SEASONAL POPULATION DYNAMICS OF GLASSY-WINGED SHARPSHOOTER EGG PARASITOIDS: VARIABILITY ACROSS SITES AND HOST PLANTS

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ABSTRACT
The California Department of Food & Agriculture (CDFA) has a number of sites in southern California where they are releasing egg parasitoids of Glassy-winged Sharpshooter. To date, species released include *Gonatocerus ashmeadi, G. fasciatus, G. morrilli,* and *G. triguttatus*. Two South American *Gonatocerus* species are scheduled for release in 2005 (pending host specificity studies and release permits) and a strain of *Anagrus epos* from Minnesota may also be included in future releases (also pending such work). CDFA monitors for parasitoid establishment and population dynamics at release sites. This project is intended to complement and expand the scope of this monitoring with an eye towards improving our understanding of the benefit of releasing alternative parasitoid species and how well they are surviving, dispersing, and impacting GWSS populations.

INTRODUCTION
One of CDFA's parasitoid release sites in southern California is Field 7H on the UC Riverside campus. A two-year field study in and around this release site was conducted to examine the temporal and host plant distribution of *Homalodisca* oviposition and associated egg parasitism (Al-Wahaibi 2004). In the current project, we plan to expand on this study and monitoring done by CDFA to attempt to improve our understanding of the population dynamics of endemic and released parasitoids in and around release sites. Although control programs appear to be effective at reducing Glassy-winged Sharpshooter populations, biological control is a more sustainable and environmentally friendly means of contributing to vector reduction and may have to suffice in much of California where chemical control is either impractical (e.g., urban areas) or economically unfeasible.

OBJECTIVES
Monitor GWSS egg parasitoids in several areas in southern California in and around CDFA's parasitoid release sites and across several host plants.

RESULTS
In the two-year study around CDFA's release site on the UC Riverside campus by Al-Wahaibi (2004), parasitism was due to a total of eight parasitoid species with *Gonatocerus ashmeadi*, *Ufens principalis* (previously *Ufens* A, Al-Wahaibi et al. 2005), *Ufens ceratus (*previously *Ufens* B), and *G. morrilli* being the most abundant. *Ufens* spp. were dominant on jojoba while on other plants, *Gonatocerus* species tended to dominate. Across all ten host plants sampled, ranked percent parasitism was *G. ashmeadi* (27.4%), *U. principalis* (19.8*%), U. ceratus* (2.9%), *G. morrilli* (2.1%,), *G. incomptus* (0.4%), *G. novifasciatus* (0.3%), *G. triguttatus* (0.1%), and *G. fasciatus* (0.01%). Note, however, that these data may be biased by the proximity of nearby hosts harboring smoketree sharpshooter and high levels of Ufens spp. on jojoba.

We are in the process of expanding our sampling program outside of the UC Riverside campus.

CONCLUSIONS
We are only 3 months into this project so it is too early to draw conclusions at present.

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FUNDING AGENCIES

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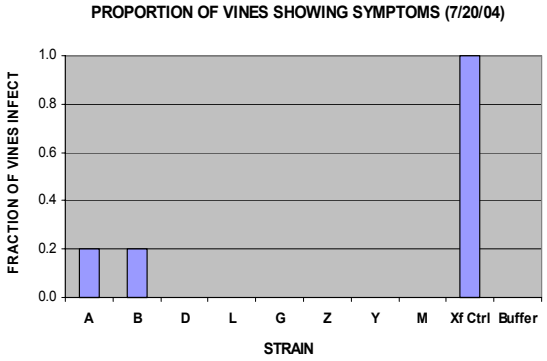
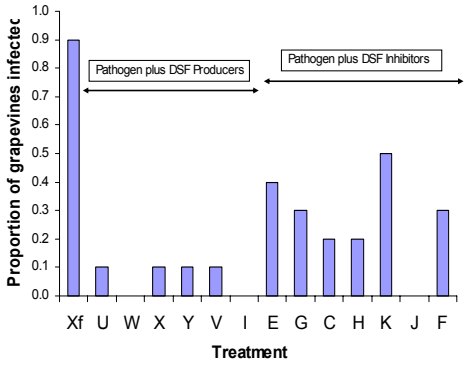
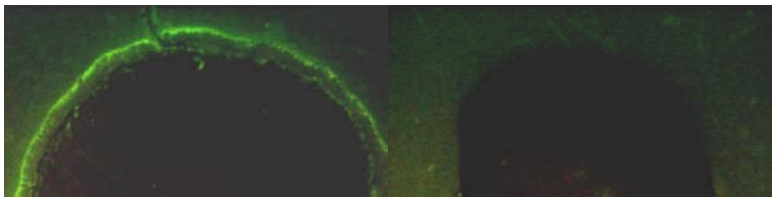




Given that DSF production by endophytes greatly reduces disease incidence and that DSF overproduction in *Xf* also reduces virulence, we have initiated studies to express *rpfF* in plants to achieve production of DFS in plants as a means of disease control . The *rpfF* gene from *Xf* as well as from *Xcc* was cloned into the plant transformation vector pCAMBIA to yield pKLN119. This plasmid carries a T-DNA that includes both hygromycin resistance and the *X. fastidiosa rpfF* gene driven by the CMV 35S promoter and followed by the NOS poly-A signal sequence. pKLN119 and the empty vector pCAMBIA1390 were electroporated into *Agrobacterium* strain GV3101. *Nicotiana benthamiana* plants were transiently transformed by infiltration with suspensions of *Agrobacterium* harboring T-DNA construct pKLN119 or pCAMBIA1390.
Disks of infiltrated leaves were removed after two days, placed on KB agar plates and oversprayed with the DSF bioreporter strain 8525 (pKLN55). Substantial green fluorescence was observed in leaf disks of the plants into which pKLN119 was introduced (left), suggesting that *rpfF* conferred DSF production in *N. benthamiana*.



CONCLUSIONS

Substantial data now show that cell-cell signaling plays a major role in the epidemiology and virulence of *Xf* and that disruption of cell signaling is a promising means of controlling Pierce's disease. Strikingly, *Xf* strains that cannot signal are also not transmissible by nor colonize an efficient insect vector. This result reveals an important and previously unappreciated connection between cell-cell signaling and transmission as well as the requirement for biofilm formation for transmission. These new findings will be helpful for those interested in targeting transmission as a means of disease control. We also found that mutants unable to signal are hypervirulent. Conversely, strains of *Xf* that overproduce DSF have low virulence and do not move within grape. This suggests that, it will be more efficient to elucidate and target *Xf*'s colonization strategies rather than traits predicted to contribute to virulence based on studies of other plant pathogens. We have identified bacterial strains that can interfere with *Xf* signaling. These strains proved very effective as protective agents for grapevines when co-inoculated with *Xf*. Both positive and negative interference with DSF signaling reduced disease in grape suggesting that signaling is normally finely balanced in the disease process; such a finely balanced process might be readily disrupted. Since in bacteria *rpfF* is sufficient to encode a synthase capable of DSF production, expression of DFS directly in plants is a attractive approach for disease control. Preliminary results are very encouraging that DSF can be made in plants. Alternatively, the use of various bacteria to express DSF implants may prove equally effective in altering *Xf* behavior and hence disease control.

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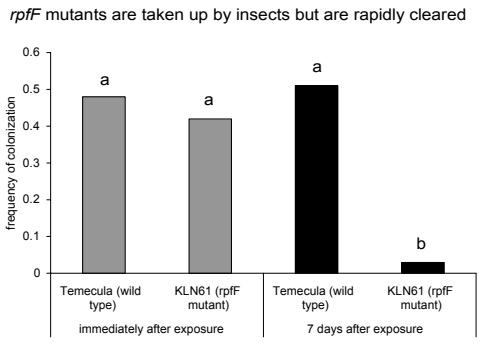


We have isolated a variety of bacteria from grapevines from vineyards affected by Pierce's disease as well as tomato and cruciferous crop plants infected with the signal-producing pathogens *Xanthomonas campestris* pv. *vessicatoria* and *Xcc,* respectively and tested them for their ability to interfere with cell-cell signaling in *Xf* in an assay using the signal-sensing strain described above. We found several strains that negatively affected signaling in *Xcc* while several strains were found to produce DSF. By adding purified DSF to either cell-free extracts of the strains with a negative influence on signaling or to whole cells we found that at least two mechanism of interference with signaling could be observed. Some strains such as strains C,E,G, H, and J are able to degrade DSF while other inhibitor strains did not do so, and apparently have another means of interfering with DSF perception by *Xcc*. The several strains that produced DSF were all identified as *Xanthomonas* species. We sequenced the 16S rRNA gene from these strains to determine their species identity.

Strain	Genus	Origin	Mechanism of DSF Interference
A	*Paenibacillus*	Grape	Unknown inhibition
B	*Paenibacillus*	Grape	Unknown inhibition
C	*Pseudomonas*	Cabbage	Enzymatic digestion
D	*Staphylococcus*	Grape	Unknown inhibition
E	*Bacillus*	broccoli	Enzymatic digestion
G	*Pseudomonas*	Cabbage	Enzymatic digestion
H	*Pseudomonas*	Cabbage	Enzymatic digestion
J	*Pseudomonas*	Tomato	Enzymatic digestion
L	*Staphylococcus*	Grape	Unknown inhibition
I	*Xanthomonas*	Tomato	DSF production
U	*Xanthomonas*	Broccoli	DSF production
V	*Xanthomonas*	Broccoli	DSF production
W	*Xanthomonas*	Broccoli	DSF production
X	*Xanthomonas*	Broccoli	DSF production
Y	*Xanthomonas*	Tomato	DSF production
Z	*Xanthomonas*	Grape	DSF production

Interfering strain G, typical of strains that apparently degrade DSF, was subjected to transposon mutational analysis of the interfering activity. Several insertional mutations that block degradation of DSF have been identified and sequence analysis of the genes required for DSF degradation are being performed. We expect this analysis to reveal the identity of the gene responsible for the interfering activity. This gene can then be introduced into other organisms, such as plants.

To test the ability of bacteria that alter *Xf* signaling to alter the process of disease in plants, we co-inoculated grapevines with *Xf* and strains that either inhibit or activate cell-cell signaling in greenhouse studies. The incidence of Pierce's disease was greatly reduced by all of the signaling interfering strains that we tested. As we had expected, DSF-producing strains generally reduced disease severity more than did strains that interfered with signaling in *Xf*. These results were highly repeatable, having been observed in 2 separate experiments. We find these results to be very exciting in that they suggest that alteration of signal molecules within plants can have a profound effect on the disease process.

but probably slightly different than the DSF of *Xcc*. Based on our knowledge of density-dependent gene regulation in other species, we predict the targets of Rpf regulation would be genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel. Similarly, we would expect the density-dependent genes to be expressed during the time when a population of *Xf* is ready to move into uncolonized areas.

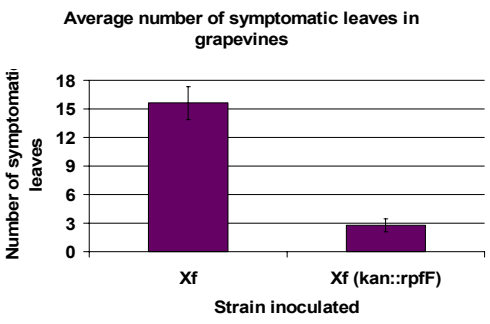
Other organisms can apparently interfere with the density-dependent behaviors of *Xf*. Several recent studies indicate that other organisms can disrupt or manipulate the cell-cell signaling system of bacteria (4, 5). We have found that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf*, but until this study we did not know of the manner in which the interaction occurred nor whether such strains had the potential to affect the virulence of *Xf* in grape. In this period we have extensively investigated both the role of DFS-production by *Xf* on its behavior within plants and insects as well as the manner in which other bacterial strains affect such cell signaling and determined the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling.

