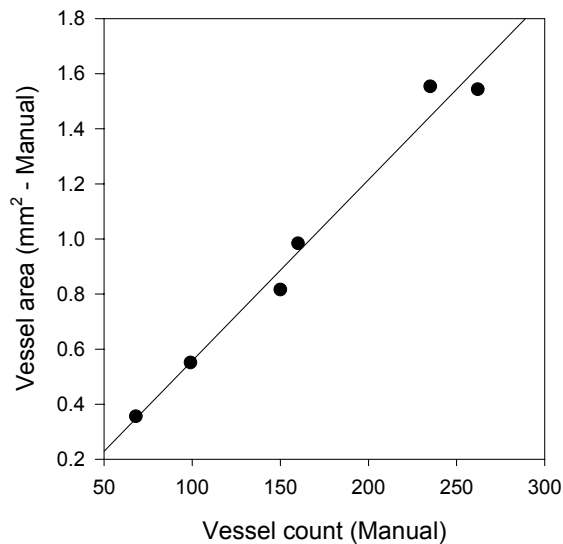


Figure 10. The number of functional vessel (vessel count) is a good predictor of the total area occupied by those vessels. Individual vessel areas were marked on the digitized MRI and summed automatically by ImageJ. Linear regression line $r^2 = 0.98$.

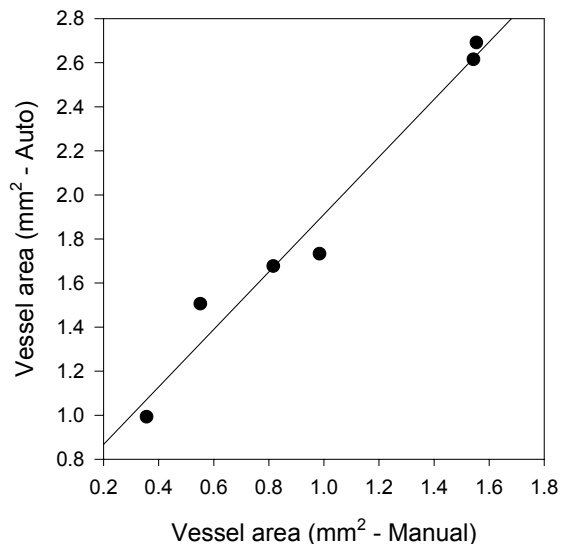


Figure 11. The area of functional xylem (the summation of the areas of individual vessels, see Figure 10 legend) is well correlated with the area calculated using an automated algorithm ($r^2 = 0.97$). A_f is the area calculated using the algorithm.

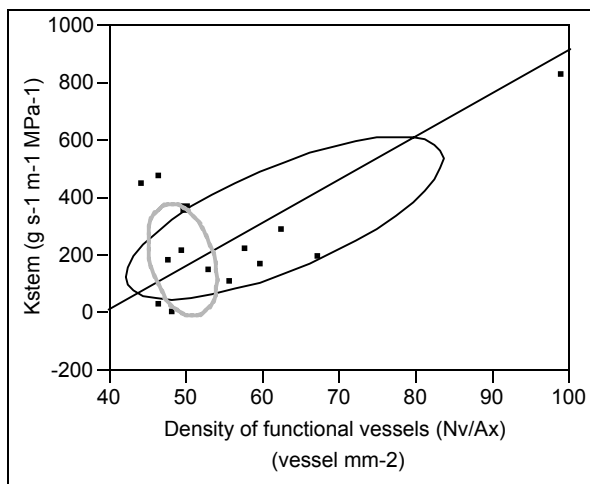


Figure 12. Principal component analysis plotting stem conductivity (y-axis) vs functional vessel density calculated as vessel number divided by total xylem area (x-axis). Ellipses enclose values for healthy vines (dashed, light line) and infected vines (heavy, grey line).

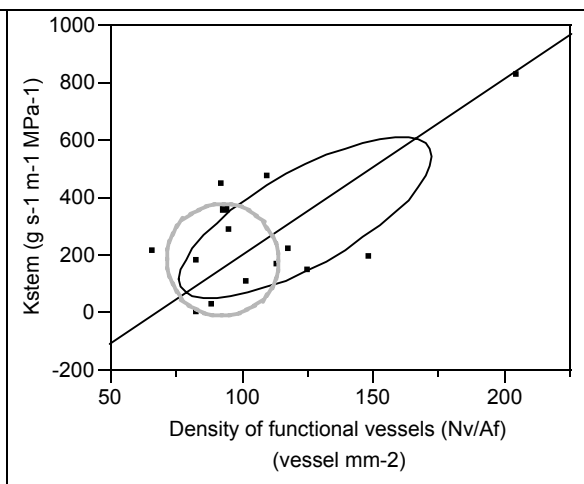


Figure 13. As in the Figure 12 legend, except that functional vessel density is calculated as vessel number divided by functional xylem area.

CONCLUSIONS

MRI will be a powerful adjunct to other, more conventional approaches for characterizing the changes that occur in grapevine xylem following introduction of *Xf*.

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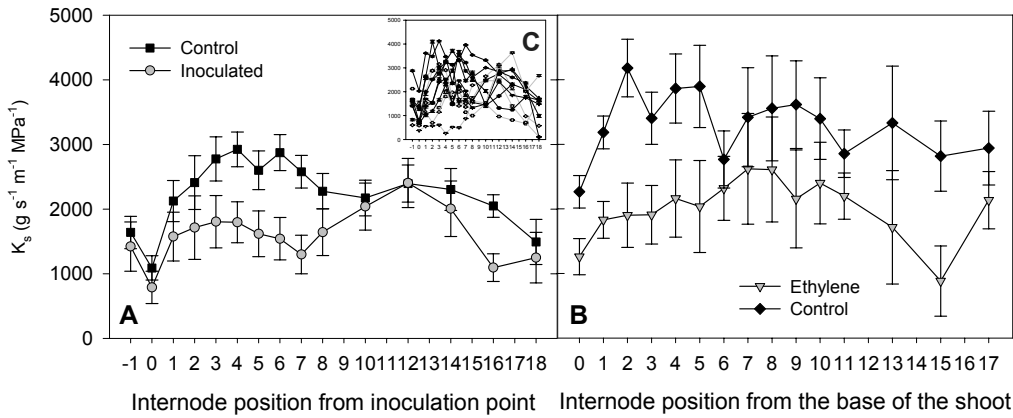


Figure 7. Specific hydraulic conductivities (K_s) for individual internodes of vines (a) inoculated with *Xf* and (b) exposed to ethylene (± 1 SE). Control plants show maximum K_s in middle third of the stem. In contrast, infected plants show a decrease in K_s in the middle portion of the stem. Panel (c) shows $K_s \pm 1$ SD for all the plants analyzed in the inoculation experiment. Although the variation among different plants is high, the error associated with the measurements is negligible. **Note:** These measurements reflect the contribution of water flowing through cavitated vessels because the embolized vessels are filled by the pressurized water that is used in the test.

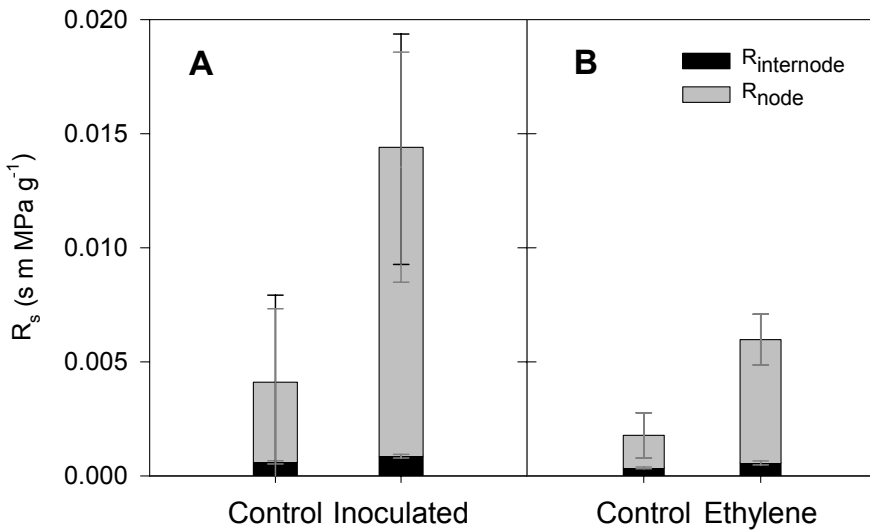


Figure 8. Specific hydraulic resistivity (R_s) for (a) vines inoculated with *Xf* and (b) exposed to ethylene. Total bar height represents $R_s \pm 1$ SE (in black). R_s components, R_{node} and $R_{internode}$, are also shown (± 1 SE in gray). The nodes are a major component of stem hydraulic resistivity (the inverse of conductivity). It can be noted that R_s is about 3 fold higher for stems of infected plants than for controls, even when infected plants have no external symptoms. This observation agrees with the information provided by MRI.

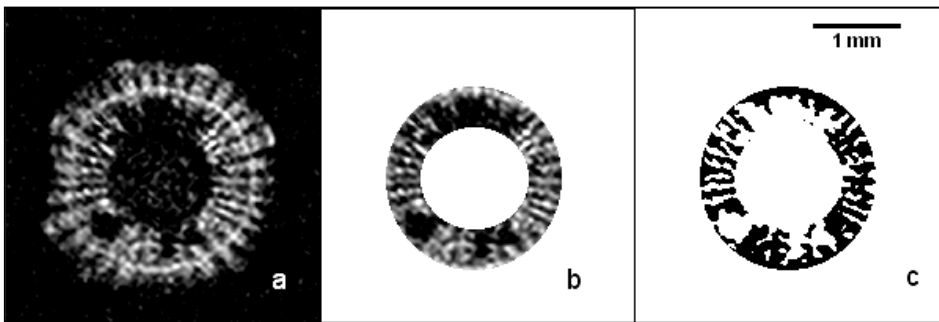


Figure 9. Example of the digital processing and analysis performed on MRIs to evaluate quantitatively the development of dark spots. (a) Original cross section MRI of an infected plant showing dark spots. Individual functional vessels are counted using this type of image. (b) Isolation and quantification of the cross sectional area of the stem that is normally xylem tissue (A_x). (c) Binary analysis of the xylem ring to determine the area of functional xylem (A_f), the black area represents the pixels that are above the threshold defined as the minimum value for a water-filled pixel. The program allows us to vary the threshold value.

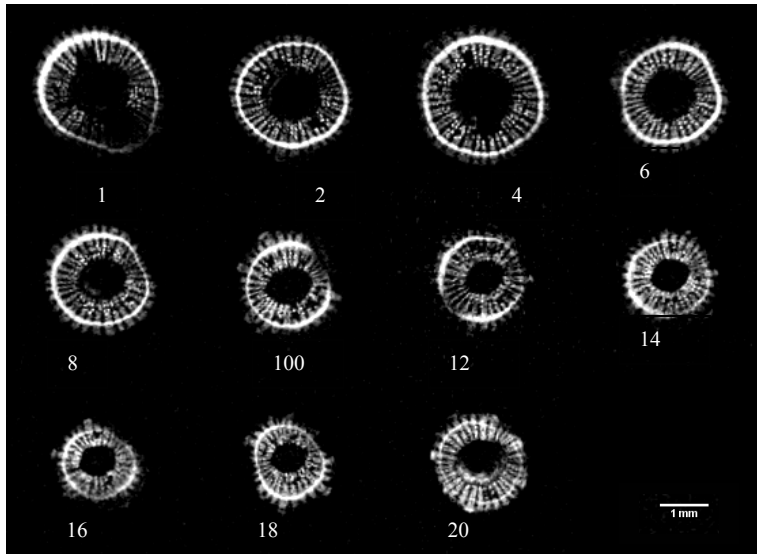


Figure 4. Stem cross section MRIs of a Control (water-inoculated) plant. The numbers indicate the internode position, counting from the base of the stem. In internodes 1-3 it is possible to observe the disruption of the xylem caused by the needle inoculation. The xylem disk looks normal in the other internodes. Note that individual vessels are easily observed as bright spots.

Figure 5. Stem cross section MRIs of an infected plant. This plant was not showing external symptoms after 6 months of inoculation. The effect of needle inoculation can be seen in internode 2. Dark sectors of embolized vessels can be observed from internodes 10 to 20. Note that in this image it is more difficult to distinguish anatomical features and individual vessel than in MRIs of a Control plant (Figure 4).

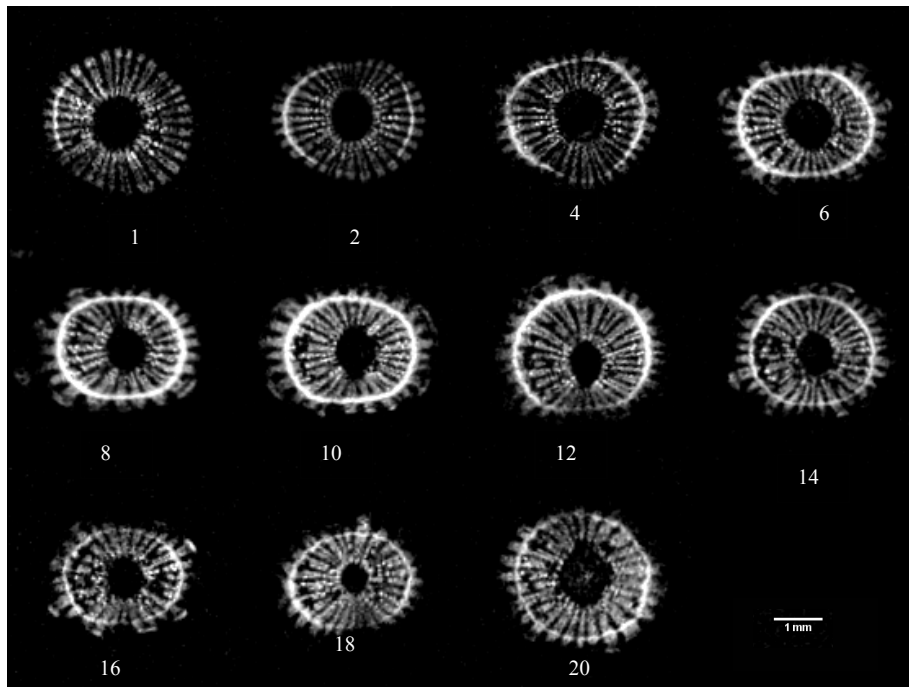
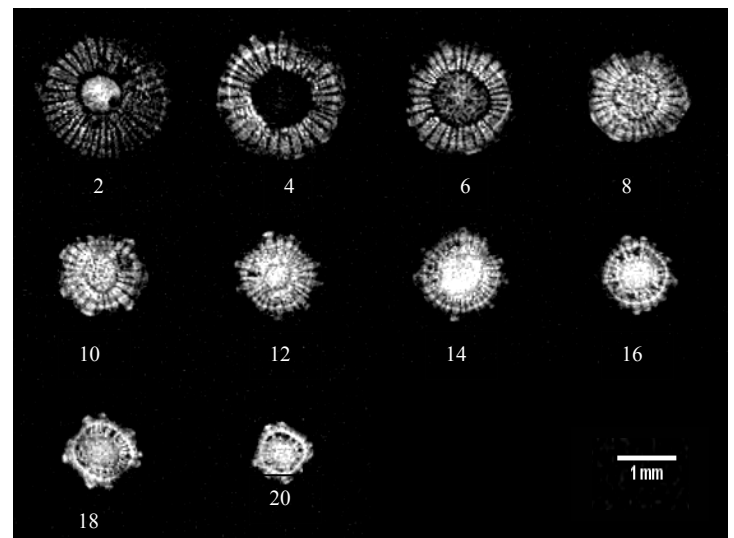


Figure 6. Stem cross MRIs of a plant exposed to ethylene. Numbers indicate the position of the internodes, numbered from the base of the stem. “Dark spots” that show non-functional vessels can be seen increasing in size from the base of the stem. The xylem disk appears to be compromised the most at internode 16, which was approximately the youngest internode in the stem (i.e., in the growing tip) at the time of ethylene treatment.

clearly in the lower range of that response. We have shown that cavitated vessels that are air-filled can be re-filled (including restoring an image showing that they are water-filled, see Figure 3). However, attempts at refilling segments of PD-infected stems that showed “dark sectors” in the MRIs generally failed. This indicates that “dark sectors” in MRIs of infected vines are likely a sign of a relatively permanent deterioration of the water movement capacity in the stem, probably a consequence of tylose formation and/or vascular gel development.