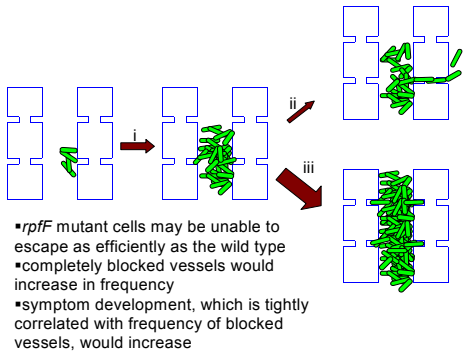
OBJECTIVES

1. Determine role of signaling factors on virulence and transmissibility of *Xf*.
2. Identify degraders and producers of diffusible signaling factors used by *Xf*.
3. Perform Pierce's disease (PD) biocontrol tests on grapevines using DSF-interfering bacteria
4. Isolation of mutant strains of DSF-degrading and DSF activating bacteria that no longer interfere with cell-cell signaling in *Xf*. to verify that disease control is linked to cell-cell signal interference
5. Creation of grapevines expressing gen4s conferring DSF-degradation and DSF-synthesis activites to test for PD resistance
6. Engineer grapevine endophytes such as *Alcaligenes xylosoxidans denitrificans* to express genes conferring DFS-degradation or DSF-synthesis activities and test whether the resulting transgenic endophytes are capable of disease control

RESULTS

We have constructed a strain of *Xf* Temecula in which the *rpfF* gene, which is required for production of the signal in *Xcc,* is knocked out. This mutant was constructed using exchange of the wild-type allele for a deleted copy carrying an antibiotic resistance gene on a suicide plasmid. The *rpfF* mutant of *Xf* does not make DSF as determined using previously constructed "signal-sensing" strains of *Xcc* to determine DSF production by *Xf* and other bacterial strains. *rpfF* mutants strains were tested for their ability to infect and move within host plants and to cause Pierce's disease symptoms. The *rpfF* gene appears to play a role in modulating disease progress because the timing and severity of symptom development are greatly exacerbated in grapevines infected with *rpfF* mutants when compared to the wild type. We have investigated the mechanism behind these differences. We have found no detectable difference in populations or movement between the wild type and *rpfF* mutants, although our sampling methods would not be able to detect small increases in colonization if they existed. We hypothesize that *rpfF* mutants may be causing increased vessel blockage in the grapevine, leading to increased symptom expression. We have recently made a green fluorescent *rpfF* mutant to investigate the pattern of colonization by the mutant and compare it to that of the wild type. Importantly, when rpfF was over-expressed in *Xf* under the control of a high and constitutive promoter, the severity of disease in plants was greatly reduced (below). The *Xf* strain that overproduced DSF caused disease symptoms in grape, but only at the site of inoculation. The mutant cells did not move within the plant as did wild-type strains. These results all support our model that DFS regulates genes required for movement of *Xf* from colonized vessels.





Such results suggest that elevating DSF levels in plants should reduce movement of *Xf* in the plant.

We have tested transmissibility of the *rpfF* mutant strain by an insect vector. The *rpfF* mutant was virtually non-transmissible. This defect in transmissibility by the signaling-deficient mutant reveals the importance of cell-cell signaling in insect transmission. Leafhoppers fed on *rpfF*-infected plants ingested *rpfF* cells but were able to rapidly clear themselves whereas the wild type is never cleared.

MANAGEMENT OF PIERCE'S DISEASE OF GRAPE BY INTERFERING WITH CELL-CELL COMMUNICATION IN *XYLELLA FASTIDIOSA*

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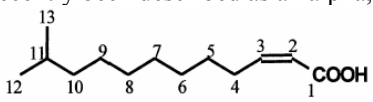
ABSTRACT
Xylella fastidiosa (Xf) is an endophyte that is restricted to the xylem, a network of vessels for water transport, in which it forms an aggregated biofilm. It is transmitted from plant to plant by xylem sap-feeding insects, and forms a polar biofilm in these insects' foreguts. In other systems, biofilms are characterized by community behavior under the control of cell density-dependent gene expression, which requires cell-cell signaling. *Xf* has homologs of the cell-cell signaling genes found in the important plant pathogen *Xanthomonas campestris* pathovar *campestris (Xcc)* and produces a similar alpha,beta unsaturated fatty acid signal molecule called DSF that coordinates gene expression in a community (2, 7). We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease. We have determined that the *rpfF* gene is necessary and sufficient for DSF signal synthesis and that *rpfF* mutants of *Xf* are hypervirulent and non-transmissible. Lack of transmissibility was linked to an inability of the *rpfF* mutant to form a biofilm in the insect foregut; while taken up by insects, the mutant strain is not retained. *Xf* strains that overproduce DSF produce disease symptoms in grape, but only at the site of inoculation and the cells do not move within the plant as do wild-type strains. Thus elevating DSF levels in plants should reduce movement of *Xf* in the plant and also reduce the likelihood of transmission by sharpshooters. We screened several collections of bacterial strains isolated from plants and identified bacterial strains that can interfere with *Xf* signaling both by producing large amounts of DSF, by degrading DSF, or by in some way interfering with recognition of DSF. When co-inoculated into grape with *Xf*, both DSF-producing strains and DFS degrading strains greatly reduced the indicidence of disease in grape; DSF-producing strains consistently were the most effective in reducing disease. Given that DSF appears to mediate an attenuation of virulence in *Xf* we are in the process of transforming grape with the *rpfF* gene to enable DSF production in planta. Preliminary results indicate that transient expression of *rpfF* in *Nicotiana benthamiana* following infiltration with appropriate *Agrobacterium tumefaciens* strains resulted in high levels of DSF production, suggesting that it is likely that grape cells will produce DSF when transformed with the bacterial *rpfF* gene. Non-endophytic bacterial species were also established in high numbers inside grape leaves and petioles following spray application to plants with a high concentration of a silicon-based surfactant with a low surface tension, suggesting that it may be possible to produce protective compounds such as DSF in plants by a variety of bacteria.

INTRODUCTION
Endophytic bacteria such as *Xylella fastidiosa (Xf)* colonize the internal tissues of the host, forming a biofilm inside the plant. A key determinant of success for an endophyte is the ability to move within the plant. We expect activities required for movement to be most successful when carried out by a community of cells since individual cells may be incapable of completing the feat on their own. Cells assess the size of their local population via cell-cell communication and coordinately regulate the expression of genes required for such processes. Our study aims to determine the role of cell-cell communication in *Xf* in colonization and pathogenicity in grapevines and transmission by the insect vector.

Xf shares sequence similarity with the plant pathogen *Xanthomonas campestris* pathovar *campestris (Xcc)* (7). In *Xcc,* expression of pathogenicity genes is controlled by the Rpf system of cell-cell communication, enabling a population of cells to launch a pathogenic attack in a coordinated manner (1). Two of the Rpf proteins, RpfB and RpfF, work to produce a diffusible signal factor (DSF) (1) which has recently been described as an alpha,beta unsaturated fatty acid (9):



As the population grows, the local concentration of DSF increases. Other Rpf proteins are thought to sense the increase in DSF concentration and transduce a signal, resulting in expression of pathogenicity factors (8). The *Xf* genome not only contains homologs of the *rpf* genes most essential for cell-cell signaling in *Xcc*, but also exhibits striking colinearity in the arrangement of these genes on the chromosome (2). We now have shown that *Xf* makes a molecule that is recognized by *Xcc*

OBJECTIVES

1. Study the behavior of strains of *A. xylosoxidans* subsp. *denitrificans* (Axd and Raxd) when grown under various biotic influences and,
2. Investigate and compare the growth of *A. xylosoxidans* subsp. *denitrificans* (wild type) and Raxd to that of Axd modified to express a short chain antibody against *X. fastidiosa* (Axd 7.7) that infects grape under different physiological conditions, such as in response to nutrient availability and energetic demands.

RESULTS

We have found that Axd and Raxd do not establish when introduced into soil, but can be recovered from soil that was sterilized before inoculation with Axd and Raxd. Axd and Raxd, when applied to leaf surfaces, can be recovered from established phylloplane communities of basil, strawberry, and sage, although recovery is scant to low. Co-culture experiments showed that Axd and Raxd growth are negatively affected by the presence of *E. coli* and *P. aeruginosa.* The growth of Axd modified to express an S1 scFv (single chain antibody variable region fragments) antibody (Axd 7.7) that binds specifically to a strain of *X. fastidiosa* that infects grape was compared to that of the wild type Axd and Raxd. Axd, Raxd, and Axd 7.7 exhibited similar growth patterns in tryptic soy broth (TSB). Axd, Raxd, and Axd 7.7 also demonstrated longer lag phases in Luria Bertani medium (LB) than for TSB. Cell numbers remained fairly constant for each strain at each growth phase. Growth studies are underway that monitor the growth of Axd, Raxd, and Axd 7.7 in dilute, R2A medium. Current studies also underway include using enzyme linked immunosorbent assays to monitor the expression of S1 scFv from Axd 7.7 under environmental challenges, such as poor nutrient availability and energetic demands.

CONCLUSIONS

From earlier work we have found that Raxd establishes within the mouthparts of *H. coagulata* (Bextine et al. 2004a) and within the xylem of several of this sharpshooter's host plants (Bextine et al. 2004b). The bacterium, however, does not establish within soil if soil communities are in place. If the soil is sterilized and biotic competition is eliminated, then Axd and Raxd grow relatively well. Conversely, Axd and Raxd can survive and be retrieved from the leaf surfaces of plants other than citrus, such as basil, sage, and strawberry plants for up to two weeks. These data suggest that Axd and Raxd are more suited to the plant environment than to a soil environment. We conclude that Axd and Raxd will remain in the plant environment long enough to exert is anti-*Xylella* effect with little to no disruption of any relevant ecosystem. Raxd did not grow well in the presence of *E. coli* and *P. aeruginosa* compared to Raxd grown in pure culture. Thus, compared to a ubiquitous bacterium and a pathogen, respectively, Raxd is not as fit under standard growth conditions.

Axd 7.7 growth compared to Axd and Raxd differed little under our experimental conditions. All data collectively suggest that Axd 7.7 shows potential for delivery of an anti-*Xylella* product with little impact on nontarget bacterial ecosystems. This statement is qualified by the fact that field tests must be implemented to assess the true behavior of strains of Axd in the environment. Laboratory studies are not suitable for a genuine assessment of risk assessment and environmental impact; nevertheless, they provide important insight.

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FUNDING AGENCIES

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SYMBIOTIC CONTROL OF PIERCE'S DISEASE: THE BIOLOGY OF THE SHARPSHOOTER SYMBIONT, *ALCALIGENES XYLOSOXIDANS* SUBSP. *DENITRIFICANS*

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

ABSTRACT
Alcaligenes xylosoxidans denitrificans (Axd) is closely associated with *Homalodisca coagulata*, the glassy-winged sharpshooter (GWSS), and xylem fluid of host plants. The bacterium has long been characterized as a nitrogen and hydrogen recycler in nature, and was recently recognized as an important decomposer of cyanogenic glycosides in plant material (Ingvorsen et al. 1991). Few studies exist that describe the fitness of Axd when it is introduced to competitive environments, such as established soil or plant microbial communities. Such studies lend important information for assessment of the potential use of Axd for symbiotic control of *Xylella fastidiosa*, the causal agent of Pierce's disease. We have found that Axd and Axd containing DsRed fluorescent protein (Raxd) do not establish when introduced into soil, but can be recovered from soil that was sterilized before inoculation with Axd or Raxd. Axd and Raxd can also be recovered from established phylloplane communities of basil, strawberry, and sage, although recovery is scant to low. Current studies underway include the recovery of Axd and Raxd from lake water microbial communities. Co-culture experiments showed that Axd and Raxd growth is negatively affected by the presence of *Escherichia coli* and the pathogen *Pseudomonas aeruginosa.* Raxd was modified to express an S1 scFv (single chain antibody variable region fragments) antibody (Axd 7.7) that binds specifically to a strain of *X. fastidiosa* that infects grape. Axd 7.7 growth in culture was compared to that of the wild type Axd and to Raxd. All strains exhibited similar growth patterns in tryptic soy broth (TSB). All strains demonstrated longer lag phases in Luria Bertani medium (LB) than for TSB. Cell numbers remained fairly constant for each strain at each growth phase. Growth studies are underway that monitor the growth of Axd, Raxd, and Axd 7.7 in dilute, R2A medium. Current studies also include using enzyme linked immunosorbent assays to monitor the expression of S1 scFv from Axd 7.7 under environmental challenges, such as poor nutrient availability and energetic demands.

INTRODUCTION
Alcaligenes xylosoxidans subsp. *denitrificans* (Axd) is currently being tested for use in symbiotic control of Pierce's disease. While the bacterium naturally resides in terrestrial and aquatic environments, little is known about the fitness of Axd when it is artificially introduced to either allocthonous or autocthonous environments with established microbial communities. Therefore, some indication of the fitness of Axd in competitive biotic scenarios must be acquired to begin to assess the potential of Axd to control *Xylella fastidiosa* (*Xf*) under natural conditions. This point also holds true for any strain of Axd that is modified to express anti-*Xf* products. In most cases, a genetically modified bacterium (GMB) is less fit than the wild type counterpart (Velicer, 1999). In an ideal case, a GMB should remain in an ecosystem for a limited but effective period of time and cause minimal or no disruption to a host or ecosystem. Here we report on the recovery of Axd and Raxd when introduced onto plant surfaces and in soil using semi-natural experimental conditions. In addition, we provide information regarding the growth of Axd and Raxd when grown under strict laboratory conditions in the presence of human and plant-associated bacteria. We also provide a comparison of the growth of Axd, Raxd, and Axd genetically modified to express a synthetic antibody construct on its cell surface (Axd 7.7) under different growth conditions.

vary in fitness is an important aspect of paratransgenesis since we are interested in providing *Axd* reagents that vary in their level of persistence.