Table 1. Mean values for calculated functional vessel densities in healthy and infected grapevine stems.

Treatment	Vessel density \pm 1 SE	
	N_V/A_x	N_V/A_f
Control	63.03 \pm 4.81	124.88 \pm 11.93
<i>Xf</i> -inoculation	49.78 \pm 4.81	93.25 \pm 11.93

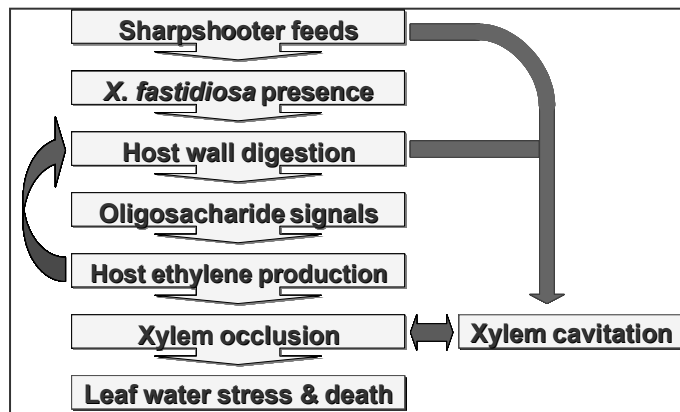


Figure 1. Hypothetical model for PD development. PD starts with a local infection caused by the glassy-winged sharpshooter’s introduction of *Xf* locally (i.e., into one or a few vessels). Once *Xf* is in the xylem the bacteria become systemic, which implies that *Xf* must be able to cross (digest away?) the cell wall in the pit membranes that separate two neighboring vessels. The digestion of the cell wall by bacterial enzymes would generate transient oligosaccharides with biological activity. The presence of these oligosaccharides is detected by the plant triggering a series of defensive responses, including a raise in ethylene production. Ethylene has been shown to induce tylose formation. Cavitation of vessels may be also important for the disruption of water transport in the plant. Cavitations may happen during insect feeding or during PD progression. The “bottom line” of our thinking is that PD is primarily caused by the grapevine’s responses (local and systemic) to *Xf* presence.

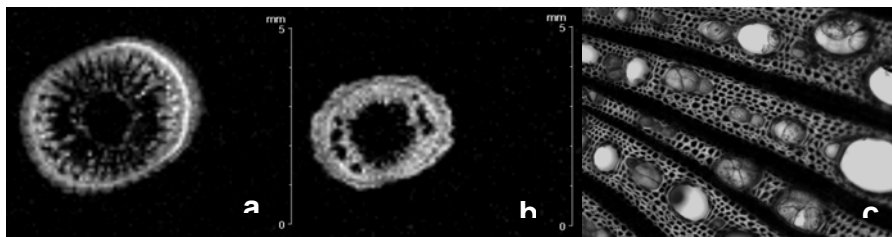


Figure 2. MRI of a PD-infected stem in a basal internode (a), and closer to the apex (b). Bright spots between the central pith (dark) and the ring of vascular cambium show functional vessels. Image b shows dark pockets within the vascular tissue that indicate areas in which vessels are not water-filled (compare the image to the healthy stem in Figure 3a). Tyloses (cellular-physical blockages of the vessels) are often associated with dark spots in MRIs of infected xylem, Tyloses are shown as accumulations of dark, bubble-like structures in vessel seen in the light microscope of an infected stem (c).

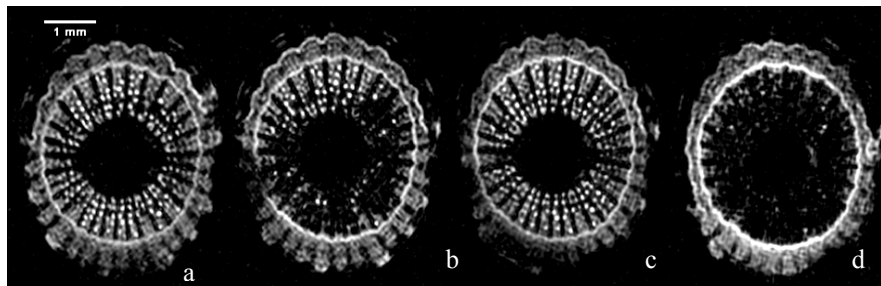


Figure 3. (a) MRI of an intact stem segment in a healthy shoot. (b) Image of the same stem portion after an important part of the cross section below has been severed, thus causing cavitation of many vessels. (c) The same stem segment after it has been refilled with water. (d) Stem segment after flushing with air to completely empty the xylem vessels.

potential importance in PD development. Our analysis can reveal vessels that have cavitated. Figure 3 shows functional vessels in an intact stem, and empty vessels after the stem is severed to cause cavitation, and that cavitated vessels can be re-filled with water under pressure. When we have the optimized MRI probe we will develop a series of image sets taken along the lengths of vines at intervals following water (control) and *Xf* inoculation to give a time course of PD development. However, at this point we do not have images for a full time course.

MRI is capable of showing xylem disruption and non-functional vessels well before external symptoms appear in infected plants. Figures 4 and 5 show images for the length of control (buffer-inoculated) and infected (*X. fastidiosa*-inoculated) vines six months after inoculation. MRIs of the control-inoculated vine show defined xylem rays, in which individual vessels can be clearly observed. As in previous experiments, stem cross section MRIs of infected plants (Figure 5) show that major sectors of the xylem appear dark, indicating that they are no longer water-filled (Note: the magnetic signal is lost in cavitated vessels). Furthermore, MRIs of plants infected with *Xf* become less sharp, making it more difficult to discriminate structure, particularly of individual, probably still functional, vessels. Efforts to explain this will be a feature of the work as this project continues. MRI also has been used to follow changes in the functionality of the xylem of plants exposed to ethylene in enclosed chambers (10 ppm for 48 hours). We previously described the progressive development in time of “dark sectors” in the xylem of ethylene-gassed, presumably indicating vessels no longer involved in water transport. This new set of experiments has allowed us to confirm that, after 6 months of exposure to ethylene, gassed plants show progressive xylem disruption along the stem (Figure 6). Most of the damage is localized close to nodes/internodes that had just developed in the stem growth tip at the time of ethylene treatment and had then expanded in the intervening six months prior to our observations. The MRIs show “dark sectors” in those internodes. These sectors decrease are less extensive in internodes below and above the internodes that were in the growth tip at the time of treatment; that is, internodes formed after the time of treatment and already partially elongated, respectively when ethylene was applied. As in *Xf*-infected plants, MRIs of ethylene-treated plants are less sharp than images of control plants (Figure 6).

The impression of a loss in xylem function that is given by the MRIs of *Xf*-inoculated and ethylene-gassed vines can be correlated with a decrease in the hydraulic conductivity of internodes. This is tested by determining the rate of movement of pressurized water through stem segments (Figure 7). Similarly, stems of treated vines showed an increase in the hydraulic resistivity (the inverse of conductivity) relative to the controls (Figure 8), although this difference was statistically significant only for the ethylene experiment. The lack of statistical difference in the inoculation experiment is mainly due to the great variability found in the hydraulic resistivity of inoculated plants. In turn, this might be explained because these vines were in a gradation of early stages of PD infection when examined (they were not showing external symptoms). While there is some correlation between the MRIs showing localized areas of empty vessels and reduced hydraulic conductivity in regions of infected stems, the correlations are not perfect. This is due to at least two factors that will be tested more fully in our continuing work. First, an empty vessel shown in the MRI at one level in the plant’s stem could be the result of a vessel obstruction or cavitation above or below the point on the stem where the MRI observation was made. There may be no actual impediment to water flow in the empty vessel at the level at which it is being imaged. Thus, a test of water flux at the imaged level may reveal no water flux difficulty. Second, while cavitation may be an important factor in PD development, because the tests of water conductivity are carried out using water under pressure, cavitated vessels will be re-filled during the test and no reduction in water flux would be revealed. Destructive anatomical work will define which kind of vessel disruption (tylose, gel or air embolism) exists in stems with non-functional vessels as revealed by MRI.

A more quantitative analysis of the MRIs has been attempted in order to characterize objectively the presence of “dark sectors” in the images. For this purpose, the MRIs were processed and analyzed using the ImageJ program (developed at the U.S. National Institutes of Health and available at <http://rsb.info.nih.gov/ij>). First, the number of functional vessels (N_f) was counted in the MRIs of inoculated and control vines (like the one in Figure 9a), based on the assumption that a bright (hence, water-filled) vessel was functional. Next, the xylem-cross sectional area (A_x) was measured by isolating in the MRIs (Figure 9b) the ring of tissue that is usually occupied by the xylem. Then, the digital image of the xylem-ring was converted to a binary image (Figure 9c) using a built-in algorithm in ImageJ, in which all the pixels above a set grey intensity threshold are black and the pixels below this value remain white, and the functional xylem-cross sectional area (A_f) was determined by measuring the black area. To confirm that the threshold area correctly estimated A_f , the area of individual functional vessels was selected by hand and measured in a series of MRIs, some with clearly delimited vessel images and others with less distinct (“fuzzy”) images such as those often seen when PD-infected grapevine stems are examined. The images from infected vines often do not show vessels as bright or dark spots, rather the images of individual vessels are fuzzy, making determination of vessel functional status difficult. The area of functional xylem measured manually was then correlated with the number of functional vessels (Figure 10), and with the results of the automated routine (Figure 11). The regressions confirmed that both the number of functional vessels and the threshold areas depicted in the binary images, are excellent estimators of A_f . Preliminary results of the quantitative analysis described above, in which all the images for an individual plant were averaged; indicate that *Xf*-inoculated vines have a lower mean density of functional vessels (Table 1) than that of controls. Figures 12 and 13 show that the vessel density also correlates positively with the hydraulic conductivity for whole stems, suggesting that the visual assessment of MRIs conveys information about the actual water movement capacity of grapevine stems. Principal components ellipses ($p = 0.5$) in Figures 12 and 13 show that, in both, inoculated and control vines, the hydraulic conductivity for the whole stem is a function of the vessel density, but infected the vines tend to localize

MAGNETIC RESONANCE IMAGING: A NONDESTRUCTIVE APPROACH FOR DETECTION OF XYLEM BLOCKAGES IN *XYLELLA FASTIDIOSA*-INFECTED GRAPEVINES

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Reporting period: The results reported here are from work conducted from April 4, 2004 to October 1, 2004. (**Note:** we are now in the second year of a project originally approved in 2003.)

INTRODUCTION

Results from Pierce's disease (PD) research programs led by Matthews, Rost and Labavitch (reported in 2001, 2002 and 2003 in San Diego) have provided substantial support for the idea that obstructions in the vine's water-transporting xylem tissue develop rapidly post-inoculation, before an appreciable bacterial population has been established. The results also strongly suggest that these obstructions, and likely other aspects of the PD "syndrome", result from the grapevine's active responses to the presence of *X. fastidiosa* (*Xf*), rather than to direct "action" by the bacterium. Thus, careful analysis of the timing of changes in xylem element anatomy and function relative to *Xf* introduction, as well as to external symptoms of disease development, is important for establishing reliable indicators of the "stage" of PD development. The analyses done thus far have been based on destructive tissue sampling. Such sampling can be particularly "blind" when it is done on vines in which (based on our earlier results) internal symptoms of PD are present but external, visible symptoms are not yet present.

In the report of the year 1 work of our study (Shackel and Labavitch, 2003), the success of Mr. Pérez and Dr. Walton in imaging non-functional vessels in the stems of PD-infected and ethylene-treated grapevine stems was demonstrated. In this report we elaborate on those studies, showing that locations of reduced vine water transport capacity, as determined by non-destructive MRI analysis, is correlated with the locations of PD and ethylene effects on vessel functionality (destructive analysis). In addition, because interpretation of the meaning of the MRIs with respect to the anatomy and functioning of vessels is a crucial aspect of our work, we have described the methodology used to validate our approach to obtaining the relevant information from the MRIs.

OBJECTIVES

1. Optimize the use of MRI (Magnetic Resonance Imaging) and to spatially visualize altered water movement in grapevines.
2. Test correlations of observed vascular system obstructions (based on grapevine dissection and microscopy techniques) with predictions based on MRI data.
3. Use MRI to follow the development of grapevine obstructions over time in vines infected with *X. fastidiosa* or treated with ethylene, bacterial wall-degrading enzymes or plant cell wall oligosaccharides, all of which may be important intermediates in regulating the vine's response to infection and the eventual development of PD symptoms.
4. Use NMR imaging to determine whether localized xylem cavitation occurs at the site and time of *X. fastidiosa* inoculation or introduction by the glassy-winged sharpshooter.

RESULTS

Optimization of the Use of MRI for Visualizing Water Transport Deficiencies in PD-Infected Grapevines.

Progress on this objective has been delayed because a supplier for a key electronic element of the new MRI probe that has been designed for use with grapevines no longer provided a key part. The parts are all now available and development of the new probe is underway. We are proceeding with the testing of aspects of the PD model using the NMR instrument in its more conventional configuration.

MRI Will Show Non-functional Sections in the Xylem of a PD-infected Grapevine Stem.

Usually the techniques to evaluate xylem function are destructive. Magnetic Resonance Imaging (MRI) allows us to visualize vessels that are functional and full of movable water. Functional vessels appear as bright spots in an MRI view of the stem cross-section; non-functional vessels lack water and appear as dark spots in the area of the stem where water-conducting cells are found. Figures 2a & 2b show the difference in the distributions of functional vessels in an infected vine at a point where leaf symptoms of PD are apparent (Figure 2a) and nearer to the stem apex at a point where the leaves show no sign of PD symptoms (Figure 2b). Compare these images with that for a healthy vine (Figure 3a). Cavitation of xylem vessels is also of

B. Green Islands

Green islands arise from the incomplete development of the deep-seated phellogen (cork cambium) in *V. vinifera*. In regions of the stem where the phellogen arises and produces subsequent phellem (cork), external tissues (phloem, cortex, epidermis) are cut off from their nutrient sources and begin to die and brown. The juxtaposition of stem regions with active phellogen, and the juvenile character of no phellogen, creates green islands. It is unknown whether green regions are delayed in their development, or whether brown regions display advanced development. No obvious correlation was seen in the level of vessel occlusion proximal to green or brown regions. Additionally, periderm formation was observed in *M. rotundifolia*. Periderm formation in this species is subepidermal (vs. deep-seated) and consequently green islands may not form in this species (Stevenson et al. 200x). This is important point for researchers using green islands as an indicator of PD resistance.

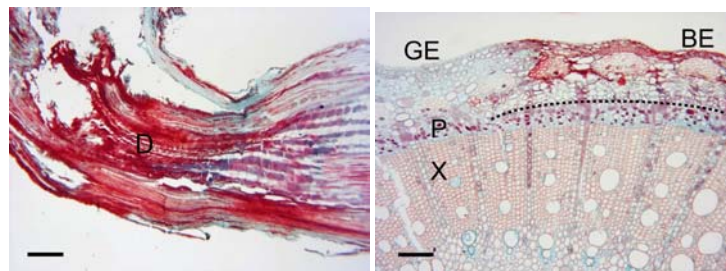


Figure 4. Longitudinal section through a matchsticked petiole (left) displaying basipetal dehydration (D) following pseudoabscission. Transverse section through a stem with green island (right) showing regions of green epidermis (GE) and brown epidermis (BE) created by presence of absence of phellogen initiation.

CONCLUSIONS

1. The development of tyloses and gums in response to *Xf* infection were qualitatively similar in the resistant *M. rotundifolia* cv Cowart and the susceptible *V. Vinifera* cv Chardonnay, although the resistant species tended to form fewer tyloses.
2. The only observable difference in hydraulic architecture was that the resistant species had narrower vessels.
3. Fluorescent beads were loaded into stems of both species. Beads moved approximately the same distance (~1.6-1.8 nodes) and in both cases did not enter into petioles.
4. Tyloses were first seen about 24 hours after pruning in both species. After four days about 50% of vessels were blocked. By eight days most vessels were blocked in both species.
5. Matchsticks formed in *V. vinifera* leaves after several days of *Xf* infection. This symptom consisted of the pseudoabscission of the petiole from the leaf blade. Green islands are green areas of the stem created by incomplete formation of periderm in infected plants.

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that some pit membrane disruption, is required for bacteria to colonize petiole and leaf tissue (Stevenson, Matthews and Rost, 2004a).

B. Resin-casting and Macerations

Resin casts were made of the internal spaces of vessel lumina and pit surface morphology in both *V. vinifera* and *M. rotundifolia* (Figure 2). Superficially, no differences were seen in pit patterns, pit integrity, or relative pit surface area between the species. Further study is required to investigate subtle characters of pit membranes (ex. total pit membrane area, dimensions of pit apertures) that may facilitate pit membrane disruption by bacteria.

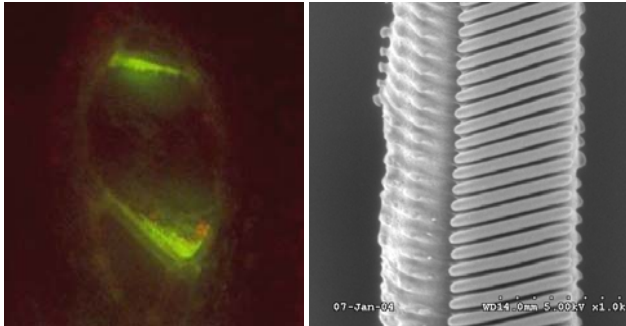


Figure 2. Fluorescent beads within stem xylem of *V. vinifera* used to mimic movement of passive bacterial cells (left), and resin casts of xylem vessels from *M. rotundifolia* to show fine detail of pit surfaces (right).

4. TYLOSE DEVELOPMENT

A. Rate of Tylose Development

A working hypothesis was developed that differential susceptibility to PD among grapevine species may involve differences in the rate of tylose development. The rate of tylose development was studied in both resistant and susceptible grapevines following wounding (pruning) injury. Tylose development was then observed allowing one, four, and eight days for tyloses to develop. Initial tylose development was found within a day, about half of the vessels were occluded by day four, and at day eight, most vessels of the stems were observed to be significantly blocked by tyloses (Figure 3). No superficial difference was seen between the rate of tylosis in PD susceptible *V. vinifera* and resistant *M. rotundifolia* at any of the time intervals, however, further quantitative analysis is necessary.

B. Vitality of Tyloses and Paratracheal Parenchyma

The presence of living cells surrounding the vessels during tylose formation following pruning was studied using the vital stain fluorescein diacetate. This technique was used to discern a correlation between the amount of tylose occlusion found in the vessel and the number of vital paratracheal cells surrounding that vessel, and whether the number of vital paratracheal cells was significantly greater in PD susceptible grapevine species. Both resistant and susceptible grapevines were observed in this manner over the eight-day time course described in 4A. No superficial differences were seen in the vitality of paratracheal parenchyma surrounding vessels in the two species, however greater quantitative analysis is required. Overall, tyloses fluoresced greatly, indicating vital development, whereas paratracheal cells fluoresced only occasionally (Figure 3). These results suggest that very few active paratracheal cells are required to result in significant tylose development.

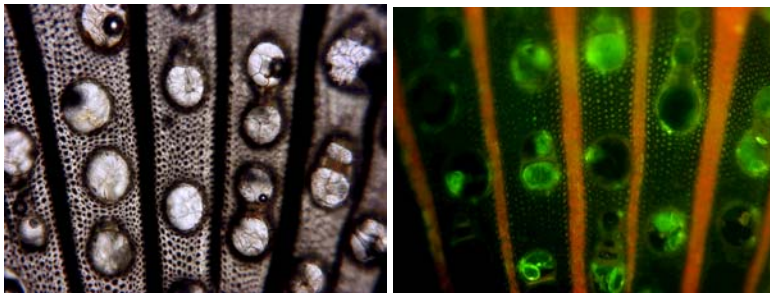


Figure 3. Micrographs of similar grapevine stems eight days following pruning. A bright field light micrograph (left) shows significant occlusion by tyloses at this interval. A fluorescence micrograph (right) shows fluorescent green vital staining predominantly by tyloses, but occasionally by paratracheal cells.

5. DEVELOPMENTAL ANATOMY OF MATCHSTICKS AND GREEN ISLANDS

The development of the external visual PD symptoms of matchsticks and green islands was studied from an anatomical perspective (Stevenson, Matthews and Rost 2004b).

A. Matchsticks

Matchsticks result from pseudoabscission of the leaf lamina from the petiole. Following significant leaf scorching, the lamina breaks from the petiole at a predictable fracture zone. No separation zone develops as is common with typical leaf abscission, and hence this process is described as pseudo-abscission. Following pseudoabscission, exposed petiole tissues dehydrate and blacken to take on the appearance of a burnt matchstick. Occasionally, a wound periderm will form near the fracture zone following pseudoabscission. When this periderm forms, dehydration of the petiole is minimal. The process of matchsticking has never before been described anatomically.

It is generally accepted that the fatal nature of Pierce's Disease is a result of the bacteria becoming systemic and water stress becoming increasingly severe until the plant is no longer able to function (Goodwin et al., 1988). However, the classic PD symptoms: patchy leaf chlorosis, persistent "green islands" on stems, and "matchsticks" (leaf abscission at the petiole/blade junction) are not generally observed in vines exposed to water stress alone. If the symptoms of PD are not, in fact, a result of water deficit, then studies relying on the assumption that water stress is the ultimate killer of plants suffering from PD, may result in misleading information and add years to finding solutions to the PD problem. Our second annual report addresses these concerns.

OBJECTIVES

1. Study the progression of anatomical symptoms created by *Xf* over a time-course in a PD resistant grapevine species, *Muscadina rotundifolia* cv Cowart.
2. Determine the hydraulic architecture of a PD resistant species, *M. rotundifolia*.
3. Study the integrity of pit membranes of both PD susceptible *Vitis vinifera* cv Chardonnay and resistant *M. rotundifolia* by following the in situ movement of fluorescently tagged beads.
4. Determine the rate of tylose development from wounding in both PD *V. vinifera* and *M. rotundifolia*.
5. Study the developmental anatomy of green island and matchsticks in *V. vinifera*.

RESULTS

1. PROGRESSION OF PD SYMPTOMS IN RESISTANT SPECIES

The progression of anatomical symptoms created by infection by *Xf* was studied along a time-course as was previously conducted with *V. vinifera* (Stevenson, Matthews and Rost, 2004). Similar experiments were conducted with PD resistant *M. rotundifolia* in an attempt to discern quantitative or qualitative anatomical differences in a six-month post-inoculation period. The development of symptoms in the resistant species was qualitatively similar to that in resistant species (development of tyloses in stems, development of gums in petioles), however the rate of development and overall occlusion created by these symptoms was dramatically lower. In the resistant species overall occlusion was minimal (<5% of vessels) after nearly four months (Figure 1), whereas in susceptible species overall occlusion was great (~50% of vessels).

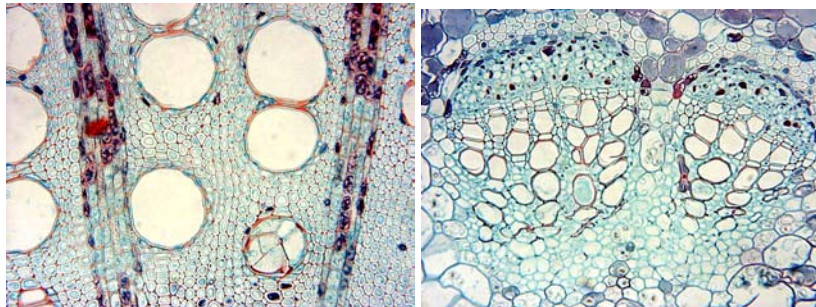


Figure 1. Minimal vessel occlusion in stem (left) and petiole (right) xylem of *M. rotundifolia* 122 days post-inoculation with *Xf*.

2. HYDRAULIC ARCHITECTURE OF RESISTANT SPECIES

The general hydraulic architecture of PD susceptible *V. vinifera* has been presented (Stevenson et al. 2004). Similar studies were conducted with PD resistant *M. rotundifolia* in an attempt to elucidate anatomical differences that may explain PD susceptibility or resistance. Regions of grapevine stem were serially sections to follow xylem arrangement in the node and internode. No significant differences were observed in the organization of stem xylem or in the divergence of xylem to lateral organs between resistant and susceptible species. The only difference found between the species was that *M. rotundifolia* possessed significantly narrower vessels than were found in *V. vinifera*. The difference may be contribute to restricting bacterial movement. Narrow vessels may cause bacterial conglomeration closer to the point of inoculation and prevent long distance bacterial seeding. Additionally, narrower vessels have less overall pit surface, which may further reduce the number of alternative pathways available to bacteria. Both of these proposals require further investigation.

3. PIT PROPERTIES OF SUSCEPTIBLE AND RESISTANT SPECIES

Preliminary investigations were conducted towards the study of the characteristics and integrity of pit membranes in susceptible and resistant grapevine species. The movement of *Xf* bacteria in the host is potentially facilitated by damaged pit membranes of grapevine, compromised either in development, or as a result of frequent cavitation/refilling cycles (Hacke et al. 2001, Sperry et al. 1987).

A. Movement of Fluorescent Beads

Fluorescent beads of similar size to *Xf* bacterial cells were injected into stem xylem of *V. vinifera* and *M. rotundifolia* (Figure 2). The distance of bead travel from the inoculation point was recorded as an indicator of vessel length and pit membrane integrity. Beads were observed to travel similar distances in both species (*V. vinifera* 1.6 ± 0.5 nodes, *M. rotundifolia* 1.8 ± 0.4 nodes). The relatively short distance that these beads traveled indicates a general integrity within the vessel pits and is evidence against pit damage commonly occurring. Beads were never observed to pass into petiole xylem, which suggests

**MECHANISMS OF PIERCE'S DISEASE TRANSMISSION IN GRAPEVINES:
THE XYLEM PATHWAYS AND MOVEMENT OF *XYLELLA FASTIDIOSA*.
PROGRESS REPORT NUMBER TWO: GREEN ISLANDS AND MATCHSTICKS**

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Reporting Period: The results reported here are from work conducted from October 2003 to September 2004.

ABSTRACT

During this period our focus was the comparative xylem anatomy of a resistant species, *Muscadinia rotundifolia* cv Cowart and a susceptible species, *Vitis vinifera* cv Chardonnay. When infected by *Xylella fastidiosa* both species produced tyloses (parenchyma ingrowths into tracheary elements) and gums; *M. rotundifolia* tended to have fewer tyloses. The resistant species also had narrower vessels, but otherwise xylem anatomy was similar to *V. vinifera*. Fluorescently tagged beads were loaded into both species. Beads traveled through the stem xylem in both, but did not move into petioles in these experiments. Tyloses were first apparent 24 hours after pruning in both species and most vessels were blocked in both after eight days of pruning. This suggests that the mechanism to form tyloses in both species is similar, although the resistant species tended to show fewer tyloses in response to *Xf*. Two symptoms, green islands and matchsticks are reported in this study. Green islands formed as a result of incomplete initiation of the phellogen. In regions of the stem where a phellogen and subsequent periderm arose, immediately exterior tissue was cut off, causing it to brown. In regions of the stem where no periderm is formed, the exterior tissues remained green. Consequently, the stem is mottled with both green living epidermis and brown dying epidermis as determined by the presence or absence of an underlying periderm. Matchsticks formed when the leaf lamina separated from the petiole, and the petiole remained attached to the stem. Lamina broke off from the petioles consistently in a fracture zone where xylem from the petiole anastomoses into the five major veins of the leaf. No separation layer was found to explain this pseudoabscission.