D. Determining the target of the anti-Xylella scFv

We attempted to determine the target of the anti-Xylella scFv we isolated previously. We used a combination of 1-D and 2-D SDS-PAGE gels and western blotting to determine a size range for the target protein.

CONCLUSIONS

We have created multiple transgenic strains of the plant and insect symbiotic bacterium, *Alcaligenes xylosoxidans* (*denitrificans*) that carry a surface expressed anti-*Xylella* antibody. These strains carry chromosomal insertions of the genes for the scFv and we were able to recover strains that varied in fitness and in their expression level for the scFv on their outer membranes. These initial strains are currently being tested for their ability to interfere with the transmission of *X. fastidiosa* by sharpshooters.

The future goals of this project are to isolate new anti-*Xylella* factors that can be expressed on the surface of *Axd*, to incorporate genetic systems aimed at preventing horizontal gene transfer of the transgenes, and to improve expression levels of the transgenes on the surface of the cell. All of these features are aimed at developing strains of *Axd* that can interrupt the spread of *Xylella* from the glassy-winged sharpshooter to uninfected grapevines.

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anchor it in the outer membrane (short-*inaZ*)[8]. Each of these was placed on a *Himar1 mariner* transposon and random chromosomal insertions were obtained for each generating multiple strains[9] (see Table 1).

B. Expression of scFv Fusions on the Surface of Axd.

We determined the degree of surface expression of the scFv fusions on *Axd* by two methods. The first was a "spun cell ELISA"[7]. This method uses a suspension of cells that express a target epitope as the substrate for an ELISA. Detection of the scFv was accomplished before and after induction of the *lac* promoter by either reaction with Protein L-conjugated HRP (which detects scFv light chains) or with a HPR conjugated antibody that reacts with the haemagglutinin epitope tag on the scFv. Results of spun cell ELISAs on different strains are shown in Table 1. Strains varied considerably in their scFv surface expression levels, presumably due to the site of insertion. Most strains of short-*inaZ* fusions, for example were poor expressers when induced and strain AL8.2 only showed appreciable levels of surface expression when uninduced.

Table 1. Characteristics of transgenic *A. xylosoxidans* strains expressing an anti-*Xylella* single chain antibody as an outer membrane protein fusion.

Strain	scFv fusion	Surface expression[1]	Relative Fitness[2]	Insert location[3]
AL7.2	*P. syringae inaZ*	++	G	- major facilitator superfamily transporter
AL7.5	""	+++	G	- inorganic pyrophosphatase
AL7.7	""	++	S	-fructose transport system repressor
AL7.10	""	+	G	ND
AL8.2	*P. syringae* short *inaZ*	+++ (uninduced only)	G/P	-probable transporter
AL8.3	""	BK	P	ND
AL9.1	*E. coli lpp-ompA*	+	S	ND
AL9.4	""	+++	S/G	ND
AL9.5	""	+	S	ND

[1] These values are relative to background as measured in a spun cell ELISA: BK = background levels; + noticeable expression, ++ strong expression; +++ very strong expression.
[2] Fitness values are measured as growth rates in liquid culture relative to that of wild-type *A. xylosoxidans*. S (= strong, essentially wild type); G (= good, but slower than wild type); P (= poor)
[3] Most likely identity of genes where transgenes were inserted. These were obtained using tblastx with flanking insertion sequences against the microbial nucleotide database from Genbank. ND= not determined.

The second method used to determine whether expression was occurring in the outer membrane of *Alcaligenes* was a test for ice nucleation. Wild-type *Axd* cannot nucleate ice (unpublished observations). We tested whether or not AL7 and AL8 strains could nucleate ice. All of the AL7 strains could nucleate ice while neither of the AL8 strains did so. This is consistent with surface expression of the full-length *P. syringae* ice nucleation protein on the surface of the AL7 strains. AL8 strains express a form of inaZ that has the internal repeat region removed. This is the region that is responsible for ice nucleation in these proteins.

C. Fitness of transgenic Axd strains

Our strains are built via transposon insertion and so should vary in fitness depending on the site of insertion in the chromosome. We measured the fitness of each strain compared to wild type by measuring their growth rates in log phase in liquid culture. These relative fitness values are shown in table 1 along with the most likely site of insertion of the transposon used to make the strain. We determined the site of insertion by sequencing outward from the transposable element inverted terminal repeats into the flanking genomic DNA and then using tblastx against the microbial genomic database in Genbank. There are no *Axd* sequences in Genbank, so the matches we obtained were typically to species in the genus *Pseudomonas*, another basal beta proteobacterial group.

Strains were highly variable in their fitnesses. Some strain fitnesses were indistinguishable from wild type (e.g., AL7.7 and AL9.5), while others were obviously affected in their growth rates (e.g., AL8.3). There was no obvious correlation between fitness and ability to surface express the scFv fusions. Indeed, one of our best expressing strains was only a modest grower (AL7.5) while other strains grew well and expressed the transgene poorly (e.g., AL9.5). The ability to isolate strains that

SYMBIOTIC CONTROL OF PIERCE'S DISEASE: CONSTRUCTION OF TRANSGENIC STRAINS OF *ALCALIGENES XYLOSOXIDANS DENITRIFICANS* EXPRESSING SURFACE ANTI-*XYLELLA* FACTORS AS MICROBIAL PESTICIDES FOR PIERCE'S DISEASE CONTROL

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INTRODUCTION

The glassy-winged sharpshooter (GWSS) is the principle vector of the xylem-limited bacterium *Xylella fastidiosa* (*Xf*), which causes Pierce's disease (PD) in grapes. Limiting the spread of this pathogen by rendering GWSS incapable of pathogen transmission (paratransgenesis) is a promising method of pathogen control. Paratransgenesis seeks to modify the phenotype of an organism indirectly by modifying its symbiotic bacteria to confer vector-incompetence

Paratransgenic approaches to disrupt pathogen infection of humans are being developed by several groups. These include interference with the ability of triatomid bugs to transmit pathogens causing Chagas' disease [1], interference with HIV attachment to its target cells in the reproductive tracts of humans [2], and the elimination of persistent *Candida* infections from biofilms in chronically infected patients [3]. Paratransgenesis has also been applied to deliver cytokines mammalian guts to relieve colitis [4; 5]. Thus, the method has wide applicability.

Alcaligenes xylosoxidans denitrificans (Axd) is Gram negative, beta proteobacterial species that can colonize the GWSS foregut and cibarium, as well as various plant tissues, including xylem. It is non-pathogenic in insects, plants and healthy humans. Given these characteristics, *Axd* has become the focus of our paratransgenesis efforts to control PD in grapes. Over the past two years we developed the technology to stably modify *Axd* by inserting genes into its chromosome and also isolated as single chain antibody that recognized an epitope on the surface of the Pierce's Disease strain of *Xf*. [6].

We report here the construction of strains of *Axd* that express an anti-*Xylella* single chain antibody (scFv) on the outer surface of *Axd* as fusions to three different heterologous outer membrane proteins. In each case, strains of varying fitness were recovered as measured by growth rate as compared to wild-type strains.

OBJECTIVES

1. Construct anti-*Xylella* scFv-membrane protein fusions;
2. Construct strains of *Axd* that express the scFv-membrane protein fusions in the outer membrane;
3. Construct transgenic *Axd* strains of varying fitness.

RESULTS

A. Membrane Protein-scFv Gene Fusions

We fused an anti-*Xylella* scFv gene to three different outer membrane protein genes in order to display the scFv on the outer membrane of *Axd*. These were a lipoprotein-outer membrane protein A (*lpp-OmpA*) fusion from *E. coli* [7]; the ice nucleation protein Z (*inaZ*) from *Pseudomonas syringae* (a gift of Steven Lindow); and an internally-deleted form of *inaZ* that eliminates the internal ice nucleation repeat sequence but retains the N and C terminus of the protein necessary to export and

concentrations of 1.5 x 10^7 bacteria per mL of potassium phosphate buffer. One mL of suspension was then placed into each 1.5 mL microcentrifuge tubes and placed at -5ºC. Samples were diluted and plated out onto PD3 and allowed to grow for seven days. After seven days colonies were counted to determine the effect of pH on the viability of the *Xf* cells. *Xf* survived the best in potassium phosphate at pH 6.6 and 6.8 and the poorest survival occurred at pH 5.0. There was significant variation between reps of these experiments so they are now being repeated; however it is interesting that these initial trends are consistent with the pH values of xylem saps extracted from Placerville, where PD is not know to occur, and saps from vines growing at Davis where *Xf* can overwinter in grapevines.

Objective 4

Previous research has shown that herbaceous and woody plants exposed to sub-lethal cold conditions have significantly elevated levels of plant hormones, such as abscisic acid (ABA), which induces the synthesis of a number of cold shock proteins (Bravo, et al., 1998; Thomashow, 1998). Preliminary studies, involving samples of Pinot noir and Cabernet sauvingnon field materials collected from Placer and Yolo counties in February, 2004, showed abscisic acid concentrations were lower in the Placerville, cold-exposed vines, that vines from Davis. ABA concentrations were lower in Pinot than Cabernet for both Placerville and Davis vines. Again, it will be important to verify these initial findings using vines grown under more controlled environments in growth chambers during 2005.

We will determine the concentration of ABA in cold-stressed and control vines growing both in the growth chamber using the temperature regimes determined in Objective 1 and in the field-grown plants in the four sites described in Objective 1. We will also determine the pH, osomolarity and protein profiles of xylem sap from ABA-treated vs. non-treated vines and assess the potential of this sap for anti-*Xf* activity.

During the spring, summer and fall, Cabernet and Pinot vines will be sprayed with 100uM solutions of ABA, a concentration that elicited cold-shock proteins at 23ºC in winter wheat (Kuwabara, et. al 2002). Additional concentrations up to 500uM may also be evaluated if no response is noted at 100uM. The pH and osmolarity of xylem sap from the treated vines will be determined as described above. The concentration of ABA in the sap will be determined using a commercially available immunoassay that has a sensitivity of 0.02-0.5 picomole/0.1 mL (Plant Growth Regulator Immunoassay Detection Kits, Sigma Chemical Co.). Preliminary work has shown that ABA concentrations in grapevine xylem sap are detectable using this kit. Xylem sap proteins will be collected, concentrated and analyzed by 1 and 2 dimensional PAGE as previously described. Unique proteins expressed in ABA-treated vines will be removed from the gels and end terminally sequenced and analyzed as previously described.

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house, plants were watered by drip irrigation and supplemental fertilizer application until the first week of October 2004. Twelve weeks after inoculation, the plants were rated for symptom development.

During October/November, 2004, 11 inoculated and 11controls of each variety (44 plants total) were transported to 3 sites that were selected because of their relatively cold winter temperatures, as well as University of California, Davis which was the control. Plot sites include: Fall River (Shasta County), University of California Hopland Research Station (Mendocino County), and University of California, Blodgett Forest Research Station (Placer County). Potted grapevines were planted in the ground to the top of the pot in order to maintain uniform soil type, prevent roots in the pots from exposure to abnormally cold temperatures, and to prevent the plants from falling over. Plants were irrigated as needed until rain provided adequate moisture for the vines. Vines will be allowed to undergo natural dormancy during the fall and experience ambient temperatures during the winter. Temperature, ETo, and other weather data for each plot are being monitored using CIMIS weather data (http://wwwcimis.water.ca.gov/cimis/data.jsp). This data, and previous temperature profiles at these sites, will be used to determine a growth chamber temperature regime that can consistently cure PD affected grapevines without causing unacceptable plant mortality. Additional grapevines, using the same varieties and inoculated as described above, but grown in 6 inches standard pots will be exposed to different temperature regimes in cold rooms located at the Department of Pomology, University of California, Davis during the winter/spring of 2005.

Objective 2

Preliminary work from Pinot noir and Cabernet sauvingnon field materials collected from Placer and Yolo counties showed some differences in xylem sap pH and osmolarity. These results were obtained from Pinot noir and Cabernet sauvingnon vines growing in one Placerville vineyard and at a vineyard at University of California Davis. Both varieties were grown in the same manner at each site, however management practices at the two sites were not identical. It is also important to note that the University of California Davis vines were grown on 5C rootstocks while the Placerville vines were not grown on rootstocks and that these vines were not the same clones. Dormant cuttings were collected in late February and xylem sap was extracted using a custom-made pressure bomb. Differences were noted in xylem sap pH, abscisic acid concentration, and osmolarity. These same parameters will be further examined in 2005 in the field sites and growth chamber experiments. Although only preliminary findings, we found that the pH of xylem sap collected in late February was lower, 5.37 for Pinot and 5.23 for Cabernet vines at the Placerville site (colder winter temperatures) than vines growing at University of California Davis, 6.35 and 6.06, respectively. Small differences in osmolarity were also noted in xylem sap from Placerville, 55.2 and 55.5, versus the osomolarity of xylem sap from Davis vines, 58.3 and 60.8 respectively. The significance and reproducibility of these differences needs to be confirmed this winter using the more controlled experimental units.

During the 2005 winter months, field grown and growth chamber plants will be sampled for potential changes in pH, osmolarity, total organic acids, proteins and other constituents that occur in xylem sap. Our hypothesis is that changes in xylem sap components in vines that undergo cold treatment may have significant effects on *Xf* viability. Previous research on several plant species has shown that a number of plant genes are expressed in response to freezing temperatures (reviewed by Thomashow, 1998). In some plants, these freeze-induced proteins are structurally related to proteins that plants produce in response to pathogens, i.e. pathogenesis-related proteins (Hon, et al. 1995; Kuwabara, et al, 2002). Thus it maybe possible that cold-stressed grapevines could produce proteins that are deleterious to *Xf*. To investigate this possibility, xylem sap will be expressed from cold-stressed and control vines using the pressure bomb, concentrated by freeze drying, and protein profiles determined by 1 and 2 dimensional polyacrylamide gel electrophoresis (PAGE). If unique proteins are found in the cold stressed plants these proteins will be cut from the gel, end terminally sequenced by the University of California Molecular Structure Facility and their sequences compared to others in the database. The potential effect of these proteins on *Xf* viability will be assessed as described in Objective 3.

Objective 3

We have been assessing the effect of many of the physical, physiological and biochemical parameters we determined in Objective 1 and 2 on *Xf* viability. We have been assessing the effect of pH and osmolarity on the viability of *Xf* cells *in vitro* using various buffers and media such as PD3 and new chemically defined media (Leite, et al., 2004). The liquid solutions used for these viability experiments included: water, extracted xylem sap, PD3, the Leite medium, HEPES, sodium and potassium phosphate buffers. In order to further examine these parameters, cultures of *X. fastidiosa* Stagg's Leap strain were grown at 28°C on PD3 for 11 days. Cells were scraped from the culture plates and suspended at concentrations of 1.5 x 10^7 bacteria per mL of liquid medium. One mL of the suspension was then placed into each 1.5 mL microcentrifuge tubes and placed at various temperatures. Samples were diluted and plated out onto PD3 and allowed to grow for seven days. After seven days, colonies were counted to determine the potential effect each treatment had on the viability of *Xf* cells. Results of these experiments indicate that *X.f.* can survive at -5ºC for 8 weeks. At lower temperatures, our results were similar to those found by Feil (2002). *Xf* survived the best in HEPES and sodium phosphate buffers and the worse survival occurred in waters and xylem sap at –5ºC. At –10 and –20ºC *Xf* rapidly died in all liquid media tested.

We also adjusted the pH of potassium phosphate buffer to the values determined for cold-stressed and control xylem saps collected from Placerville and University of California, Davis vines described previously. Cultures of *X. fastidiosa* Stagg's Leap strain were again grown at 28°C on PD3 for 11 days. Cells were harvested from culture plates and suspended at

IDENTIFICATION OF MECHANISMS MEDIATING COLD THERAPY OF *XYLELLA FASTIDIOSA*-INFECTED GRAPEVINES

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ABSTRACT
Preliminary xylem sap composition studies were conducted in February 2004 using Cabernet sauvignon and Pinot noir grapevines growing in Placerville (cold winter temperature) and UC Davis (warmer temperatures). The pH of xylem sap from both varieties was almost a full unit lower in vines grown in cold temperatures versus warm. A similar trend also occurred with sap osmolarity, however the differences were not as great. Because these vines were grown under different management practices and on different rootstocks these results must be considered preliminary. In 2004 we established four field sites in Shasta, Placer, Mendocino and Yolo counties to repeat these measurements on clonal vines that were grown in 5-gallon pots at University of California, Davis. One-half of the vines were inoculated with *Xf* while the other half is un-inoculated controls. Sap will be collected from the vines during the late winter and pH, osomolarity, carbohydrates, organic acids and abscisic acid (ABA) will be measured and compared. The vines will be returned to University of California, Davis at bud break and observed for the development of PD symptoms and tested by PCR to determine if any of the vines were "cold cured" of their infection. Similar experiments using potted vines that will be exposed to defined cold temperature regimes in cold storage facilities located at University of California, Davis will be conducted in 2005. Proteins present in the collected xylem sap will be analyzed by PAGE and the identity of major or unique xylem sap proteins will be determined by sequencing them. *Xf* viability studies using buffers of various pHs, xylem sap from warm- and cold-treated vines will also be studied. The goal of this research is to understand the physiological/biochemical basis of cold therapy that was first documented by A.H. Purcell.

INTRODUCTION
The geographical distribution of Pierce's disease (PD) in North America is strongly associated with the severity of winter temperatures, i.e. PD does not occur in New York, the Pacific Northwest nor at high altitudes in S. Carolina, Texas and even California (Hopkins and Purcell, 2002). Sandy Purcell demonstrated that relatively brief exposures to sub-freezing temperatures can eliminate *Xylella fastidiosa* in some percentage of cold treated *V. vinifera* grapevines, however some of the coldest temperatures he used killed the vines (Purcell 1977, 1980). He also found that a higher percentage of vines that were moderately susceptible to PD such as Cabernet sauvignon, were cured by cold therapy treatments compared to susceptible varieties such as Pinot noir. Purcell's group also showed that whole, potted vines exposed to low temperatures had a higher rate of recovery than PD-affected, detached bud sticks exposed to the same cold temperatures (Feil, 2002). Clearly, some factor(s) that were expressed in the intact plant, but not in detached bud sticks, helped eliminate *Xf* from the plants. Our objective is to elucidate the physiological/biochemical basis that mediates cold therapy and to identify the physiological/biochemical factor(s) that occur or are expressed in cold treated vines that eliminate *Xf*. If such factor(s) are found, it may be possible to induce their expression under non-freezing temperatures and potentially provide a novel approach for managing PD.

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

RESULTS AND CONCLUSIONS
Objective 1
The same varieties used by Purcell (1977, 1980) and Feil (2002) in previous cold therapy studies, Pinot noir (PD-susceptible) and Cabernet sauvignon (moderately resistant to PD) grapevines grafted on 101-14 rootstock were inoculated with *Xf* in the spring of 2004 using a pinprick inoculation procedure (Hill and Purcell, 1995; Purcell and Saunders, 1999). The vines were grown in five gallon pots in a greenhouse using a nutrient-supplemented irrigation regime. Treatment vines were inoculated with the Stagg's Leap strain of *Xylella fastidiosa*, whereas control vines were inoculated with water. During late summer and fall, the plants were moved into a screen house in order to acclimatize them to decreasing temperatures. While in the screen

Figure 2. GWSS killed by *B. bassiana* and *M. anisopliae.*

Bioassay 3

This assay was conducted using only 10^9 conidia/mL concentration and 10 laboratory-reared GWSS per isolate. All the isolates from the previous bioassay were used in this assay except for PcBb1, which was replaced by the *B. bassiana* isolate from *S. festinus* (SfBb1). This assay had also suffered from very high mortality and all the insects died within 5 days after the treatment. Fungal infection was seen in only one GWSS cadaver treated with SfBb1.

CONCLUSIONS

The fact that GWSS is susceptible to entomopathogenic fungi such as *B. bassiana* is promising. Although infections occurred only at relatively high concentrations, there is enough variability in *B. bassiana* as a species to suggest other isolates may be more virulent. Efforts will continue to obtain isolates from collaborators and from likely GWSS host habitat in California for further laboratory evaluation and eventual field application.

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FUNDING AGENCIES

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a

b

Isolation of Fungal Pathogens

Soil samples were collected from an organic citrus orchard and a conventional pomegranate orchard in Tulare Co, CA and a citrus orchard at AgOps at UC Riverside. Fungal pathogens were isolated using larvae of the greater wax moth, *Galleria mellonella* L. and by soil plating on selective media. Waxworms were incubated in Petri plates with moist soil samples and fungal pathogens were isolated from cadavers. Alternatively, aliquots of soil suspensions were plated on media selective for *B. bassiana* and *Metarhizium anisopliae* (Metschnikoff) Sorokin. So far, 140 *B. bassiana* isolates and 4 *M. anisopliae* isolates have been isolated (Table 1). Additionally, *B. bassiana* was also isolated from the California harvester ant, *Pogonomyrmex californicus* Buckley, collected in Shafter, CA and the three-cornered alfalfa hopper, *Spissistilus festinus* (Say), collected in Parlier, California. Fungal isolates were cultured on selective and non-selective media to multiply the inoculum.

Table 1. Fungal pathogens isolated from citrus and pomegranate orchards and infected insects

Source	Method	*B. bassiana*	*M. anisopliae*
Organic citrus in Tulare Co	Waxworm bait	37	-
Pomegranate in Tulare Co	Waxworm bait	3	4
Riverside citrus	Waxworm bait	78	-
Riverside citrus	Selective media	22	-
California harvester ant	Selective medium	1	N/A
Three-cornered alfalfa hopper	Selective medium	1	N/A

Pathogenicity of Entomopathogenic Fungi to GWSS

Laboratory-reared or field-collected GWSS adults supplied by CDFA, Arvin were used for the bioassays. GWSS were either placed at -5^{o} C for 5 min or exposed to CO_2 for 15 sec to immobilize them and were inoculated by rolling them in a 10 µL drop of conidial suspension. Controls were treated with 0.01% of SilWet, an adjuvant used for preparing conidial suspensions. GWSS were individually incubated in a Petri plate with an excised citrus leaf and a moist filter paper. Petri plates were placed in a plastic box with moist paper towels and incubated at 27^{o} C and 16:8 L:D photophase. GWSS were observed daily for mortality. Dead GWSS were surface sterilized in 3% sodium hypochlorite solution followed by rinsing in deionized water and incubated in sealed Petri plates on water agar or moist filter paper at 27^{o} C in the dark.

Bioassay 1

The isolate of *B. bassiana* from *P. californicus* (PcBb1) was tested against laboratory-reared GWSS at four concentrations 10^1, 10^3,10^5, and 10^7 conidia/ml in comparison with controls. Each treatment and control had 10 adult GWSS. Infections were observed only at higher concentrations with 50% infection in GWSS treated with 10^7 conidia/ml and 10% in those treated with 10^5 conidia/ml.

Bioassay 2

Five *B. bassiana* isolates and a *M. anisopliae* isolate were tested against field-collected GWSS at four concentrations of 10^3, 10^5,10^7, and 10^9 (or 10^8 in case of *M. anisopliae*) conidia/ml along with untreated and SilWet (0.01%) treated controls. Isolates of *B. bassiana* included one from *P. californicus* (PcBb1), two from soil samples from citrus orchards in Tulare (GmBb25) and Riverside (GmBb41) counties, CA, one from *H. coagulata* in Weslaco, TX (TxBb) and a commercial isolate (designated GHA). The isolate of *M. anisopliae* (GmMa1) was from a soil sample from the pomegranate orchard in Tulare Co, CA. Each treatment and controls had 20 GWSS. Although all tested isolates were infective (Figures 1 and 2), all GWSS in this bioassay, including controls, suffered from a high mortality.