INTRODUCTION

Xylella inoculation of stem xylem precedes a relatively rapid movement of bacteria through the hydraulic network (system of xylem) to the leaves. Once bacteria moving in the transpiration stream enter regions of the hydraulic network that contain narrow tracheary elements and terminal tracheary elements (i.e. shorter vessels in petioles and leaves), bacteria may be 'filtered out', accumulate, and become embedded in a gel which effectively blocks water flow in that conduit. Tyloses are cell wall extensions of xylem parenchyma cells into tracheary elements. Tylose formation in the stem coincides with bacterial infection, but at least initially, is not present to such a degree that bacterial movement is apparently prevented or that the water supply to distal tissues is restricted to levels causing visual symptoms. Additionally, bacteria can move relatively quickly from an inoculated shoot to another shoot via the subtending trunk.

A similar understanding of the progression of events is needed for resistant varieties and species in order to localize investigations into the mechanism(s) of resistance. The anatomical symptoms of PD, xylem occlusions of gums and tyloses, are well documented in both susceptible (Esau 1948) and resistant plants (Mollenhauer and Hopkins 1976). However, it is not clear whether these occlusions are related to susceptibility or resistance. Only the susceptible plants express leaf scorch and eventual death, and these disease symptoms are widely understood to be water stress (Hopkins, 1989). Sufficient occlusions would produce water deficits downstream. Plants resistant to PD may remain healthy despite systemic populations of *Xylella* present in the vascular tissue because tylose and gum formation are not induced compared to susceptible varieties. Alternatively, the occlusions may prevent the movement of the bacteria, and comparative studies report that the frequency of occlusions is greater in resistant than in susceptible varieties (Fry and Milholland, 1990). Thus, resistant varieties or species may restrict *Xf* to regions of the hydraulic network proximal to the point of inoculation, either by occlusions or other mechanisms described below. In the reported experiments, we have initiated those studies. Regardless of whether resistance is dependent upon controlling the movement of *Xf*, Pierce's Disease is fatal because *Xf* becomes systemic. Host species in which *Xf* is confined to specific tissues, or is otherwise prevented from becoming systemic, do not display symptoms of PD (Hill and Purcell, 1995).

Merlot, high endochitinase concentration in roots (35S promoter)
Merlot, low endochitinase concentration in roots (35S promoter)

Additional controls will include own-rooted transgenic vines to be used to test for presence of foreign protein in the xylem sap.

CONCLUSION

The success of this project will rest on the careful, methodical characterization of foreign gene products. This project will not involve the speculative and lengthy creation of novel transgenic grapevines, but rather uses pre-existing transgenic grapevines in order to investigate the potential for transgenic rootstocks to deliver proteins to their non-transgenic scions.

Based on the evidence from the movement of imidacloprid and PGIP in grafted grapevines, it is likely that transgenic grapevine rootstocks will transmit transgenic proteins to their non-transgenic scions. However, it is premature to speculate concerning the time frame for reduction to practice in the form of a novel PD management strategy. We emphasize that this study is intended to investigate the biological principles of protein transport via xylem in grapevines, a topic that has been studied very little in the past. By understanding the potential of a transgenic grapevine rootstock to move proteins into a non-transgenic scion, scientists will be better equipped to investigate and develop novel PD management strategies.

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FUNDING AGENCIES

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

By studying non-transgenic scions grafted on transgenic rootstocks in the course of this project, we expect to learn whether the transgenic proteins can move from the rootstock to the scion, whether molecule size affects transport, and whether substance concentration in the rootstock affects levels found in the scion.

OBJECTIVE

Determine the relationship between protein molecule size and concentration in grapevine roots and its ability to move from a grapevine rootstock to a scion across a graft union.

RESULTS

This project is just getting underway, thus, rather than present non-existent research results, an outline of our research plan is presented here.

The following transgenic grapevines are available for use:

1. Two lines of Chancellor transformed with an NPT-II/GUS gene fusion producing a fused protein product. One line strongly expresses the *gus* reporter gene (*uidA*) in all tissues, while the other line shows no GUS expression, even though the gene is present.
2. Multiple lines of Chardonnay and Merlot producing both NPT-II and endochitinase.
3. A series of lines of Chardonnay producing NPT-II along with one of three antimicrobial peptides (AMPs).

All of these lines produce transgenic products under control of constitutive promoters. In cases 1 and 2 above, the CaMV 35S promoter was employed, whereas in case 3, NPT-II was downstream of an *Arabidopsis* ubiquitin promoter. The CaMV 35S promoter was used by Meredith and Dandekar (2003), who showed that PGIP protein from rootstocks could be detected in xylem sap. The NPT-II/GUS gene fusion product in Chancellor was shown to express in root tissues (Striem et al. 2000), but will require re-testing to make sure that protein production has not been lost since these tests were run. We will need to test the other lines (2 and 3 above) to determine the transgenic protein concentration in their roots. The size of the transgenic product molecules varies: NPT-II is ~280 amino acids (aa) (29 kDa); endochitinase is 424 aa (42 kDa); the NPT-II/GUS bifunctional fusion protein has 885 aa (97 kDa).

We will examine root tissues from separate lines of each of the three types of transformed vines listed to determine gene transcription and transgenic protein concentration via established procedures. To test for gene transcription we will use semi-quantitative RT-PCR (Vidal et al. 2003). Transgenic protein concentrations will be determined using standard methods already in use in our lab. We will identify lines with high and low concentrations of transgenic proteins for further use in this project.

The transgenic lines with high and low concentrations of transgenic proteins, along with negative controls, will be bench grafted as rootstocks to non-transgenic Chardonnay scions. The grafted vines will be grown in a greenhouse. Once the grafted vines have been established and their shoots have grown to 50 cm, the non-transgenic Chardonnay scions will be examined for presence of transgenic proteins. Leaf tissue as well as xylem sap will be tested. Samples will be collected under sunny, warm conditions conducive to transpirational pull through the xylem.

Outline of rootstock/scion combination planned:

13 rootstock/scion combinations planned, including control

10 vines of each combination x 13 combinations = 130 vines total planned

Control rootstock: Non-transgenic Chardonnay (to be grafted to non-transgenic Chardonnay)

Experimental rootstocks:

(Each rootstock will be grafted to non-transgenic Chardonnay scions.)

Chancellor, high NPT-II/GUS fused protein product concentration in roots (35S promoter)

Chancellor, transformed vine with no GUS expression in roots (35S promoter)

Chardonnay, high NPT-II concentration in roots (Nos promoter)

Chardonnay, low NPT-II concentration in roots (Nos promoter)

Chardonnay, high NPT-II (*Arabidopsis* ubiquitin promoter)

Chardonnay, low NPT-II (*Arabidopsis* ubiquitin promoter)

Chardonnay, high endochitinase concentration in roots (35S promoter)

Chardonnay, low endochitinase concentration in roots (35S promoter)

Merlot, high NPT-II concentration in roots (Nos promoter)

Merlot, low NPT-II concentration in roots (Nos promoter)

IMPROVING OUR UNDERSTANDING OF SUBSTANCE TRANSPORT ACROSS GRAFT UNIONS

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ABSTRACT

Researchers seeking to genetically-engineer grapevine rootstocks in order to affect Pierce's disease (PD) resistance in scion cultivars know very little about the transport of substances produced by foreign genes across the graft union. Our project seeks to understand how protein size and concentration may affect protein transport from a rootstock to a scion. We possess genetically engineered lines of Chardonnay, Merlot and Chancellor that produced proteins ranging in size from 29 to 97 kDa. These proteins can be readily detected by established techniques. Lines will be identified with low and high protein production potential in their root tissues, and graft combinations will be created with non-transgenic Chardonnay scions. Xylem sap will be collected from the scion and tested for the presence of the transgenic proteins. Given that *Xylella fastidiosa* causing plugging of xylem tissues, the results of xylem sap testing will be directly applicable to efforts to develop PD resistance inducing rootstocks.

INTRODUCTION

One approach being utilized to develop a long-term solution to Pierce's disease is the development of transgenic PD resistant versions of important wine and table grape varieties. The development of each transgenic cultivar will require a concentrated effort and significant amounts of technical expertise, testing, and funding. To bring each successful product to market, and to pass regulatory agency approval for transgenic crops, also will require a great deal of time and funding. This would be required for each of dozens of scion varieties.

A rootstock-based approach provides a potentially excellent alternative. In theory, a transgenic rootstock would confer PD resistance to its non-transgenic scion. Advantages include: 1) many fewer rootstocks will need to be transformed as compared to the dozens of table grape and wine grape varieties that would need to be altered, 2) consumers might be more accepting of wines produced from non-transgenic scions even if they are grafted on transgenic stocks; and 3) in general, it has been technically easier to transform rootstocks than scion varieties. Before this approach is successful, however, our understanding of the biology of the graft union and the types of substances that can be successfully transported from rootstocks to scions must be improved.

Water, mineral nutrients, hormones, carbohydrates, and other compounds are all known to move, via both xylem and phloem, from rootstocks across graft unions into scions of woody plants. To date, however, there is little evidence available to show whether a transgenic protein can move from the rootstock into the scion in a grafted woody plant. In recent work with grapevines, Meredith and Dandekar (2003) showed that pear polygalacturonase inhibiting protein (PGIP), with a size of 36.5 kDa, could be detected in xylem sap of non-transgenic scions grafted on transgenic stocks engineered to produce this protein. Of great relevance to this proposal, we noted that protein movement into the xylem occurred even without a specific signal targeting it to the extracellular spaces or to the xylem. Imidacloprid (a small compound with molecular weight of approximately 0.25 kDa) and other systemic insecticides applied to the soil are taken up by the roots of grapevines and move from root systems into the scion (Toscano et al. 2003). The present project will investigate aspects of plant physiology critical to determining the potential for deploying transgenic rootstocks for PD management.

It is possible that the size of a transgenic protein produced in a rootstock influences its transport to the scion. For example, large proteins might be less likely to be transported than small proteins. Understanding the relationship between size and movement will allow us to more efficiently test anti-PD compounds. If transgenic proteins are transported across the graft union, their concentration in the roots might be higher than their concentration in the scion. Since there is likely to be a threshold concentration for PD control provided by a given compound, it will be critical to understand the relationship between concentration in the rootstock and concentration in the scion.

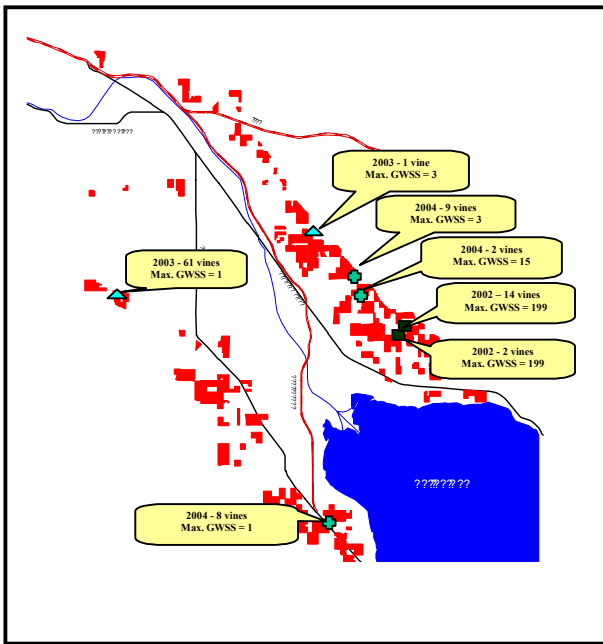


Figure 4. Sites with PD and maximum GWSS numbers in the Coachella Valley from 2001-2004.

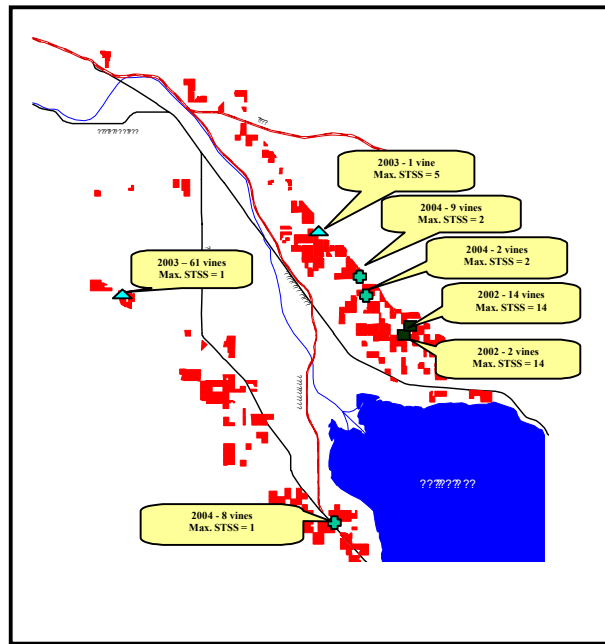


Figure 5. Vineyards (red) in the Coachella Valley, and sites where PD was confirmed in 2002, 2003, and 2004.

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FUNDING AGENCIES

Funding for this project was provided by the California Desert Grape Administrative Committee, the University of California Pierce's Disease Grant Program, and the California Department of Food and Agriculture.

GWSS abundance was altered by the CDFSA-sponsored vector control program being implemented through the Riverside County Agricultural Commissioner's Office. Treatments from this program were initiated in the winter of 2003, and since then, very few GWSS adults have been caught on our traps (Figure 3).