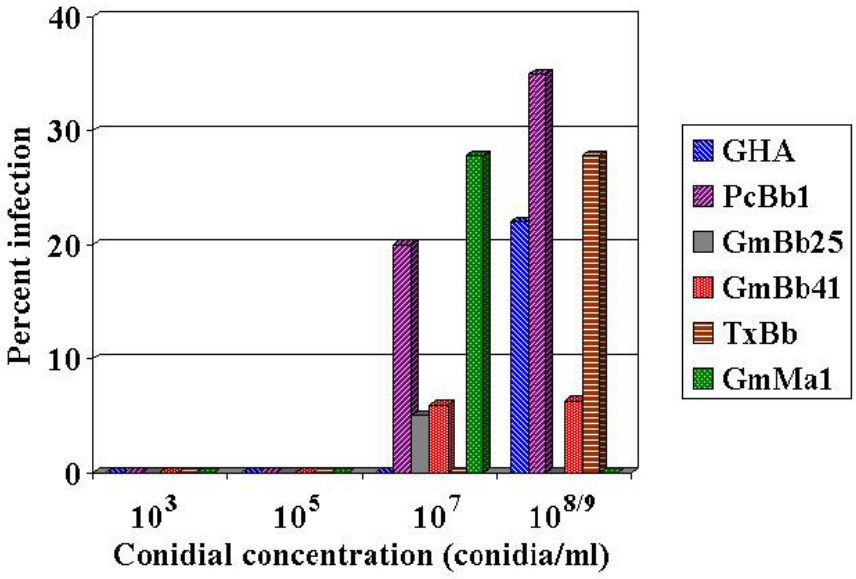

Figure 1. Pathogenicity of *B. bassiana* and *M. anisopliae* to GWSS

MICROBIAL CONTROL OF THE GLASSY-WINGED SHARPSHOOTER WITH ENTOMOPATHOGENIC FUNGI

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

ABSTRACT
Objectives of our study were to search for fungal pathogens of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say) and evaluate their potential against the host. Searches within citrus orchards in Tulare and Riverside counties revealed no natural infections of entomopathogenic fungi in GWSS populations. Entomopathogenic fungi were also absent in cadavers of GWSS periodically collected from Riverside citrus orchards (courtesy CDFA) when incubated in the laboratory under ideal conditions for fungal emergence. However, about 140 isolates of *Beauveria bassiana* (Balsamo) Vuillemin and four isolates of *Metarhizium anisopliae* (Metschnikoff) Sorokin, both hyphomycetous fungi, were isolated from soil in GWSS habitats and other insect hosts. Some of these isolates along with a Weslaco isolate of *B. bassiana* from GWSS and a commercial *B. bassiana* isolate have been tested against GWSS. Preliminary results indicate that GWSS is susceptible to high concentrations of these fungi.

INTRODUCTION
The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say), native to the southeastern United States, is a serious pest of the California grape industry because it vectors *Xylella fastidiosa* (Wells et al. 1987), a xylem-limited bacterium that causes Pierce's disease (PD). Although PD has been in California for a long time, the introduction and rapid spread of GWSS made the situation worse. In addition to grapes, GWSS has a wide host range and spreads various diseases in those hosts caused by *X. fastidiosa*. Vector control or avoidance has been a key tactic in controlling PD. Widely practiced chemical control with imidacloprid and application of kaolin particles have their limitations. While kaolin particles, although non-toxic, can leave unwanted deposits on the harvested grape bunches, chemical insecticides have undesirable effects including human health, impact on non-target organisms, and environmental concerns. Moreover, use of chemical insecticides in citrus disrupts the successful, long-term control afforded by IPM of many different citrus pests (Grafton-Cardwell and Kallsen 2001). Use of microbial agents, such as entomopathogenic fungi, can be a viable alternative that is compatible with IPM practices. Entomopathogenic fungi invade the host by penetrating through the integument and are appropriate candidates for GWSS that has piercing and sucking mouthparts.

Entomopathogenic fungi have been isolated from GWSS (Mizell and Boucias 2002, Jones - personal communication) and other cicadellids (Galaini-Wraight et al. 1991, Hywel-Jones et al. 1997, Magalhaes et al. 1991, Matsui et al. 1998, McGuire et al. 1987). The purpose of our study is to discover additional isolates of entomopathogenic fungi active against GWSS.

OBJECTIVES
1. Conduct surveys to find fungal infections in GWSS populations or insects closely related to GWSS and isolate soilborne entomopathogens from GWSS habitats.
2. Culture and isolate the fungi and evaluate their pathogenicity against GWSS.
3. Evaluate the host range of fungi that infect GWSS.
4. Conduct small-scale field tests to evaluate selected pathogens against GWSS on citrus in fall and winter.

RESULTS
Natural Infections in GWSS Populations
Citrus orchards in Tulare and Riverside counties were surveyed, in vain, for infected GWSS. GWSS cadavers from CDFA collections in the Riverside area were periodically obtained and incubated in the laboratory for fungal development. No entomopathogenic fungus has so far been found from these cadavers. However, cultures of *Beauveria bassiana* (Balsamo) Vuillemin from infected GWSS collected in Texas by Jones and *Hirsutella* spp collected in Florida by Mizell and Boucias were received in the past two months for testing against California GWSS.

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4. mature cecropin A with no signal peptide sequence

The authenticity of the PCR-amplified sequences was confirmed by nucleotide sequencing in both directions and the constructs are currently being used to generate transgenic *A. thaliana* by standard procedures.

Table 1. Effect of cecropins and kanamycin against the growth of *X. fastidiosa*

	Concentration (μM)	Increase in bacterial concentration in comparison to cultures lacking antibiotic		
		Week 1 (% ± s.d.)	Week 2 (% ± s.d.)	Week 3 (% ± s.d.)
cecropin A	0.5	69 ± 3	47 ± 47	64 ± 42
	0.25	72 ± 10	80 ± 21	117 ± 5
	0.1	103 ± 13	68 ± 2	87 ± 25
	0.05	110 ± 46	50 ± 1	91 ± 22
cecropin B	0.5	69 ± nd	114 ± 6	87 ± 45
	0.25	63 ± 31	75 ± nd	110 ± 15
	0.1	72 ± 101	128 ± 63	90 ± nd
	0.05	93 ± 17	101 ± 18	74 ± 10
cecropin P1	0.5	98 ± 18	70 ± 40	70 ± 62
	0.25	82 ± 18	98 ± nd	120 ± 17
	0.1	111 ± 52	93 ± 24	72 ± 24
	0.05	93 ± 10	99 ± 22	73 ± 18
kanamycin	2	11 ± 3	9 ± 8	16 ± 2
	1	19 ± 8	32 ±39	33 ± 22
	0.5	42 ±16	77 ± 9	103 ± 16
	0.25	60 ± 13	72 ± 17	105 ± 12

nd = not determined

Table 2. Effect of recombinant cecropin A on the growth of *E. coli*

Source of recombinant cecropin A	Inoculum dose (bacteria/mL)	Inhibition (%)
Sf21 cell pellet (1 x 10^5 cells)	1.1 x 10^3	3.1 ± 13.2
Sf21 cell supernatant (undiluted)	1.1 x 10^3	99.7 ± 0.1
Sf21 cell supernatant (undiluted)	1.0 x 10^4	57.9 ± 1.6
Sf21 cell supernatant (undiluted)	8.5 x 10^4	51.6 ± 0.2
Sf21 cell supernatant (undiluted)	7.3 x 10^5	13.1 ± 0.1
Sf21 cell supernatant (1:5 diluted)	7.0 x 10^5	11.1 ± 0.2
Sf21 cell supernatant (1:10 diluted)	7.0 x 10^5	2.5 ± 0.1

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some Gram(+) bacteria, but are inactive against eukaryotic cells at concentrations that are antimicrobial [4, 9, 11] and possibly at concentrations up to 300 times higher [8]. *X. fastidiosa* is a Gram(-) bacterium [12]. In Gram(-) bacteria, the antibacterial activities of cecropins A, B, and P1 are up to ten-times greater than tetracycline [9, 13]. Cecropins have a unique combination of characteristics (specificity, gene basis, small size, potency against Gram(-) bacteria, etc.) that may make them potentially ideal substances for the control of *X. fastidiosa* in GWSS.

OBJECTIVES

I. Identify peptide antibiotics (cecropins) that are effective against *Xylella fastidiosa*
   i. Determine the antibiotic sensitivity of *X. fastidiosa* to chemically synthesized cecropins
   ii. Produce recombinant cecropins using baculovirus expression vectors
   iii. Determine the toxicity of cecropins against GWSS cells grown in culture
II. Analyze the effectiveness of cecropins produced in transgenic *Arabidopsis*
   i. Generate transgenic *Arabidopsis* expressing cecropin that is active against *X. fastidiosa*
   ii. Determine the localization, yield, activity, and stability of plant-expressed cecropin
   iii. Analyze the effect of cecropin expression on the transgenic *Arabidopsis*
   iv. Analyze the effectiveness of plant-expressed cecropin for the control of *X. fastidiosa* transmission

RESULTS AND CONCLUSIONS

In order to establish the optimal conditions for the growth, storage, and assay of *X. fastidiosa* (Temecula strain) in our laboratory, we tested three different media (PD3, PW, and GYE; see [14]) and various inoculation routines. In general, our procedures were modified from protocols established in the Bruce Kirkpatrick laboratory at U.C. Davis. Optimal conditions for the generation of bacterial (*X. fastidiosa*) lawns for agar disc diffusion assays were also determined. Of the three media that were tested, PD3 gave the fastest growth of *X. fastidiosa* in liquid medium (roughly 20-and 135-fold increases in the OD_{600} at 7 and 14 days post inoculation, respectively) and on agar plates (formation of a lawn by 7-10 days post seeding). In order to generate a lawn, 150 µL of a 14 day-old culture (OD_{600}=0.48-0.5) was spread onto a 10 cm-diameter plate containing PD3 agar medium.

Using the optimal growth conditions with PD3 medium, we examined the minimal inhibitory concentration (MIC assay) at which cecropins A, B, and P1 (commercially purified peptides) were effective in inhibiting the growth of *X. fastidiosa*. We found that cecropins A, B, and P1 were effective at partially inhibiting the growth of *X. fastidiosa* at concentrations that were equal to or greater than 0.05, 0.25, and 0.5 µM, respectively, at two weeks post inoculation (Table 1). In general, cecropin A was the most effective against *X. fastidiosa*. The effectiveness of the cecropins as well as kanamycin was reduced by three weeks post inoculation. This was speculated to be the result of antibiotic degradation.

Once the sensitivity of *X. fastidiosa* to the various cecropins was established, a codon-optimized (for *A. thaliana*) cecropin A gene (pro gene including the insect-derived signal peptide sequence) was synthesized using commercially synthesized oligomers. A comparison of the *A. thaliana*-optimized (upper) and authentic (lower) cecropin A gene sequences is as follows:

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ATGAACTTCTCTAGAATCTTCTTCTTCGTGTTCGCTTGCCTCACTGCTCTCGCTATGGTGAACGCTGCTCCTGAGCCTAAGTGGAAGCTCTTCAAGAAGA 100
ATGAACTTTTCGAGGATCTTTTTCTTCGTCTTCGCTTGCCTGACGGCTCTAGCAATGGTCAATGCGGCGCCGGAACCTAAATGGAAGTTATTCAAGAAGA 100
******** ** ** ***** ******** *********** ** ***** ** ***** ** ** ** ** ** ***** ****** * **********

TCGAGAAAGTGGGTCAGAACATCAGAGATGGAATCATCAAGGCTGGACCAGCTGTGGCTGTGGTGGGACAGGCTACACAGATCGCTAAGGGTTGA 195
TTGAGAAAGTCGGTCAGAACATTCGAGATGGCATCATCAAAGCTGGCCCAGCCGTCGCTGTTGTAGGCCAGGCAACACAGATTGCTAAGGGTTAA 195
**  ******* ******** ***** ***** ** ***** ** ** ***** ******* ******** *********** ********** *
```

Of the 195 nucleotides that encode the pro gene, 33 nucleotides were mutated for optimal expression in *A. thaliana* (and putatively in grape stock). The synthesized gene was directionally cloned into the baculovirus transfer vector pAcUW21 at the *Bgl*II and *Eco*RI sites. Subsequently, the recombinant transfer vector was used to generate a recombinant baculovirus (vAcCecA) expressing the cecropin A gene using standard procedures. Expression of biologically active cecropin A was confirmed by minimal inhibitory concentration assays using *E. coli* by comparison of vAcCecA- or wildtype AcMNPV-infected insect Sf-21 cell culture supernatants or cell extracts (Table 2). These experiments confirmed that the synthetic gene encoded a functional peptide and that this peptide was correctly processed in insect-derived cells. vAcCecA expressed high levels (roughly 90 mg/liter of insect cell culture (2×10^6 cells/mL)) of cecropin A.

Following confirmation that the synthetic gene produces biologically active cecropin A, the synthetic gene was inserted into the pCAMBIA1305 series of plasmid vectors in order to express the cecropin A in transgenic *A. thaliana* (and eventually grape stock). Four different recombinant pCAMBIA1305 vectors were generated by PCR-amplification as follows:

1. pro cecropin A sequence with authentic insect signal peptide sequence
2. pro cecropin A sequence with rice glycine rich protein and authentic insect signal peptide sequences
3. mature cecropin A sequence with rice glycine rich protein signal peptide sequence

DEVELOPMENT OF PEPTIDE ANTIBIOTIC-BASED CONTROL STRATEGIES FOR *XYLELLA FASTIDIOSA*

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ABSTRACT
Peptide antibiotics are short (generally less than 70 amino acid residue-long), pore forming peptides encoded by single genes. Because peptide antibiotics are 'gene-based' they can be produced directly at the target location where they are needed (e.g., grape stock). In this project, we are testing the hypothesis that peptide antibiotics such as cecropins A, B, and/or P1 can be used as an effective means to control or reduce the spread of *Xylella fastidiosa*-induced disease. During the reporting period, we have established the optimal growth and assay conditions for the *X. fastidiosa* bacterium. Under these optimal conditions, we found that cecropin A can effectively inhibit *X. fastidiosa* growth over a two-week period (initial concentration of 0.05μM). Longer-term growth inhibition was seen only when higher concentrations of cecropin A were used suggesting that the cecropin A is being degraded under the conditions of our assay. On the basis of the effectiveness of cecropin A against *X. fastidiosa*, a synthetic plant codon (i.e., *Arabidopsis thaliana*) optimized, cecropin A gene was synthesized. The product of this synthetic cecropin A gene was expressed using the baculovirus expression vector system (BEVS) in insect cells. In insect cells roughly 90 mg/liter of culture of biologically active cecropin A was produced by the recombinant baculovirus. Following confirmation of biological activity of the insect cell produced cecropin A, the synthetic cecropin A gene was inserted into the pCAMBIA1305 series of plasmid vectors in order to express the cecropin A in transgenic *A. thaliana* and eventually grape stock. Four different recombinant pCAMBIA1305 vectors were generated (carrying either the pro or mature cecropin A gene fused to either an authentic insect- or plant- (rice glycine-rich protein) derived signal peptide sequence). We are currently in the process of generating transgenic *A. thaliana* using these pCAMBIA vectors. We believe that continuous expression (although potentially at relatively low levels) of cecropin A will be effective for reducing or inhibiting the growth of *X. fastidiosa* within the plant.

INTRODUCTION
Traditional antibiotics are natural or chemically synthesized small molecules that can selectively kill or stop the growth of bacteria. Antibiotic inhibition of *X. fastidiosa* (at least 17 isolates tested) has been analyzed for six different antibiotics (ampicillin, kanamycin, neomycin, penicillin, streptomycin, and tetracycline) [1, 2]. These studies demonstrate that antibiotic treatment is potentially an effective method for the control of *X. fastidiosa*. Under field conditions, however, barriers between the antibiotic and bacterium, and degradation effects will require significantly higher application doses than those found effective in the laboratory. Such doses may be impractical especially for broad-spectrum antibiotics due to secondary effects (e.g., toxicity against mammalian red blood cells) and the risk of increasing resistance. Thus, although traditional antibiotics such as tetracycline are highly active, an effective delivery system to bring them in contact with *X. fastidiosa* in the plant or insect vector is not available.

Recently, a great deal of scientific effort is being put into the study of a second type of antimicrobial agent called peptide antibiotics. Peptide antibiotics have been identified from a wide range of organisms including bacteria, fungi, plants, insects, birds, crustaceans, amphibians and mammals. In general, peptide antibiotics are small (less than 50 amino acids), have a net positive charge, and are composed of 50% or more of hydrophobic amino acids [3, 4]. One class of peptide antibiotic is composed of so-called ribosomally synthesized peptides [5]. These peptides are encoded by single genes and synthesized by a protein complex (ribosome) that is found in all cells and processed following synthesis via common pathways [3, 6]. In other words, unlike traditional antibiotics, peptide antibiotics have the potential to be easily produced by common protein expression systems or in transgenic organisms (e.g., plants). Furthermore, because peptide antibiotics are "gene-based", they can be produced directly at the location where they are needed and their synthesis can potentially be regulated by using appropriate gene promoters.

Some of the best-characterized peptide antibiotics are the cecropins. Cecropins were the first peptide antibiotics to be identified in an animal, the giant silkmoth *Hyalophora cecropia* [7, 8]. At least ten different cecropins have been isolated from lepidopteran (moths and butterflies) and dipteran (flies) insects [9, 10]. Cecropins are composed of a single chain of 35-39 common L-amino acids and do not contain disulfide bonds [10]. Cecropins are active against many Gram(-) bacteria and

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South Carolina whereas GWSS could not be found in the forested hills and mountains of northern Georgia, eastern North Carolina, Kentucky, and Tennessee, where only a few adult *O. orbona* as well as its old egg masses (all with evidence of parasitization) were collected.

Our survey also benefited greatly from the exploratory work by Roman Rakitov, who reared mymarid and trichogrammatid egg parasitoids of several species of the genus *Cuerna* (other than *C. costalis*). Particularly, the mymarid *Anagrus epos* Girault was reared by Roman Rakitov near Glyndon, Clay Coounty, Minnesota, from egg masses of a *Cuerna* sp. and sent to UCR quarantine facility under a permit. This is the first representative of the genus *Anagrus* ever reared from eggs of a proconiine sharpshooter. We were able to establish a quarantine colony of this species on eggs of GWSS, which is a fictitious host for *A. epos* (GWSS does not occur in Minnesota). *Anagrus epos* is a gregarious species: 3-5 adult wasps emerged from smaller eggs of the original host, *Cuerna* sp., whereas up to 10-12 adult wasps emerged from larger eggs of GWSS. Under quarantine laboratory conditions (temperature 24°C, RH ca. 50%), the first two generations of *A. epos* developed from egg to adult within 20-21 days; for unknown reasons, it took the next two generations much longer (more than 30 days) to develop under the same conditions. Currently, this species is under quarantine evaluation as a potential biocontrol agent against GWSS in California.

Table 1. Species of egg parasitoids collected during 2004 and sent to University of California, Riverside quarantine.

Genus and species of egg parasitoid	Originally from: (State: locality)	Original or probable sharpshooter host	Propagated on GWSS at UCR quarantine (Yes/No)
Acmopolynema sema Schauff (Mymaridae)	GA: nr. Centerville	*?Homalodisca insolita* (Walker)	No
Gonatocerus ashmeadi Girault (Mymaridae)	GA: nr. Centerville	*H. coagulata / O. orbona*	No
	GA: Byron	*H. coagulata / O. orbona*	Yes
	NC: Garner	*H. coagulata*	No
	NC: North Myrtle Beach	*? H. coagulata*	No
	NC: nr. Warsaw	*H. coagulata*	No
	SC: Charleston	*H. coagulata*	Yes
	SC: nr. Yemassee	*H. coagulata / O. orbona*	Yes
Gonatocerus fasciatus Girault (Mymaridae)	GA: nr. Centerville	*H. coagulata / O. orbona*	No
	GA: Byron	*H. coagulata / O. orbona*	No
	NC: Garner	*H. coagulata*	No
	NC: nr. Greensboro	*?O. orbona*	No
	NC: nr. Warsaw	*H. coagulata*	No
Zagella spirita (Girault) (Trichogrammatidae)	GA: Byron	*H. coagulata / O. orbona*	No (failed)
Ufens new species (Trichogrammatidae)	GA: Byron	*H. coagulata / O. orbona*	No (failed)
Paracentrobia acuminata (Ashmead) (Trichogrammatidae)	GA: nr. Centerville	*?H. insolita/ ?Cuerna costalis*	No

Objective 2

As a result of the exploratory work conducted during the reported period, numerous specimens of proconiine sharpshooters and of their egg parasitoids were collected and preserved in ethanol with appropriate labels; many of these were critically point-dried from ethanol, point- or card-mounted, labeled, and identified to genera and species. Representatives of some species (of both sexes) were selected, dissected, and slide-mounted. The specimens were deposited in the collections of Entomology Research Museum, UC Riverside.

CONCLUSIONS

This is the next step in the development of a "classical" biological control program for the reduction of glassy-winged sharpshooter (GWSS) densities in California as a cornerstone for an IPM program to manage GWSS. As the result of our surveys conducted during 1997-2004, several previously unknown proconiine sharpshooter host associations were discovered for various species of Mymaridae and Trichogrammatidae. We concluded searching for egg parasitoids of GWSS in the Nearctic part of its distribution range. Next year, our exploratory efforts will focus on the southernmost part of the distribution range of GWSS in southern Mexico, which is in the Neotropical region.

SEARCHING FOR AND COLLECTING EGG PARASITOIDS OF GLASSY-WINGED SHARPSHOOTER IN THE CENTRAL AND EASTERN USA

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Reporting Period: The results reported here are from work conducted from January 1, 2004 to October 6, 2004.

ABSTRACT
Search for egg parasitoids of proconiine sharpshooters (Hemiptera: Clypeorrhyncha: Cicadellidae: Cicadellinae: Proconiini) in central and eastern USA during 2003 and 2004 resulted in rearings of several species of Mymaridae and Trichogrammatidae (Hymenoptera) (Table 1). Cultures of some species, notably of *Anagrus epos* Girault, were established at UCR quarantine.

INTRODUCTION
Presence of the proconiine sharpshooters *Homalodisca coagulata* (Say) (GWSS - the Glassy-winged Sharpshooter) and its close relative *Oncometopia orbona* (Fabricius) (the Broad-headed Sharpshooter) in central and eastern United States justified conducting a survey of their principal natural enemies, egg parasitoids in the families Mymaridae and Trichogrammatidae. No such surveys have ever been conducted North of central Georgia, Mississippi, Louisiana, and Texas. Prior research showed presence of the mymarid *Gonatocerus fasciatus* Girault there (Triapitsyn et al. 2003). A number of trichogrammatid genera and species were recognized in southeastern USA from eggs of a grass-inhabiting *Cuerna costalis* (Fabricius), also a proconiine sharpshooter, as well as from *H. coagulata* and *O. orbona* (Triapitsyn 2003).

OBJECTIVES
1. **Exploratory work** - Search for and collect egg parasitoids of proconiine sharpshooters in the northern- and eastern-most home range of GWSS, *Oncometopia* spp., and *Cuerna* spp. for introduction into California, establishment of cultures in UCR quarantine, and a following evaluation.
2. **Curatorial work** – Curate the collected voucher specimens of mymarid and trichogrammatid egg parasitoids.

RESULTS
Objective 1.