STSS Seasonal Abundance

Generally, trap counts of STSS peaked at about 1/3 the densities of GWSS in 2001 and 2002 (Figure 3). However, in 2003, average densities equaled GWSS, and at certain sites, there were far more STSS than GWSS (Figure 2B). Since STSS have non-citrus hosts throughout the Valley, they have not been affected by the treatments in citrus. It is unclear at this time what role this species may play in the epidemiology of PD in the Coachella Valley, but we will be investigating this as we continue data analysis

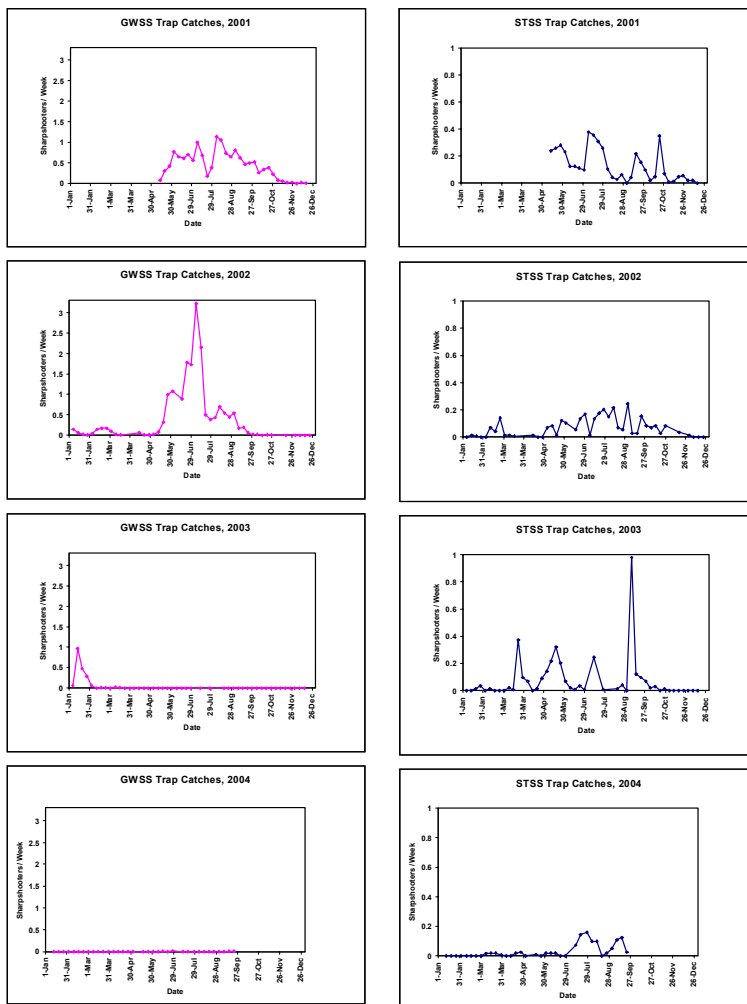
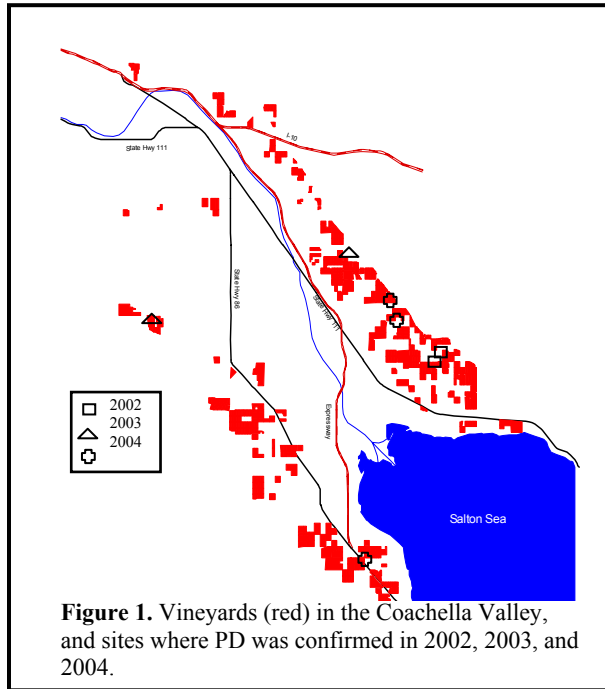


Figure 3. Average number of GWSS (pink) and STSS (blue) trapped per week from 2001 – 2004 in the Coachella Valley displayed for each year.

Describe the epidemiology of PD in the Coachella Valley

Since we have so few sites infected with PD, and the number of infected vines at each site is low, it is difficult to draw conclusions about the epidemiology of PD in this area. However, we calculated the maximum numbers of GWSS and STSS adults caught on yellow traps within one mile of the 7 fields in which we have found PD, to determine if any relationships were apparent. From this exercise, we present several preliminary observations. First, we observe the highest incidence of PD was not in an area where we caught large numbers of GWSS (Figure 4) or STSS (Figure 5). In fact, the heaviest PD vineyard, found in the northwest part of the Valley, has had maximum numbers of GWSS and STSS of 1 per week since we started trapping in 2001. In this field, we suspect other sharpshooter species are involved with PD spread, or our trapping program is too coarse to detect GWSS and STSS. Second, the two vineyards in which we identified PD in 2002 were in areas that were heavily infested with GWSS (Figure 4). If the trend of increasing GWSS from 2001 to 2002 (see Figure 3) had been allowed to continue in 2003 (in the absence of the CDFSA spray program) one might have predicted spread of PD from these fields to neighboring vineyards. Because this did not materialize, the evidence suggests that the areawide program effectively impeded PD spread in this area of the Coachella Valley. Finally, while the number of fields in which we have found PD remains low, relative to other areas of the state, each year we have found additional vines with PD. Having learned from the epidemic that occurred in Temecula, we suggest continuing the sharpshooter and PD monitoring efforts to insure that this scenario is not repeated in the Coachella Valley.

1 infected vine in an adjacent field. Both fields were in the southeast corner of the Valley (Figure 1). The PD-strain of *X. fastidiosa* was confirmed in these plants with selective-media plating and PCR. These were the first post-GWSS PD finds in the Valley.



Intensive sampling in these 2 fields over the past 2 years has found 13 additional vines infected with *X. fastidiosa*. In 2003, we visually inspected an estimated 616,400 vines and samples from 478 vines with suspected PD were subjected to ELISA. Five of these 478 vines were positive for PD. Four of these vines were at one field site and the 5th vine was at another site. Interestingly, neither vineyard was near the infected vineyards identified in 2002, and the fields were not near each other (Figure 1). One of the vineyards was in a fairly isolated location on the west side of the Valley. Further searches of the two infested vineyards found no additional PD infection at one of the sites, however work at the site on the west side of the valley has identified a total of 61 infected vines. We are in the process of characterizing this field to determine the spatial pattern of infection. In the 2004 survey, we observed an estimated 571,861 vines and collected 187 samples to assay for PD. From these assays we identified 5 infected vines, adding 3 vineyards to our list. These vineyards were located in the east-central part of the valley with an additional find in the far southwest corner of the Valley (Figure 1). Further research has identified a total of 19 infected vines from these three vineyards. We are in the process of determining the distribution of PD-infected vines in these vineyards.

Spatial and temporal abundance of sharpshooters

Yellow sticky cards have been used to trap GWSS and STSS adults from May 2001 to the present. These 156 traps are distributed uniformly at one-mile intervals throughout the Coachella Valley. Traps are checked weekly and the total numbers of sharpshooters are recorded.

We discuss the trap data in two distinct time periods. The first, from May 2001 through January 2003, preceded the CDFA treatment program in citrus while the second period from February 2003 to the present has been during the implementation of this areawide program. During the early part of this period, GWSS vastly outnumbered STSS (Figure 2A). While average densities did not exceed 3 GWSS per week, some sites had very high GWSS catches; up to 160 insects per week were trapped (Figure 2B). During the second period of trapping, STSS numbers remained consistent with previous years, and even increased in 2003 (Figure 2B). A few sites reached high densities of STSS, nearly as abundant as the GWSS peaks in 2002. Presently, STSS outnumber GWSS in the Valley. The reason for these seasonal dynamics is that the CDFA treatment program specifically targets citrus, a preferred host of GWSS during certain times of the year. STSS, on the other hand, utilizes a number of desert scrubs and riparian plants, thus its densities have been largely unaffected by the treatment program. STSS is a known vector of PD, but it is not clear how important it is in the epidemiology of the disease.

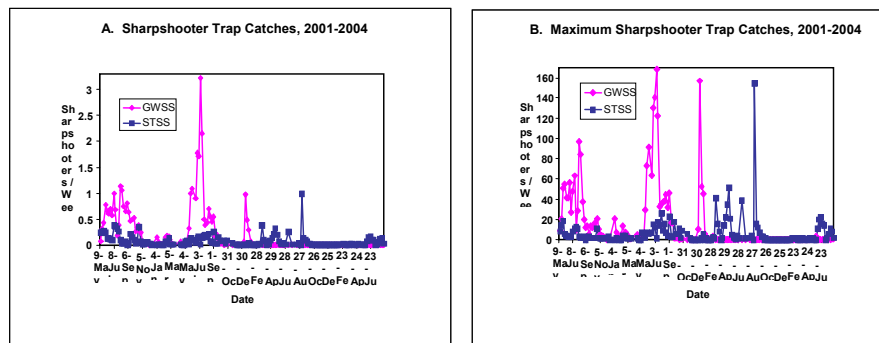


Figure 2. (A) Average number of GWSS (pink) and STSS (blue) trapped per week from 2001 - 2004 in the Coachella Valley. (B) Maximum number of GWSS (pink) and STSS (blue) trapped per week.

GWSS Seasonal Abundance

From 2001-2003, two peaks of adult activity were identified; a broad-peak centered around a maximum abundance in July and a second smaller period of activity in January and February (Figure 3). Summer densities in 2002 were higher than the same time in 2001 and winter counts in 2003 were higher than winter densities in 2002. This apparent general increase in

AREA-WIDE EPIDEMIOLOGY OF PIERCE'S DISEASE IN THE COACHELLA VALLEY

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REPORTING PERIOD: The results reported here are from work conducted from May 1, 2001 to September 30, 2004.

ABSTRACT

This is a continuation of the epidemiology project that was initiated in 2001 in the Coachella Valley. Surveys in 2001 did not detect any Pierce's disease (PD). In 2002, we identified 2 infected vines in one vineyard and 1 infected vine in an adjacent vineyard. These were the first finds of PD in the area since 1983. Intensive surveys in these vineyards over the past 3 years have revealed a total of 16 infected vines. In June 2003, we found PD-infected vines in 2 additional vineyards. Further work in these vineyards has identified a total of 62 vines infected with PD. This past summer (2004), we again surveyed all vineyards in the Valley, finding PD-infected vines at 3 additional sites. Additional searches have identified a total of 19 infected vines in these three vineyards. With the finds this past summer, we now have identified 97 PD-infected vines from 7 vineyards. Except for the two infected vineyards identified in 2002, sharpshooter densities have been low near the sites that have PD.

Since the inception of this project in May 2001, we have used yellow sticky traps to monitor the spatial and temporal abundance of adult glassy-winged sharpshooters (GWSS), *Homoladisca coagulata* (Say) and native smoke tree sharpshooters (STSS), *Homoladisca liturata* Ball in the Valley. In 2001-2003, two peaks were identified in abundance; a broad-peak around a maximum abundance in July and a second smaller peak in winter. Summer densities in 2002 were higher than the same time in 2001 and winter counts in 2003 were higher than winter densities in 2002. This apparent increase in GWSS abundance was altered by the CDFA-sponsored vector control program being implemented through the Riverside County Agricultural Commissioner's Office. This program was initiated in the winter of 2003, and since then, very few GWSS adults have been caught on our traps. Relative densities of the STSS have remained constant throughout the 4-year study period.

INTRODUCTION

The Coachella Valley is home to 11,345 acres of table grapes; in 2003 harvested grapes from this region were valued at \$115,939,900 (Riverside County Agricultural Commissioner, 2003). Pierce's disease first was identified in the Valley in 1983 (Goheen 1984), and from that time until recently, it has not been a concern to growers. When the GWSS was identified from the Valley in the early 1990s (Blua et al. 1999), growers became concerned, since this insect had been shown to be instrumental in the devastating spread of PD in the Temecula Valley in the late 1990s. At the request of the table grape growers, we initiated a study in 2001 to determine the spatial and temporal distribution of GWSS, and to identify the distribution of PD in the Valley. From that point in time to the present, we have continued our monitoring efforts, with the intention of describing the epidemiology of GWSS-transmitted PD in this area.

OBJECTIVES

The goal of our studies in the Coachella Valley is to describe the epidemiology of PD in the presence of GWSS, and to use this information to design management strategies to reduce disease spread.

Three objectives are pertinent to this report:

1. Determine the incidence and distribution of PD in the Coachella Valley.
2. Determine the spatial and temporal abundance of sharpshooters in the Coachella Valley.
3. Describe the epidemiology of PD in the Coachella Valley.

RESULTS AND CONCLUSIONS

Determine the incidence and distribution of PD in the Coachella Valley

For the past 4 years, we have searched for PD in the Coachella Valley. In 2001, we visually inspected 300 plants in each of 25 vineyards and all vines in a 60-acre vineyard proximal to an area that had PD in 1983. We collected 233 symptomatic samples and analyzed them with ELISA. None of these plants were positive for *Xylella fastidiosa*, the causal agent of PD. In 2002, we visually sampled 300 plants in each of 25 vineyards, and visually inspected 35,000 vines distributed throughout the Valley. We analyzed (by ELISA) 268 plants from these surveys, and found 2 infected vines in one field and 1 infected vine in an adjacent field. We analyzed (by ELISA) 268 plants from these surveys, and found 2 infected vines in one field and

information related to two of the four epidemiological components (i.e., GWSS abundance and the agricultural environment). Data from the other two components (i.e., PD and grapes) also have been collected, largely by Barry Hill and Jennifer Hashim (Hill and Hashim 2002, Hashim and Hill 2003). These scientists have directed crews to survey hundreds of vineyards in Kern and Tulare counties over the past four years. Much of the data has been entered and managed in a GIS format at UC Berkeley under the direction of Maggi Kelly. We have begun the process of bringing the PD data together with the GWSS data and crop layers. Once the map databases are constructed and standardized, we will pursue the analyses phases of this project.

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FUNDING AGENCIES

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

EPIDEMIOLOGICAL ANALYSES OF GLASSY-WINGED SHARPSHOOTER AND PIERCE'S DISEASE DATA

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ASBTRACT

The progression of PD in vineyards and across a landscape is dependent upon factors related specifically to four components: GWSS, *Xylella fastidiosa* causing PD, grapes, and the environment. When conditions in all four of these areas are optimal, disease spreads with devastating consequence as in Temecula in the late 1990s. Conversely, sub-optimization within any of the four categories can slow or stop disease progress. The aggressive insecticide campaigns against GWSS are prime examples of creating this sub-optimal condition for disease spread. This single approach has been effective, but it may not be sustainable in reduced budget times. The science of epidemiology seeks to determine how the 4 components listed above interact, with the goal of creating long-term, sub-optimal conditions for disease spread. Achieving this goal will enable California producers to continue growing grapes in areas known to have PD and GWSS.

INTRODUCTION

Earlier studies pointed out the importance of the distribution of disease (Weltzien 1972, 1978) and insects (Southwood 1978), but mapping the distribution of disease and insect populations has not been applied to entomological and epidemiological studies until recently. This is mainly because there was a lack of suitable technologies or methods to map the distribution of insects and diseases in the field. Recently, the global positioning system (GPS), the geographic information system (GIS), and geostatistics have been applied to entomological and epidemiological. These technologies combined with advanced statistical methods can facilitate the making of distribution maps and the analyzing and modeling of the spatial phenomena represented on the maps.

OBJECTIVES

The overall goal of this research is to analyze the GWSS and PD data to investigate the relationship between GWSS and PD. The objectives of this research include,

1. Determine the spatial patterns and structures of GWSS and PD distributions, and use these analyses to create statistical distribution maps.
2. Analyze map correlations between GWSS abundance and incidence of PD.
3. Relate the epidemiology of GWSS-transmitted PD to environmental components, and identify characteristics of areas with rapid and slow PD infection rates.