The first exploratory trip was made to Kentucky and Tennessee by S. Triapitsyn in July 2003 (Hoddle & Triapitsyn 2003). The second trip to Illinois (the northernmost distribution range of *Oncometopia orbona* and *Cuerna costalis*), eastern Kentucky, and south-central Tennessee was made by S. Triapitsyn in April 2004, in an attempt to locate and collect the overwintered and egg-laying adults of *C. costalis*. Part of the trip (in southern Illinois) was made together with Roman Rakitov, who showed his methods of collecting *C. costalis* in known localities where this species had been collected in the past (occurrence of proconiine sharpshooters there is spotty). We were able to collect several adults of *C. costalis* in one locality in Shawnee National Forest, on a private meadow. Yellow pan traps were placed in this locality and we managed to collect a specimen of *Gonatocerus novifasciatus* Girault (Mymaridae), a known parasitoid of *H. coagulata* elsewhere. There it most probably is parasitoid of *Cuerna costalis*, the only proconiine sharpshooter occurring on that meadow. This gave us a hint what species of egg parasitoids occur there despite the fact that it is practically impossible to find egg masses of this proconiine sharpshooter when its density is so low. Also parasitoids and leafhoppers were collected there using vacuum. In several locations in southern Illinois, both methods revealed frequent presence of *Gonatocerus rivalis* Girault and its likely host, *Draculacephala antica* (Walker) (determined by Roman Rakitov). *Draculacephala* is a cicadelline (tribe Cicadellini) sharpshooter genus, which members were the most abundant leafhoppers of the subfamily Cicadellinae in all three states visited. This could be an apparent new host association for this species of *Gonatocerus*, which is a member of the *sulphuripes* species group.

Subsequent trips to Georgia, North Carolina, and South Carolina in June and August 2004 by S. Triapitsyn resulted in collections of several mymarid and trichogrammatid species, listed in Table 1, which were reared from egg masses of proconiine sharpshooters. Quarantine colonies of *Gonatocerus ashmeadi* Girault from Georgia and South Carolina were discontinued several generations following their establishment because it was shown that this species is morphologically, biologically, and genetically homogenic throughout its range (Vickerman et al. 2004). Both GWSS and to some degree *O. orbona* were found to be abundant almost everywhere in the lowlands (especially coastal) in Georgia, North Carolina, and

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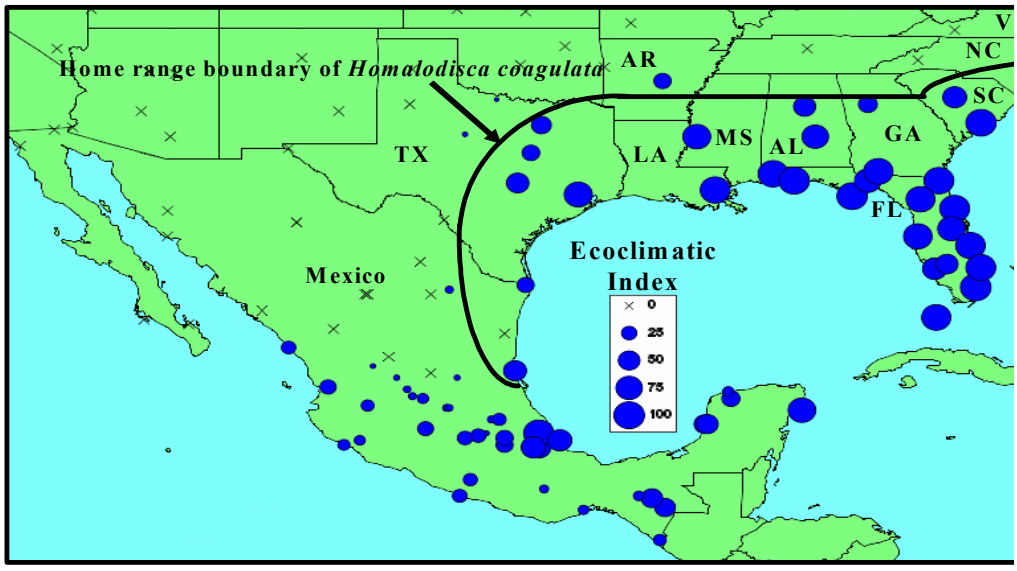
Triapitsyn, S. V. and P. A. Phillips. 2000. First host record of *Gonatocerus triguttatus* (Hymenoptera: Mymaridae) from eggs of *Homalodisca coagulata* (Homoptera: Cicadellidae), with notes on the distribution of the host. Florida Entomologist 83: 200-203.

FUNDING AGENCIES

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

the proposed exploratory work; these will need to be critically point-dried from ethanol, point- or card-mounted, labeled, slide-mounted, and identified to genera and species. DNA analyses will be conducted if necessary.

Figure 1. Current and CLIMEX-predicted geographical range of GWSS. Large blue dots indicate good climatic conditions for GWSS. Small blue dots are marginal habitats. x on map indicate unsuitable areas.



CONCLUSIONS

Research to be conducted in the course of this project will be of benefit primarily to the CDFA GWSS Biological Control Program as well as to other biocontrol specialists and agencies conducting projects against GWSS in California such as the USDA-APHIS. Ultimately, we hope that this project will be beneficial to California's agriculture.

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SEARCHING FOR AND COLLECTING EGG PARASITOIDS OF THE GLASSY-WINGED SHARPSHOOTER AND OTHER *HOMALODISCA* SPECIES IN SOUTHEASTERN AND SOUTHWESTERN MEXICO

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Reporting Period: The results reported here are from work conducted from July 1, 2004 to October 6, 2004.

ABSTRACT
According to the proposed (and approved) research timetable, work on this project will commence as early as in January 2005, when we may have the first chance to collect parasitized egg masses of *Homalodisca* spp. in Mexico. This report is only for information purposes about this project.

INTRODUCTION
Egg parasitoids of the Glassy-winged Sharpshooter (GWSS), *Homalodisca coagulata* (Say), were discovered through survey activities conducted throughout USA and northeastern Mexican states of Nuevo Leon and Tamaulipas, which resulted in collection, introduction, and release in California of several species of mymarid eggs parasitoids (*Gonatocerus* spp.) (Morgan et al. 2000; Triapitsyn et al., 2002; Triapitsyn & Hoddle, 2001, 2002). During 2003 and 2004, we conducted a survey of egg parasitoids of GWSS in central and eastern USA (Hoddle & Triapitsyn, 2003, 2004). According to McKamey (2002), the native host range of GWSS also includes central and southern Mexico, well beyond the currently known range mapped by Triapitsyn & Phillips (2000). McKamey's (2002) report is supported by the CLIMEX-predicted distribution range of GWSS (Hoddle 2004; also Map below).

Here we propose the final step in the development of a classical biological control program against GWSS in California: to search new climatically suitable areas in Mexico for GWSS parasitoids. Additionally, our previous exploratory work in Mexico (in the States of San Luis Potosí, Tamaulipas and Veracruz) during 1999-2003 resulted in the discovery of at least two new, undescribed species of *Homalodisca* egg parasitoids, which are related to *G. ashmeadi* Girault and *G. morrilli* (Howard) but differ from those both morphologically (Triapitsyn et al. 2002) and genetically (D. Vickerman, unpubl. data). These parasitoids need to be recollected in Mexico and tested as potential biological control agents against GWSS in California.

OBJECTIVES
This project has two main objectives:
1. Search for and collect egg parasitoids in southern-most home range of GWSS and other *Homalodisca* species in southeastern and southwestern Mexico; and
2. Introduction and establishment of quarantine cultures of the selected species and their following initial evaluation for potential establishment in California.

RESULTS
There are no results to be reported at this time. The following experimental procedures will be used to accomplish the above objectives:

Exploratory Work.
Search for and collect egg parasitoids of southern-most home range of GWSS and other *Homalodisca* species (in the Mexican states of Tamaulipas (southern part), Veracruz, San Luis Potosí, Campeche, Oaxaca, Yucatán, and Quintana Roo) for introduction into California, establishment of cultures in UCR quarantine, and a following evaluation. Several short exploratory trips will be made to those states during winter and spring 2005 and parasitized egg masses of *Homalodisca* will be collected there and sent to UCR quarantine facility under the existing permit. The two already known egg parasitoids of GWSS from Tamaulipas and adjacent Mexican states (*G.* near *ashmeadi* and *G.* near *morrilli*) will be recollected from known localities.

Quarantine and Identification Work
Colonies of the selected parasitoids will be established in UCR quarantine using GWSS as a host (fresh egg masses will be supplied by David Morgan). Voucher specimens of the collected parasitoids will require appropriate curation as a result of

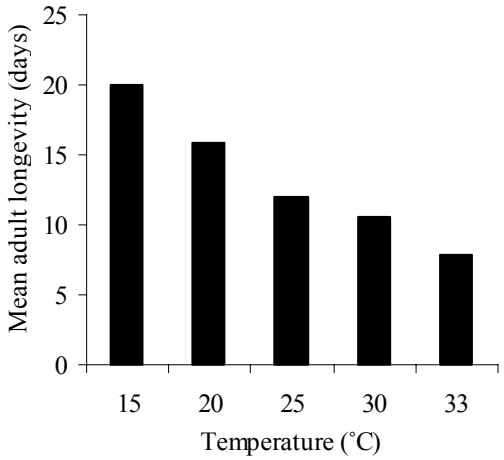

Figure 5. The average time, in days, from when mated females used in the study first emerged to when they died of natural causes.

CONCLUSIONS

The wasps at 30°C died quicker (figure 5) and laid fewer eggs (figure 3) than wasps at 25°C. This difference was offset by the findings that the individuals at 30°C successfully utilized a higher percentage of the eggs that were made available to them than those at 25°C. Whilst individuals at 30°C produced fewer viable offspring, it is possible that as a population effect greater numbers of offspring may be produced due to a faster generation turnover and higher rate of parasitism overall. Wasps at 30°C will cause a population to grow at a much faster rate due to the wasp ovipositing in, largely, an equal number of eggs. The success of the wasp at this temperature is indicative of the much shorter developmental times whereby the wasp will produce offspring that develop at much faster rates. Individual wasps surviving for extended periods of time were observed at 15°C and declining in a linear manner as temperature rose. Whilst wasps at 15°C, for example, survived considerably longer than at other temperatures their efficacy was affected by the temperature and made very little impact on the number of offspring produced.

The success of a biological control agent is measured by the mortality it inflicts on its target which is in part a function of its reproductive and developmental activity across a range of temperatures (Nahrung and Murphy, 2002). The results from this study suggest that *G. ashmeadi* operates most effectively at moderate to high temperatures. Identifying the optimal temperature for reproduction and developmental of *G. ashmeadi*, will greatly aid mass-rearing efforts, using day-degree models to predict geographic range, to assess generational turnover in various locales in comparison to GWSS and to optimize releases of natural enemies into a field environment.

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Nahrung H.F., Murphy, B.D., 2002. Differences in egg parasitism of *Chrysophtharta agricola* (Chapuis) (Coleoptera : Chrysomelidae) by *Enoggera nassaui* Girault (Hymenoptera : Pteromalidae) in relation to host and parasitoid origin. Austral. J. Entomol. 41, 267-271.

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FUNDING AGENCIES

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

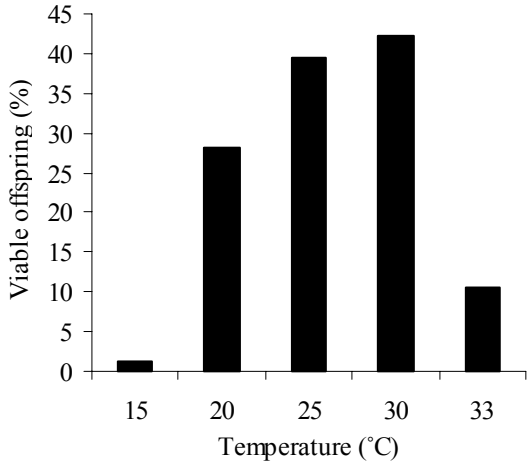

Figure 1. Mortality rates fell as temperatures rose until 30°C. Few viable offspring were produced at 33°C. The highest percentage of viable offspring from available eggs was at 30°C.

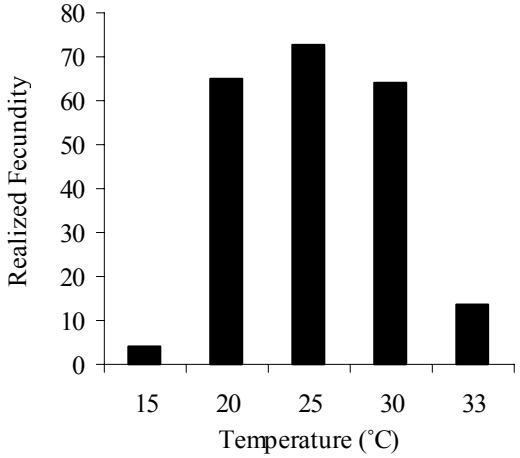

Figure 2. The average number of offspring emerging from parasitized eggs at each temperature. Parasitized eggs that did not yield viable offspring are not represented here.

The number of offspring produced by individual wasps over their lifetime was greatest at 25°C and fell sharply as temperature either increased of decreased (Figure 2). Approximately 73 offspring were produced by wasps at 25°C down to an average of around 4 and 14 at 15°C and 33°C, respectively. These data show that at constant high or low temperatures wasps fail to produce many offspring and may have little or no impact on GWSS population growth as a consequence.

There appeared to be no trends to the ratios of females produced at each experimental temperature (Figure 3). The highest percentage of females was produced at 25°C with approximately 70% of offspring being female. All other temperatures were, with the exception of 20°C, were within 10% of this temperature. These results indicate that temperature may not play an important role in the sex selection of *G. ashmeadi* offspring.

The time between eggs being made available to individual wasps and the emergence of offspring, fell from a high of approximately 39 days at 15°C to approximately 10 days for 30 – 33°C (Figure 4). As temperature rose, the time required for the development of wasp larvae was reduced. This faster development time at higher temperatures suggests that wasps will cycle through several generations in comparison to GWSS.

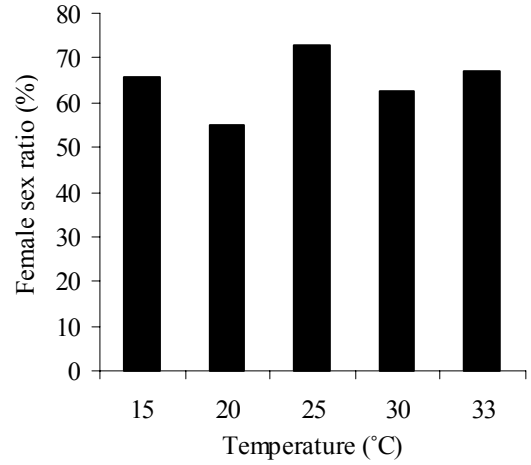

Figure 3. The percentage of *G. ashmeadi* offspring that was identified as female at each temperature.

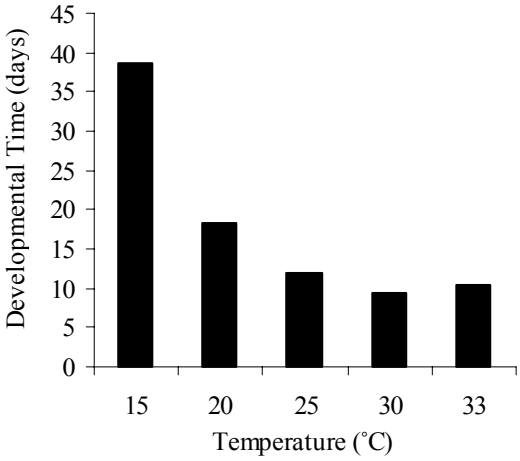

Figure 4. The period of time between oviposition by *G. ashmeadi* and the emergence of wasp offspring represented in days.

Mean adult longevity for individual mated female *G. ashmeadi* used in this study fell from an average of approximately 20 days at 15°C to approximately eight days at 33°C (Figure 5).

REPRODUCTIVE AND DEVELOPMENTAL BIOLOGY OF *GONATOCERUS ASHMEADI*, AN EGG PARASITOID OF THE GLASSY-WINGED SHARPSHOOTER

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

ABSTRACT
The reproductive and developmental biology of *Gonatocerus ashmeadi* Girault, a self-introduced parasitoid of the glassy-winged sharpshooter (GWSS) *Homalodisca coagulata* Say, was determined at five constant temperatures in the laboratory; 15; 20; 25; 30; and 33°C. Wasps at each experimental temperature were given, on average, between 10 and 15 GWSS eggs per day for its natural life for oviposition. At 30°C, immature *G. ashmeadi* sustained the highest mortality rates as adult emergence was lowest at this temperature. The largest proportion of female offspring was produced at 25°C and lifetime fecundity was greatest at 25°C. The development time was greatest at 15°C and lowest at 30°C. Mean adult longevity was inversely related to temperature with a maximum of approximately 30 days at 15°C to a minimum of approximately two days at 33°C.