RESULTS AND CONCLUSIONS

This project has just begun, so our report is preliminary at the present time. Prior to analyses, the GWSS and PD data need to be centralized into a geo-referenced database. Fortunately, there has been a tremendous and successful effort to maintain a weekly trapping effort for GWSS in areas of Kern, Tulare, and Ventura Counties. The data have been managed in a geographic information system (GIS) maintained by Rosie Yacoub of CDFA in Sacramento. We are working closely with Rosie to obtain trapping data from Kern County. Secondly, for certain areas there are crop layers that have been entered into the GIS, and we will work closely with the Kern County GIS group to obtain these layers. Within these two data sets we find

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petiole, a true abscission zone formed. At the distal end of the petiole where the lamina is attached, the tissue constricted and concurrently became necrotic. Observations at the cellular level suggest that the constriction and necrosis at this junction is not an actual abscission zone (Stevenson *et al.* 2004). Neither the abscission zone at the stem/petiole junction nor the fracture zone at the petiole/lamina junction developed until the lamina was severely chlorotic. In +*Xf* vines, a fracture zone also occurred at the petiole/lamina junction. Comparisons of the anatomy of the fracture zone at the petiole/lamina junction of +*Xf* vines and -*Xf* water-stressed vines showed that these fracture zones were identical. However, abscission zones did not develop at the stem/petiole junction of either well-watered or water-stressed +*Xf* plants.

The canes of both +*Xf* and -*Xf* water-stressed plants matured faster, becoming stiffer and more woody than those of the well-watered plants, based on the extent of periderm development up the canes. Stems of water-stressed +*Xf* plants became woody before the well-watered plants. Interestingly, in +*Xf* plants only the well-watered vines developed green islands, having an average of 2.1 ± 0.31 green islands per plant.

Vessel Blockage in Relation to Leaf Scorch Symptoms

Leaf scorch symptoms, in particular, have been considered a direct result of water deficits within the leaf, specifically due to clogged vessels limiting water transport. If leaf scorch is simply a matter of reduced water availability to the leaf margins, then we should be able to induce leaf scorch symptoms by selectively severing veins to simulate xylem vessel blockage. To this end, experiments were conducted in which all veins but one were severed such that a single secondary leaf vein connected the two halves of a lamina and was the sole water source for the nearly-severed portion of the leaf. Nearly-severed leaf halves of vines experiencing low transpirational demand in the laboratory appeared turgid and showed no signs of necrosis for up to 36 days. In the greenhouse, under medium to high transpirational conditions, sections of leaves which received water via a single vein remained green and turgid (Fig. 3) for at least 30 days after the veins were severed. This was true for leaves of +*Xf* and -*Xf* grapevines alike. Significantly, leaf scorch symptoms of PD did not develop on any of the -*Xf* nearly-severed leaves. Even when these leaf sections did eventually dehydrate after approximately two months, the symptoms were similar to water deficit, not PD.



Figure 3. Turgid leaf of non-infected Chardonnay under moderate to high transpiration 13 days after all but one vein was severed, resulting in a single leaf vein connecting and supplying water to half of the leaf. Black arrow shows secondary vein supplying water to the nearly-severed leaf half.

CONCLUSIONS

In summary, water deficit clearly had an exacerbating effect on the symptom development of PD. Water-stressed +*Xf* vines displayed more extensive PD symptoms throughout the plant than did well-watered vines. Matchstick and leaf scorch symptoms moved up the canes more rapidly than in well-watered vines implying that the bacteria spread more rapidly throughout the plant under water deficit conditions, assuming bacterial proximity is necessary for symptom development. Importantly, with the exception of green islands, extended water deficit did not affect the nature of the PD symptoms. Indeed, in water-stressed +*Xf* plants, PD masked all of the symptoms of water deficit, except green islands, which occurred only in well-watered +*Xf* vines.

Detailed comparisons of the visual symptoms of PD and water deficit revealed that conclusions reached from earlier work, stating that water deficit causes PD symptoms, were not completely correct. The visual characteristics of +*Xf* vines were unique to PD and distinctly different from -*Xf* vines experiencing extended water deficit. The fracture zone at the petiole/lamina junction, common to all treatments, appears to be a plant response to stress and not specifically induced by bacterial infection. In contrast, the lack of an abscission zone in +*Xf* plants implies that the bacteria were in some way suppressing development of an abscission zone. Conversely, water deficit overcame the influence of *Xf* to prevent the occurrence of green islands, possibly by hastening periderm development. Considering that only well-watered +*Xf* vines developed green islands, water deficit could have masked the green island symptom of PD by inducing the periderm of +*Xf* water-stressed canes to develop faster than could the conditions necessary to impair periderm activity leading to green islands. This suggests that the bacteria are in some way inhibiting periderm activity at seemingly random locations.

Finally, based on the dramatic and sudden increase in the number of nonfunctional vessels which was caused by severing leaf veins, it seems clear that xylem vessel blockage, whether due to gums, tyloses or bacterial accumulation, is not responsible for leaf scorch symptoms and that *Xf* bacteria are able to affect plant responses in ways not involving altered vine water status. While occluded xylem vessels may worsen leaf scorch symptoms, several other factors, or combination of factors, may contribute. Ultimately, however, comparison of the leaf scorch symptoms of PD and the chlorosis of extended water-stressed leaves shows that *Xf* bacteria are able to produce, alter or eliminate signals that result in leaf scorch symptoms and that these signals can, to some degree, override signals controlling plant responses to water deficit. (A manuscript containing the completed study will be submitted to a peer-reviewed journal shortly.)

ultimate killer of plants suffering from PD may result in misleading information and add years to finding solutions to the PD problem. Therefore, it is important that it be determined which PD symptoms, if any, are a result of water stress, and what role water shortage actually plays in symptom development and vine death.

OBJECTIVES

1. Evaluate the impact of vine water status on the development of the visual symptoms of PD.
2. Determine whether visual PD symptoms are a direct result of water deficits.

RESULTS

Objective 1

In the field, extended water deficit exacerbates citrus variegated chlorosis in citrus (Gomes *et al.* 2003) and PD in Virginia creeper (McElrone *et al.* 2001, 2003). Thus, it was not surprising that subjecting potted grapevines to extended water deficit also resulted in a faster and more extensive onset of PD symptoms (barring green islands) than in well-watered *Xf*-infected (+*Xf*) vines. The first clear indications of leaf scorch were seen 48 DAI. Water-stressed +*Xf* vines developed more symptomatic leaves with severe symptoms than well-watered +*Xf* vines (Fig. 1). Interestingly, the leaf scorch and matchstick symptoms in the well-watered +*Xf* plants had the same visual characteristics as in the +*Xf* water-stressed plants. There was no significant difference between well-watered +*Xf* and healthy (-*Xf*) vines in stomatal conductance (0.86 ± 0.09 & 0.69 ± 0.06 cm s^{-1}), transpiration (6.53 ± 0.83 & 5.66 ± 0.83 $\mu\text{g cm}^{-2} \text{s}^{-1}$), and leaf water potentials (-0.60 ± 0.05 & -0.73 ± 0.11 MPa, respectively). Likewise, these parameters were equivalent for water-deficit +*Xf* and -*Xf* vines (0.28 ± 0.04 & 0.34 ± 0.05 cm s^{-1} , 2.41 ± 0.31 & $2.86 \pm .39$ $\mu\text{g cm}^{-2} \text{s}^{-1}$, -1.07 ± 0.05 & -1.28 ± 0.13 MPa, respectively).

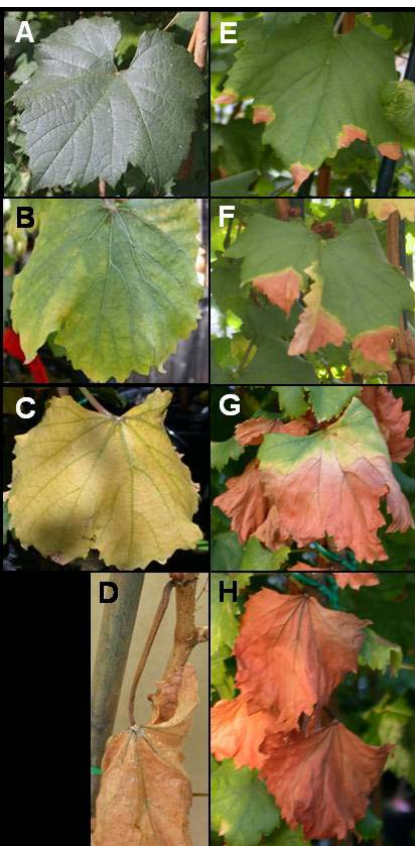
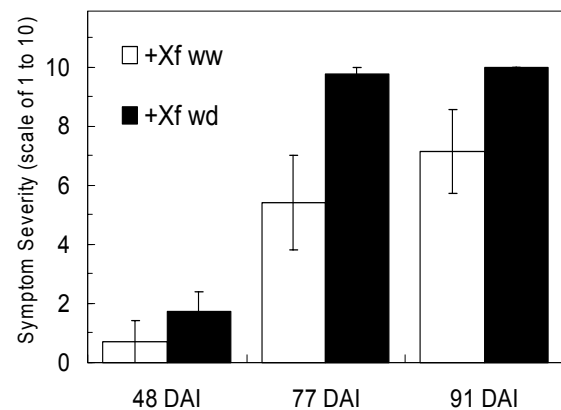


Figure 2. Progressive development of leaf symptoms of non-infected water-stressed Chardonnay leaves (A-D), and Chardonnay with Pierce's disease (E-H).

In -*Xf* water-stressed plants, two sites of constriction and necrosis developed on petioles, one at the stem/petiole junction (the basal end of the petiole) and the other at the petiole/lamina junction (the distal end of the petiole). At the basal end of the

Figure 1. Symptom development during whole plant water deficit and well-watered conditions. Severity of Pierce's disease symptoms in Chardonnay grapevines. Vines infected with *X. fastidiosa* were well-watered (+*Xf* ww; white bars) or subjected to water deficit (+*Xf* wd, black bars). On a scale from 1 to 10, 1 indicates mild symptoms, 10, the most severe PD symptoms. Non-infected values are zero due to the lack of PD symptoms and are not shown.



Objective 2

The results revealed that visual symptoms of Pierce's disease in grapevine are qualitatively and quantitatively different than those of extended water deficit. Regardless of water status, +*Xf* plants displayed symptoms unique to PD. In general, PD symptoms masked water-deficit symptoms. The PD symptoms manifested in laminae, petioles and stems often revealed an interaction between plant and bacteria in which plant responses to *Xf*-infection seemed to be either elicited or suppressed by the bacteria.

Comparison of Visual Symptoms of Water Deficit and PD

To determine whether PD symptoms are a direct result of water deficit, the visual characteristics of well-watered and water-stressed grapevines inoculated with *Xylella* (+*Xf*) or water (-*Xf*) were evaluated. Leaves of well-watered -*Xf* grapevines remained green and healthy throughout the course of the experiments (Fig. 2a). Water-stressed -*Xf* vines gradually developed leaf chlorosis in a fairly uniform pattern over the entire leaf lamina (Fig. 2b-c), with the veins staying green until leaves became necrotic. Leaves remained attached to the stem even after the leaves were apparently dead (Fig. 2d). In contrast, the first PD symptom to appear was leaf scorch. Leaf scorch symptoms started with chlorosis at the margins of the leaves and moved towards the petiole in patches such that sections of necrosis were bordered by slim regions of chlorosis (Fig. 2e-f). As symptoms progressed, laminae of +*Xf* vines became completely necrotic, while the petioles remained green (Fig. 2g-h). Eventually laminae fell from the petioles to form "matchsticks."

**MECHANISMS OF PIERCE'S DISEASE TRANSMISSION IN GRAPEVINES:
THE XYLEM PATHWAYS AND MOVEMENT OF *XYLELLA FASTIDIOSA*.
PROGRESS REPORT NUMBER ONE: COMPARISON WITH SYMPTOMS OF WATER DEFICIT
AND THE IMPACT OF WATER STRESS**

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ABSTRACT

The pathology of diseases such as Pierce's disease (PD) of grapevine (*Vitis vinifera* L.) that are caused by the xylem-limited bacteria *Xylella fastidiosa* (*Xf*) is widely attributed to vessel occlusion and subsequent water deficits. Grapevines (*Vitis vinifera* L. 'Chardonnay') were exposed to water deficits, stem inoculation with *Xf*, and combinations of both to evaluate whether symptoms of PD were a consequence of water deficits. When vines were inoculated with *Xf* and exposed to water deficits, more extensive PD symptoms developed throughout the plant than when + *Xf* vines were well-watered. However, vines infected with *Xf* exhibited symptoms unique to PD that included inhibited periderm development in stems (green islands), leaf blade separation from the petiole (matchsticks), and irregular leaf scorch. Vines exposed to water deficits and not *Xf*, displayed accelerated periderm development, basal leaf abscission at the stem/petiole junction, and uniform leaf chlorosis. Water deficits induced the development of an abscission zone, but PD did not. Pierce's disease symptoms could not be produced with any of several water deficit treatments, including severing all but one secondary vein near the leaf tip. The results indicate that factors other than water deficits are involved producing the symptoms of PD. We conclude that the widely accepted hypothesis that PD-infected plants develop water deficits that cause green islands, matchsticks, localized leaf scorch, and eventual death of vines should be reevaluated.

INTRODUCTION

The overwhelming consensus among researchers is that the fatal nature of PD is a result of the *Xf* bacteria becoming systemic and blockage occurring in xylem vessels (due to bacterial accumulation, tyloses, gums, and/or emboli), causing water transport to become progressively impaired until the plant is no longer able to function (Goodwin *et al.* 1988a, b; McElrone *et al.* 2001, 2003; Newman *et al.* 2003, 2004; California Agricultural Research Priorities 2004). Indeed, Pierce's disease has become nearly synonymous with plant water deficit. This view is largely based on correlative evidence. Hopkins (1988) showed a strong association between reduced water conductance in stems of citrus seedlings and *Xf*-caused disease symptoms. Low leaf water potential and turgor, impaired hydraulic conductance, and higher stomatal resistance were correlated with PD symptoms in grapevines (Goodwin *et al.* 1988a). While reduced leaf water potential, stomatal conductance and stem hydraulic conductivity are characteristic of water deficit, it should be noted that these same features also occur in flooded plants (Kramer & Boyer 1995), so correlations are not necessarily indicative of causality.

From our recent work we observed that, although PD symptoms have been attributed to water deficit, the visual symptoms of PD did not appear to be the same as those resulting from water deficit alone. In grapevine, typical visual symptoms of PD are "green islands," patchy or marginal leaf necrosis (often called leaf scorch), and "matchsticks" (petioles that remain attached to the stem after the laminae have fallen off) (Purcell 1986; Goheen & Hopkins 1988, 1989; Stevenson *et al.* 2004). These symptoms are not characteristic of water deficit symptoms in grapevines (Okamoto *et al.* 2004). In addition, the diagnostic symptoms of PD have never been observed in healthy grapevines exposed to water deficits, nor have they ever been reported to develop as a consequence of water deficits.

Interestingly, citrus trees already infected with *Xf* and subjected to drought displayed accelerated symptom development of citrus variegated chlorosis (Gomes *et al.* 2003). Extended water deficit also increased the severity of Pierce's disease in the woody liana, Virginia creeper (McElrone *et al.* 2001, 2003). Thus, extended water deficit (such as drought) may exacerbate the development of PD symptoms in grapevine as well. However, there are no reports describing the effects of water deficit on *Xf*-infected grapevines, nor has there been a detailed comparison of water deficit and PD symptoms. If the visual symptoms of PD are not, in fact, a result of water deficit, then studies relying on the assumption that water stress is the

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Table 1. PD symptom scores of the ten grape rootstocks during the second, third, and fourth growing seasons.

Rootstock	PD score		
	2002	2003	2004
O39-16	3.1bc	3.8b	2.3
101-14	2.2d	2.4c	1.9
110R	2.2d	1.8cd	2.3
3309C	3.6b	4.2ab	3.0
44-53	2.6cd	2.3c	---
5BB	2.7cd	1.6cd	2.9
5C	2.2d	1.9cd	2.1
Freedom	3.7b	5.0a	---
Ramsey	1.1e	1.0d	1.2
St. George	1.4e	1.7cd	0.9

Table 2. Vine survival of the ten grape rootstocks after four growing seasons.

Rootstock	Number of living vines				Survival %
	2001	2002	2003	2004	2004
O39-16	9	9	6	6	67
101-14	10	10	10	9	90
110R	10	10	9	8	80
3309C	10	10	5	1	10
44-53	10	10	3	0	0
5BB	10	10	10	7	70
5C	10	10	9	9	90
Freedom	10	8	1	0	0
Ramsey	8	8	8	8	100
St. George	10	9	9	7	70

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All rootstock vines developed PD symptoms, although the severity varied. The least severe PD scores were seen on Ramsey and St George, with average PD scores of 1.1 and 1.4 in 2002, 1.0 and 1.7 in 2003, and 1.2 and 0.9 in 2004, respectively (Table 1). The consistently low PD scores on these varieties over several years demonstrate that Ramsey and St. George are reliably resistant or tolerant of PD in north Florida.

Freedom (3.7 – 5.0 score in 2002-2003) and 44-53 (2.6 – 2.3 score in 2002-2003) did not survive through the rating period of 2004. That Freedom succumbed to PD is not surprising—this rootstock showed the worst PD symptoms of all the rootstocks in the trial in the previous two years of observations. The 44-53 showed severe PD symptoms in 2002 and 2003, but typically its symptoms were not as severe as those on O39-16 and 3309C, so it was surprising that this rootstock succumbed while O39-16 and 3309C remain in the trial.

Of the surviving rootstocks, 3309C (3.0) and 5BB (2.9) had the most severe PD symptoms in 2004. The 3309C has consistently shown heavy PD symptoms and most of the vines of this rootstock have died (Table 3). The slightly less severe average PD score for 3309C probably reflects the survivorship of this vine (heavier symptoms being related to lower survivorship). Although 5BB showed excellent survivorship in earlier years of the study, it is now beginning to develop PD symptoms. The 5C, 110R, and 101-14 showed moderate PD symptoms over the three year period (Table 1). O39-16 symptoms in 2004 were less severe than in earlier years, when it was among the most symptomatic rootstocks; however, symptom severity overall was lower in 2004.

After four growing seasons in Florida's heavy PD pressure, environment, the survival rate was very different among the rootstocks (Table 2). Only Ramsey shows 100% survival. All Freedom and 44-53 vines have been killed by PD and only one of ten 3309C vines remains alive. Vines greatly deteriorated in the third growing season; from 2002 to 2003, the vine losses of Freedom, 44-53 and 3309C were 87%, 70%, and 50%, respectively. There was less change overall in vine survival from 2003 to 2004. Although Freedom and 44-53 completed their precipitous decline, other varieties may be reaching a "steady state" of vine survival, with diminishing losses to PD. The 110R, 5C, and 101-14, noted for their moderate PD symptoms, have survival rates of at least 80%.

Fishleder (2000) examined the response of grape rootstocks to PD in a greenhouse. In contrast to this study, Fishleder inoculated vines with *Xf*. The results from this study largely coincide with and confirm Fishleder's findings. In particular, both this research and Fishleder's work found St George to show only minor PD symptoms; O39-16, 5C, 5BB, 110R were intermediate in symptom development; and 3309C and Freedom showed severe PD symptoms. However, our results contradict Fishleder's regarding Ramsey. While we observed only low levels of PD symptoms in Ramsey, Fishleder found Ramsey to be one of the most symptomatic of rootstocks tested. What accounts for this disparity in observation? It is possible that the *Xf* strain that Fishleder cultured and used to inoculate the vines growing in the greenhouse was substantially different in pathogenicity or host specificity from the naturally occurring *Xf* prevalent at Tallahassee, Florida. Another possibility is that while the *Xf* populations in the respective studies do not differ in pathogenicity or host specificity, the direct inoculation through pin prick employed by Fishleder is more difficult for the plant to resist than the natural inoculation by insect vectors that is thought to have occurred in the vineyard.

Rootstock performance in north Florida primarily is a factor of PD response. Cultivars differed in their performance and some were markedly superior—these should be further investigated for their influence on scions. Specifically we suggest Ramsey and St George for additional study. These rootstocks survive well under natural inoculation conditions in north Florida. The evaluation of rootstock cultivars in PD limited viticultural regions is important—much PD management research is focusing on augmenting PD resistance and or tolerance in scions, but rootstocks are a critical component of viticulture. As demonstrated here, several rootstocks have substantial levels of PD resistance that should permit their cultivation in PD prone regions, allowing concentration of effort on scion improvement. Additionally, testing the PD response of ungrafted rootstocks indicates the potential for rootstock varieties to be cultivated as nursery mother vines in PD prone regions. Rootstocks identified as resistant or tolerant to PD could be genetic resources for breeding improved PD resistant scion varieties, as in the case of MidSouth and MissBlue, which have PD resistant rootstocks as parents (DeGrasset and Dog Ridge, respectively). PD resistant rootstocks might be necessary for the cultivation of PD tolerant scion varieties if *Xf* spreads to the root system.

Field evaluation of PD resistance in Florida is easy due to high PD pressure resulting from high populations of vectors and bacteria in the area and should be continued as a technique to test PD management strategies and screen plant material.

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FIELD EVALUATION OF GRAPE ROOTSTOCK RESPONSE TO NATURAL INFECTION BY PIERCE'S DISEASE

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ABSTRACT

To understand the adaptation of grape rootstocks commonly used in major grape production areas worldwide to Florida, where Pierce's disease (PD) is the primary limiting factor in grape production, ten important grape rootstocks were cultivated at the experimental vineyard, Florida A&M University, Tallahassee, Florida. Disease resistance and symptoms and growing performance were evaluated. PD symptoms were scored in September and October 2002, 2003, and 2004, with leaf symptoms the basis of scoring. None of the grape rootstocks was completely resistant to PD and the severity of PD varied with rootstock cultivar. St George and Ramsey showed least PD symptoms. Freedom and 44-53 succumbed to PD by the 2004 rating period; of the surviving rootstocks, 3309C had the highest PD score. Overall vine survival, evaluated in 2002, 2003, and 2004, varied among the rootstocks. Based on the performance of ungrafted vines, St George and Ramsey are the most suitable rootstocks in this north Florida environment, where natural infection by PD is very high and vectors and inoculum are abundant.

INTRODUCTION

Rootstocks are used widely in viticulture to provide resistance against soil pests and pathogens and improve scion performance. Choice of rootstock depends on pest populations, soil, and growing conditions. The grape rootstocks in common use world wide are deployed primarily to provide phylloxera and nematode protection (Bouquet 1980, Einset and Pratt 1975, Winkler et al 1974). In contrast, Pierce's disease (PD), caused by gram-negative bacterium *Xylella fastidiosa* (*Xf*), is the primary limiting factor of growing *Euvitis* grape in the southeast United States (Lu and Ren 2002, Chen et al 2001). Pierce (1905) reported that rootstock variety affected expression of "California vine disease" (now known as Pierce's disease) in grape. Grape rootstock trials in Mississippi showed a large effect of rootstock trial on vine longevity in a region recognized for high Pierce's disease pressure (Loomis 1965, 1952, Magoon and Magness 1937). In humid and hot regions of the United States, such as Florida, bunch grapes often are highly susceptible to pests and diseases (Olien and Hegwood 1990). When the Florida hybrid bunch grape cultivar Blanc du Bois was grafted on to muscadine, which is relatively tolerant or resistant to the bunch grape pests and diseases common in North America, the scion showed a reduction in both PD and anthracnose symptoms and fruiting improved (Ren and Lu 2002). Growing conditions in Florida are harsh—a successful rootstock for grape industry in that area must be tolerant to PD and adapted to the environment. Evaluation of rootstock performance and survival in Florida would provide useful information on rootstocks performance for humid tropical and subtropical environments, especially where PD is prevalent. Greenhouse screening has been used to investigate the PD resistance, tolerance, and susceptibility of grape cultivars. However, field screening is more applicable, since conditions closely match those in a commercial vineyard. When relying on natural infection in the vineyard, there is no need to inoculate vines or maintain colonies of *Xf* or insect vectors. Field screening is cheap, requires no specialized equipment and can be accomplished quickly, with symptom expression being used as the main criterion. Northern Florida is an ideal test environment due to heavy PD pressure, with abundant vectors, including glassy-winged sharpshooter, and inoculum, in contrast to many other locations, especially California, which demonstrate substantial cycling of PD incidence.

OBJECTIVES

1. Evaluate the response of grape rootstocks to natural field infection by Pierce's disease.

RESULTS AND CONCLUSIONS

Ten grape rootstocks (five replicates of two vines each, ten vines total per rootstock cultivar) were planted in the spring of 2001. Vines were bilaterally cordon trained and spur pruned. Pierce's disease (PD) symptoms were scored in 2002, 2003 and 2004, with symptoms on leaves assessed in a numerical scale from 0 to 5. For PD, 0 represented no symptoms, 1 = minor symptoms up to 15% of leaves with marginal necrosis (MN), 2 = 15-30% of leaves with MN, 3 = 30-50% of leaves with MN, 4 = 50-75% of leaves with MN, 5 = over 75% of leaves with MN or vine dead. Vine vigor was surveyed later fall in 2002. The annual shoot and node growth was recorded from ten randomly sampled shoots per plant, and shoot diameter was taken in the middle of 4th node. Node length was calculated with total node numbers and the length of each shoot. Twenty (4 x 5) random shoots were investigated for shoot death rate from each vine: 5 shoots in each canopy quadrant area divided by the main trunk and trellis wire. A shoot was considered as dead if more than half of the shoot had died. Trunk diameters were measured 50 cm above the ground in fall 2003.

Table 5. A selective set of SSR primer pairs from the *Vitis shuttleworthii* ESTs

<i>Repeat</i>	<i>Left Sequence</i>	<i>Right Sequence</i>	<i>Product Size</i>
GTCGTCGTCGTCGTCGTCGTC	TACAAGAGCCAAGAGGGATT	GGATAACGAAGGAGACAGAGT	245
AGCAGCAGCAGC	AGGGAGATGACAAAGATGAAG	CCAAACACCGTAGGAGAGA	367
AACAACAACAACAAC	AATAATAAGAAGGAGATGCGG	GTTGTGGTGGTTCGTGAAG	367
AGCAGCAGCAGC	CAGAGTGCAGCACAGCA	GCGTTTTCTCAAGGTTCTACTT	368
AACAACAACAACAAC	TGACTGGCATACTGATTACC	CCCAATGAACTACCTTTACCT	368
CGGCGGCGGCGGCGGCGG	ACCCAATGAACTACCTTTACC	AGGAACAAGACAAACAATACTACT	113
CCTCCTCCTCCTCCTCCTCCT	TTTATCCCAACAATCAGG	CTTTCACAGCAGAAGAGTT	226
CCTCCTCCTCCTCCTCCTCCT	GCCTTGGACCGAACTATC	CCTTGGACCGAACTATCTG	226
GAGAGAGAGAGAGAGAGAGAGA	CGACCTAAGAAACACCATTC	CCTTGGACCGAACTATCTG	292
ACCACCACCACCACCACC	CGCATCAGAAGTCATCAAC	ACCCTCACTCTCACACTCAC	238
TCCTCCTCCTCCTCCTCC	ACGGAAGAAGAGAAGAAAGAG	ATCCACCGAAACAACCTTAC	133
AGAAGAAGAAGAAGAAGA	ACAAAGCAGGTAAGTAGCAAA	AAGACGGAAGAAGAGAAGAAA	233
TCTCTCTCTCTC	GTGATTGTTACCGACCTTGA	ATTCCCTTCTTCTCCTTTACC	195
TCTCTCTCTCTC	CCTCGGAAACAACCTTACA	CGAAGAAGAGAAGAAAGAGAAA	195
TGATGATGATGATGATGAGGATGATGA	AAGACCGAAGAAGAGAAGAAA	TAATACCGTGAAATCACAAA	281
ACCTACCTACCTACCTACCT	TTACCCGACACTGGACAC	ACTTACCACCGAGATGAGG	266

After the potential SNP-EST and SSR-EST are verified, PD segregating populations will be used for marker development. Several populations derived from the hybridization of Native American species/hybrids and *V. vinifera* grapes will be candidates for this purpose. For example, a 183-seedling population of N18-6 x ‘Cabernet Sauvignon’ has been evaluated for PD resistance for several years in our vineyard. ‘N18-6’ is a breeding line highly resistant to PD while ‘Cabernet Sauvignon’ is the best known wine grape variety highly susceptible to PD. Segregating analysis revealed that three dominant genes provide full resistance to the Pierce’s disease.

FUNDING AGENCIES

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Table 2. Comparison of disease resistant gene (R-gene) families in grape (*V. shuttleworthii*, *V. vinifera*) and *Arabidopsis*

R-gene Class	Number in <i>V. shuttleworthii</i>	Number in <i>V. vinifera</i>	Number in <i>Arabidopsis</i>
TIR-NBS-LRR	11	64	85
CC-NBS-LRR	9	51	41
NBS-LRR	9	64	10
TIR-NBS	3	19	17
CC-NBS	5	30	4
TIR	18	82	36

A series of experiments are being conducted to identify and isolate PD resistant genes through gene expression profiling analysis by using DNA microarrays. Specifically, a comparative analysis of transcriptional profiles of 1) unchallenged *V. shuttleworthii* grapes (control), *Xf* challenged *V. shuttleworthii* grapes (samples will be collected on different timeframes after infection).

For marker development, we are developing SNP and SSR markers from our *V. shuttleworthii* sequence data set and the *V. vinifera* ESTs in the public domain. Aligned sequences will be mined for Single Nucleotide Polymorphism. A preliminary screening of the SNP and SSR marker from the 12,056 *V. shuttleworthii* ESTs indicated that the SNP and SSR markers are abundant in *V. shuttleworthii* grapes, and around 800 candidate SSR and SNP sites have already been identified. Table 3 shows the distribution of the di-, tri-, and tetra- SSRs from *Vitis shuttleworthii* ESTs, and Table 4 shows the abundant SSRs motifs from *Vitis shuttleworthii* ESTs. We have designed and synthesized the PCR primer pairs using computer software such as Primer3 to flank the SSR loci (partially shown in Table 5). Verification of these primers with PCR amplification on selective grape DNA templates is under way.

Table 3. Distribution of EST derived SSRs from *Vitis shuttleworthii*

Number of ESTs	Number of SSR-ESTs	Motifs		
		di-	tri-	tetra-
10,995	401(3.651 ¹)	82(20.32 ²)	306(76.5)	13(3.2)

¹ SSR-EST percentage in total EST

² di-nucleotide motif percentage in SSR-EST.

Table 4. Distribution of the abundant (>5) SSR-ESTs among the *V. shuttleworthii* EST data set.

SSR Motif	Number of ESTs
GA/CT	36
AT/TA	13
CAA/GTT	90
ACC/TGG	34
TCT/AGA	19
CAG/GTC	15
AAG/TTC	14
CAC/GTC	14
CTT/GAA	13
CCA/GGT	12
CCT/GGA	12
TGA/ACT	9
TCC/AGG	8
CAT/GTA	7
GAT/CTA	6
TGC/ACG	6
CTC/GAG	5
Total	313

TOWARDS IDENTIFYING PIERCE'S DISEASE RESISTANT GENES FROM A NATIVE AMERICAN GRAPE SPECIES (*VITIS SHUTTLEWORTHII*) – A GENOMICS APPROACH

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ABSTRACT

INTRODUCTION

There are over 160,000 grape ESTs in the public data bases and the vast majority of these ESTs were generated from the European grape varieties (*Vitis vinifera*). However, the European grapes are highly susceptible to the Pierce's disease and they are not necessary possessing all the genes required for providing a full protection against the GWSS and *Xf* attack. On the other hand, PD resistant sources exist in some native North American grape species, particularly those species originated in the southeast United States. For example, *Vitis shuttleworthii*, a species originated from the southeast United States, is considered to be one of the most PD resistant grape, which has long been used for developing PD resistant grape varieties for the deep south - a most severe PD infected area. We therefore propose to search for PD resistant genes from the *Vitis shuttleworthii* grape.

The Viticulture Center at Florida A&M University and the USDA-ARS Horticultural Laboratory at Fort Pierce (Florida) jointly initiated a grape EST project from the native American grape -*Vitis shuttleworthii*, aiming to identify and isolate grape disease resistant genes including the Pierce's disease resistant genes. We have sequenced 30,000 ESTs, and have several on-going experiments for expression analysis and marker development for identifying the PD resistant genes.

OBJECTIVES

The objectives of this research are to identify/isolate PD resistant genes from *Vitis shuttleworthii* grapes and develop EST derived molecular markers for PD resistance. Specifically, the project is gearing towards to: 1) discover genes for PD resistance from *Vitis shuttleworthii* grapes; 2) conduct comparative genomics analysis between *V. shuttleworthii*, *V. vinifera* grapes and other plant species; 3) develop SSR and SNP markers for PD resistance, which will be used for accelerating the development of PD resistant grape varieties.

RESULTS AND CONCLUSIONS

We have sequenced 30,000 ESTs from a clone of *V. shuttleworthii* grape. Blasting analysis revealed that 13% of the *V. shuttleworthii* ESTs are unique when compared to the existing *Vitis vinifera* NCBI databases, and 3% of the ESTs did not find any homologous sequences among all plant ESTs reported in NCBI. Overall, approximately 7% of ESTs were related to disease / pest defense or stress tolerance genes, and it is obvious that these genes are abundant in the *V. shuttleworthii* grape (Table 1, Table 2).

Table 1. Comparison of transcription factor (TF) families in grape (*V. shuttleworthii*, *V. vinifera*), *Arabidopsis* and Rice

<i>V. vinifera</i>	<i>Arabidopsis</i>	Rice
124	190	156
69	144	143
114	105	125
67	82	71
131	72	83
34	36	21
31	28	8
121	81	75
10	6	5

Table 1. Response of grape leaves to partially purified culture filtrate from virulent (PD002), weakly virulent (PD91-2), and avirulent (PD-F1) strains of *Xf* as measured by the amount of necrosis produced. Leaf necrosis ratings were: 0 = no necrotic lesions; 1 = 25% or less of the leaf surface with necrotic lesions; 2 = 26-50% necrosis; 3 = 51-75% necrosis; 4 = 76-100% necrosis. The level of PD resistance: S = Susceptible, T = Tolerant and R = Resistant.

Virulent Strain (PD002)												
<i>Leaf Necrosis Rating by Replicate</i>												
Grape Variety	1	2	3	4	5	6	7	8	9	10	Mean	Control
Chard. (S)	1	1	1	1	2	2	1	2	2	2	1.5	0
Blc. Bois (T)	1	1	1	1	1	1	0	1	1	2	1.0	0
Carlos (T)	0	1	0	1	1	1	2	0	1	1	0.8	0
Bl. Beauty(R)	1	1	0	1	1	1	1	1	0	0	0.7	0
Alachua (R)	1	0	1	0	1	0	1	1	0	1	0.6	0
Fry (R)	1	0	0	1	1	0	1	1	0	0	0.5	0
Noble (R)	0	0	0	0	0	0	0	0	0	0	0.0	0

Weakly Virulent Strain (PD91-2)												
<i>Leaf Necrosis Rating by Replicate</i>												
Grape Variety	1	2	3	4	5	6	7	8	9	10	Mean	Control
Chard. (S)	1	1	1	0	0	1	1	2	1	1	0.9	0
Blc. Bois (T)	1	0	0	0	1	1	0	1	1	0	0.5	0
Carlos (T)	0	0	0	1	0	1	0	0	1	0	0.3	0
Bl. Beauty(R)	0	0	1	1	0	0	1	0	1	0	0.4	0
Alachua (R)	0	1	0	0	0	1	0	0	1	0	0.3	0
Fry (R)	0	0	1	1	0	0	0	0	0	0	0.2	0
Noble (R)	0	0	0	0	0	0	0	0	0	0	0.0	0

Avirulent Strain (PD-F1)												
<i>Leaf Necrosis Rating by Replicate</i>												
Grape Variety	1	2	3	4	5	6	7	8	9	10	Mean	Control
Chard. (S)	0	1	0	0	0	0	1	0	1	0	0.3	0
Blc. Bois (T)	0	0	0	0	0	1	0	0	0	0	0.1	0
Carlos (T)	0	0	0	0	0	0	0	0	0	0	0.0	0
Bl. Beauty(R)	0	0	0	0	0	0	0	0	1	0	0.1	0
Alachua (R)	0	0	0	0	0	0	0	0	0	0	0.0	0
Fry (R)	0	0	0	0	0	0	0	0	0	0	0.0	0
Noble (R)	0	0	0	0	0	0	0	0	0	0	0.0	0

Abbreviations: Chard = Chardonnay, Blc. Bois = Blanc du Bois, Bl. Beauty = Black Beauty.

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avirulent strain. The leaf necrosis ratings for Black Beauty, a PD tolerant variety of muscadine grape, were 0.7, 0.4 and 0.1 when challenged with PPCF from the virulent, weakly virulent and avirulent strains of *Xf*, respectively.

In addition, leaves from susceptible varieties of grape generally produced greater levels of necrosis than did leaves from tolerant and resistant varieties. For example, the mean percentage of leaf necrosis for ‘Chardonnay’, ‘Blanc du Bois’ (a PD tolerant Florida hybrid bunch grape), Alachua and Noble (PD resistant muscadine grapes) were 1.5, 1.0, 0.6 and 0.0, respectively, when challenged with the PPCF from the virulent strain of *Xf*. Similar and consistent trends also were observed when using PPCF from the weakly virulent and avirulent strains of *Xf*, but as mentioned before, the leaf necrosis ratings were lower, which resulted in less overall differences between susceptible and resistant varieties.

These results suggest that *Xf* may produce extra cellular “toxin(s)” that could cause necrotic lesions when applied to grape leaves and that might have potential in screening grape germplasm and hybrids for PD resistance. The “toxins” extracted from the culture filtrate of more virulent strains of *Xf* produced more necrosis than did the “toxins” from less virulent strains. Leaves from susceptible varieties of grape also reacted more strongly to these “toxins” than did the leaves from resistant grape varieties. At this time the nature of the “toxin(s)” is not known, nor is it known whether the different strains of *Xf* produce different quantities or types of these “toxins”. Future studies will attempt to answer these questions and expand the number of PD susceptible and resistant grape varieties and *Xf* strains evaluated with this test.



Figure 1. An example of the type of symptoms caused by *Xf* culture filtrate in young ‘Chardonnay’ (A, PD susceptible) and ‘Noble’ (B, PD resistant) grape leaves. Lanes 1 and 2 = control leaves treated with distilled water, lane 3 = leaves treated with undiluted culture filtrate from a virulent strain of *Xf* PD002, and 4 = leaves treated with diluted (1:2 vol/vol) culture filtrate of *Xf* PD002. Incubation time was 48 h.

CORRELATION BETWEEN RESISTANCE TO PIERCE'S DISEASE AND *XYLELLA* STRAIN VIRULENCE USING PARTIALLY PURIFIED CULTURE FILTRATE

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ABSTRACT

Previous research at the FAMU Center for Viticulture suggested that cells of a virulent strain of *Xylella fastidiosa* (*Xf*) may produce toxic compounds that could be used to determine varietal susceptibility to Pierce's disease (PD) in grapes. In the experiments reported here, when grape leaves were challenged with partially purified culture filtrate of *Xf* with different levels of virulence, positive correlations between the degree of leaf necrosis and (1) the virulence of the *Xf* strain and (2) the level of PD resistance were observed.

INTRODUCTION

Pierce's disease (PD), a lethal disease of grapevine, is caused by the bacterium *Xylella fastidiosa* (*Xf*) (Proteobacteria: Xanthomonadales) and is spread by leafhoppers known as sharpshooters. *Xylella fastidiosa* is native to the southeastern U.S., where it reproduces in ornamentals such as crape myrtle, eucalyptus, and hibiscus, but also in various crop plants including citrus, avocado and grapes (Blua et al. 1999). In Florida and other southeastern States, the abundance of *Xf* and vectors such as the glassy-winged sharpshooter (*Homalodisca coagulata*) has precluded commercial production of European grape varieties. The first evidence of PD infection usually is a drying or "scorching" of leaves. Typically, the leaves dry progressively over a period of days to weeks, showing a series of concentric zones of discolored and dead tissue. Vines develop symptoms as the bacteria multiply and begin to block the water conducting system and reduce the flow of water to affected leaves. However, Hopkins (1983) reported that only about 40% of the xylem vessels of infected plants have bacterial occlusions and plants with this percentage of non-functioning vessels typically do not show symptoms of water stress. The PD bacterium also has been reported to produce a phytotoxin or phytotoxins that may damage plant tissues and play an important role in disease initiation and development (Lee 1982).

OBJECTIVES

1. Determine whether partially purified culture filtrate from virulent, weakly virulent and avirulent strains of *Xf* would produce different levels of necrosis when applied to leaves of a given variety of grape.
2. Determine whether partially purified culture filtrate from a given strain of *Xf* would produce different levels of necrosis when applied to leaves from susceptible, tolerant and resistant varieties of grape.

RESULTS AND CONCLUSIONS

Cultures of virulent (PD002), weakly virulent (PD91-2) and avirulent (PD F1) strains of *Xf* were centrifuged to remove cells. The supernatant was filtered and then extracted with ethyl acetate, and the eluate was evaporated to dryness. The powder was then reconstituted in distilled water and applied to the surface of detached leaves of different grape varieties that had been wounded with a sharp needle. After 48 h, the leaves were scored based on the percentage of the leaf surface with necrotic lesions (Table 1).

In general, the mean percentage of leaf necrosis was greater when leaves were challenged with partially purified culture filtrate (PPCF) from the more virulent strains of *Xf*. For example, the leaf necrosis rating for 'Chardonnay', a highly PD susceptible variety of *V. vinifera* grape, was 1.5 for the virulent strain of *Xf*, 0.9 for the weakly virulent strain and 0.3 for the

Table 2. Summary of up-regulated and down-regulated transcripts between resistant and susceptible genotypes among three tissues following of *Xf* infection

Genotypes	Tissue	Up Regulated	Down regulated
Resistant (9621-67)	Stem	8	6
	Leaf	1	2
	Shoot	16	3
Susceptible (9621-94)	Stem	8	5
	Leaf	3	2
	Shoot	7	2

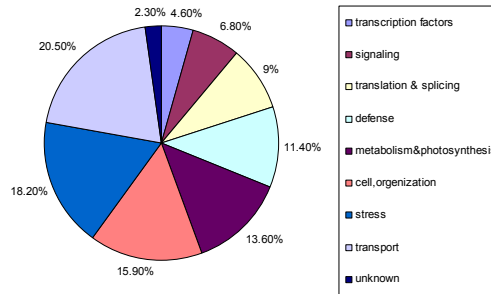


Figure 1. Functional category of putative genes of among 63 differentially expressed transcripts.

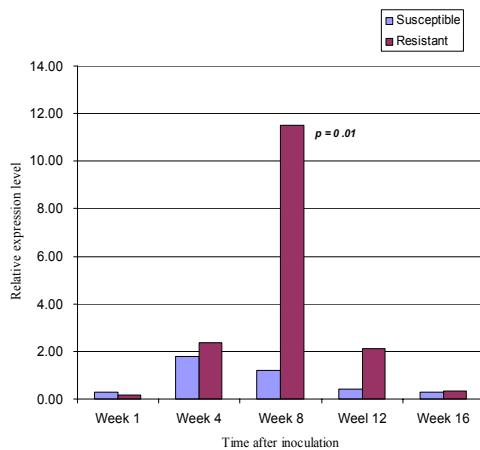


Figure 2. Taq-Man gene expression analysis was used to analyze expression during PD development. Here is an example of the putative pathogenesis-related gene, which increased more than 10 times the transcriptional levels in the 8th week after inoculation in the susceptible genotype (9621-94) as compared to the resistant genotype (9621-67).

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