INTRODUCTION
The mymarid wasp species *Gonatocerus ashmeadi* Girault, *G. triguttatus* Girault, *G. morrilli* Howard, and *G. fasciatus* Girault are the most common natural enemies associated with the insect pest *Homalodisca coagulata* in it's home range of southeastern USA and northeastern Mexico (Triapitsyn and Phillips, 2000). The wasp *G. ashmeadi* is a self-introduced resident of California and most likely came into the state in parasitized *Homalodisca coagulata* eggs (Vickerman et al., 2004) and has established widely in association with *H. coagulata*.

One factor that can limit the success of the establishment of natural enemies is mismatching the environmental conditions favored by the introduced agent with those that predominate in the receiving range (Hoddle, 2004). Quantification of the reproductive and developmental biology of a natural enemy is paramount to predicting, planning, and promoting the establishment and population growth of introduced agents. This can be enhanced by determining demographic characteristics such as day-degree requirements for immature development, population doubling times and lifetime fecundity for estimating population growth rates at various temperatures and for comparison with the target pest and other species of biological control agents. Determining the introduced control agent's reproductive and developmental biology and environmental requirements with that of the host will allow for a greater understanding of factors affecting biological control of GWSS.

The following work was undertaken to provide information on the reproductive and developmental biology of the parasitoid wasp *G. ashmeadi*. These data will provide knowledge of the insect's life cycle, in particular in relation to GWSS, and will improve the understanding of optimal timings of its release for biological control purposes in many agricultural systems as well as improve the efficiency of laboratory rearing of these insects. In addition to improving release and rearing strategies, this information will target foreign exploration of strains of *G. ashmeadi* for possible introduction into California and also identify geographical areas that will be conducive to the use of this species as biological control agent following GWSS establishment in various parts of California and in areas such as Tahiti and Hawaii where GWSS has recently invaded.

OBJECTIVES
1. Examine the developmental and reproductive biology of *G. ashmeadi* in order to determine its day-degree requirements, and demographic statistics.

RESULTS
The rates for oviposition that led to successful reproduction of offspring were highest at 30°C (Figure 1). Each wasp at each temperature, on average, had the same number of GWSS eggs made available to them for oviposition. At 30°C, approximately 42% of eggs presented to wasps produced into viable parasitoid offspring. Conversely, this rate decreased with temperature to 1% at 15°C. Higher temperatures similarly lowered the production of viable offspring with approximately 13% surviving to adult stages at 33°C. These results suggest that *G. ashmeadi* progeny survivorship was most successful when oviposition occurred at 30°C, intermediate at 20-25°C and lowest at 15°C.

next step that is now required is to test hypotheses generated from lab studies in the field. Field level assessments will evaluate our understanding of the system under investigation, and consolidate interpretations needed to determine the most important aspect of the GWSS biological control program: "How effective are egg parasitoids at controlling GWSS in California?" To get to the crux of this issue we are asking two questions in this proposal: (1) How big an impact do individual female parasitoids have on GWSS population growth via parasitization of eggs, and (2) do biotic impediments such as brochosomes affect parasitization efficacy in the field? When these two questions are addressed together we will begin to develop a comprehensive understanding of the impacts parasitoids have at the field level and factors affecting parasitization success. This will allow us to form a much better understanding of what levels of control we can expect from mymarid egg parasitoids when different ecological conditions are prevailing in the field.

OBJECTIVES

This is a new proposal that was officially funded in July 2004. This project has two objectives aimed at determining the field level impact individual female *Gonatocerus ashmeadi* have on glassy-winged sharpshooter (GWSS) egg masses. These two research objectives are complimentary:

1. Measure real life time contributions of individual female parasitoids to parasitism of GWSS egg masses under field conditions. This research objective is high priority.
2. Determine the ecological significance of brochosome deposition on GWSS egg masses and its effect on parasitism rates by *G. ashmeadi* under field conditions.

RESULTS

This project has not commenced. There are two major reasons for this: (1) Recruitment of Dr. Nic Irvin as the post-graduate researcher for this program has been held up by the excessive time it has taken to process the required visas to employ her in the USA given her alien status. (2) Dr. Irvin will start working on this project in early March 2005 when GWSS populations begin to build again. It made no sense to employ Dr. Irvin earlier than this time as at the time of notification of successful visa application GWSS populations were declining in the field and there would be few reproductive adults and parasitoids to work with. We will be formally requesting a no cost extension for this project.

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REALIZED LIFETIME PARASITISM AND THE INFLUENCE OF BROCHOSOMES ON FIELD PARASITISM RATES OF GLASSY-WINGED SHARPSHOOTER EGG MASSES BY *GONATOCERUS ASHMEADI*

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

ABSTRACT

INTRODUCTION

GWSS is an exotic pest in California having invaded and established in this state in the late 1980's. One potential reason for the inordinate numbers of GWSS in California compared to population densities in the pest's home range in southeastern USA is a lack of an efficient natural enemy fauna that has evolved to use GWSS as a resource. As part of a classical biological control program against GWSS, scientists with the CDFA and UCR have been prospecting for, importing into quarantine, and clearing for release mymarid egg parasitoids from the home range of GWSS for establishment in California. To date, two new parasitoid species have been established in CA, *Gonatocerus triguttatus* and *G. fasciatus*. It is too early to ascertain the impact on GWSS population growth that these two parasitoids will have. The self introduced *G. ashmeadi* (Vickerman et al., 2004) is the key natural enemy of GWSS egg masses in CA at present (Blua et al., 1999). Over summer, parasitism levels of GWSS egg masses and individual eggs in masses by *G. ashmeadi* approaches 100% but parasitism levels of the spring generation of GWSS are substantially lower (Triapitsyn and Phillips, 2000). Naturally occurring populations of *G. ashmeadi* in CA have been augmented with mass reared individuals from populations found in the southeastern USA and northeastern Mexico which encompasses the home range of GWSS (D. Morgan - CDFA, pers. comm. 2003).

Substantial laboratory work with *G. ashmeadi* has been conducted in an attempt to understand and parameterize basic aspects of this parasitoid's reproductive biology, and host selection behaviors. Irvin and Hoddle (2001) have evaluated oviposition preferences of *G. ashmeadi* when presented GWSS eggs of various ages. Interspecific competition between *G. ashmeadi* with *G. triguttatus* and *G. fasciatus* for GWSS egg masses of different ages has been assessed along with factors influencing the sex ratio of offspring (Hoddle and Irvin, 2002; 2003). The effect of resource provisioning and nutrient procurement on the longevity of *G. ashmeadi* has also been determined (Irvin unpublished data). Furthermore, the foraging efficacy of *G. ashmeadi* in simple and complex environments for scarce and abundant GWSS egg masses has also been completed and compared to similar data collected for *G. triguttatus* (Irvin unpublished data).
The effect of brochosomes on the foraging efficacy of *G. ashmeadi* has also been evaluated in the laboratory. Brochosomes are a chalky material produced by the malpighian tubules in many xylophagous cicadellid species (Rakitov, 1999; 2000; 2004). Brochosomes are excreted from specialized openings on the posterior of the abdomen and are collected and deposited by mated females on the forewings. During oviposition, females rub brochosomes off the forewings and deposit them on the tops of eggs masses (Hix, 2001). The adaptive significance of covering egg masses with brochosomes is uncertain (Rakitov,1999). Hix (2001) has suggested that brochosomes may protect GWSS eggs from desiccation, UV light, natural enemies (parasitoids, predators and pathogens); or they provide a signal to other female GWSS that leaves have already been oviposited in. We have investigated the effect of brochosomes on the foraging efficacy of *G. ashmeadi* in the laboratory. Data clearly demonstrate that moderate to heavy brochosome coverage of GWSS eggs is a major impediment to oviposition to *G. ashmeadi* when compared to conspecific parasitization efficiency of GWSS eggs with light or no brochosome coverage (Velema et al., 2004).

Studies currently funded by the CDFA to by conducted by this lab will look at: (1) laboratory-level fecundity rates of *G. ashmeadi* under varying temperature regimens; (2) field cage studies assessing interspecific competition between parasitoids released for the classical biological control of GWSS; (3) factors affecting sex ratio allocation during mass production of mymarid parasitoids; and (4) the effect of resource provisioning on parasitization rates and overwintering longevity of key mymarid parasitoids under field conditions. The work proposed in this grant will complement and support completed studies and work in progress.

Many factors act in concert to affect successful biological control. The GWSS-*Gonatocerus* system has benefited from intensive laboratory study to generate a basic understanding of factors influencing host selection and parasitism success. The

correspond to those in the literature for each of these genes (van Hille *et al.* 1993; Pietrantonio and Gill, 1993; Zeng *et al.*, 2002; Liu *et al.,* unpublished data).

CONCLUSIONS

The presence of more than one GWSS V-ATPase A subunit gene will be confirmed by DNA blot hybridization. We have developed a clone capture technique which will allow us to isolate all gene clones with sequence similarity from our cDNA library in a single experiment. This procedure involves the formation of a RecA-mediated triple-stranded molecule between our biotinylated partial clone and full length cDNA clones with sequence similarity. Triple-stranded molecules are then removed from the reaction using streptavidin magnetic beads. This approach will allow us to much more quickly analyze all the members of specific gene families already partially cloned. Thus far we have succeeded in isolating clones similar to the KAAT-like gene clone recently obtained (data presented in the report of a related project: Development of Glassy-winged Sharpshooter Mimetic Insecticidal Peptides, and an Endophytic Bacterial System For Their Delivery to Mature Grape.). The clones isolated are being analyzed to identify the regions best suited for antibody targeting using bioinformatics tools. We anticipate that this approach also will allow us to isolate gene families of genes identified by microarray screening as being tissue-specifically expressed. This will be important in determining that a potential target does not have similarity to genes expressed other than in the organs we want to target.

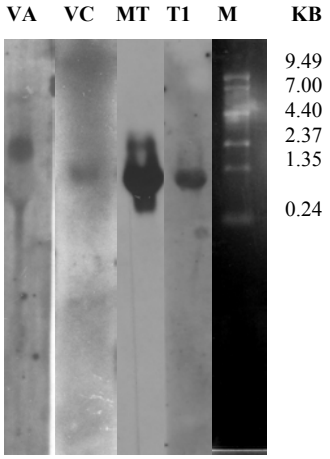

Figure 3. RNA blot hybridizations of 10μg GWSS total RNA hybridized to V-ATPase A (VA), V-ATPase c (VC) and the trypsin-like gene (T1) clones labeled with DIG and detected with chemiluminecsence and the HCMT1 (MT) gene clone labeled with 32P.

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We have dissected and identified all of the components of the GWSS alimentary canal, performed ultrastructural studies of these tissues, and developed *in situ* hybridization techniques for the localization of gene expression (Figure 2). As expected the genes encoding the V-ATPase A and c subunits and that expressing HcMT1 are all expressed throughout the GWSS gut. HcMT1 clearly also is expressed in the salivary glands. The studies localizing the expression of the trypsin-like and maltase-like genes are in progress.

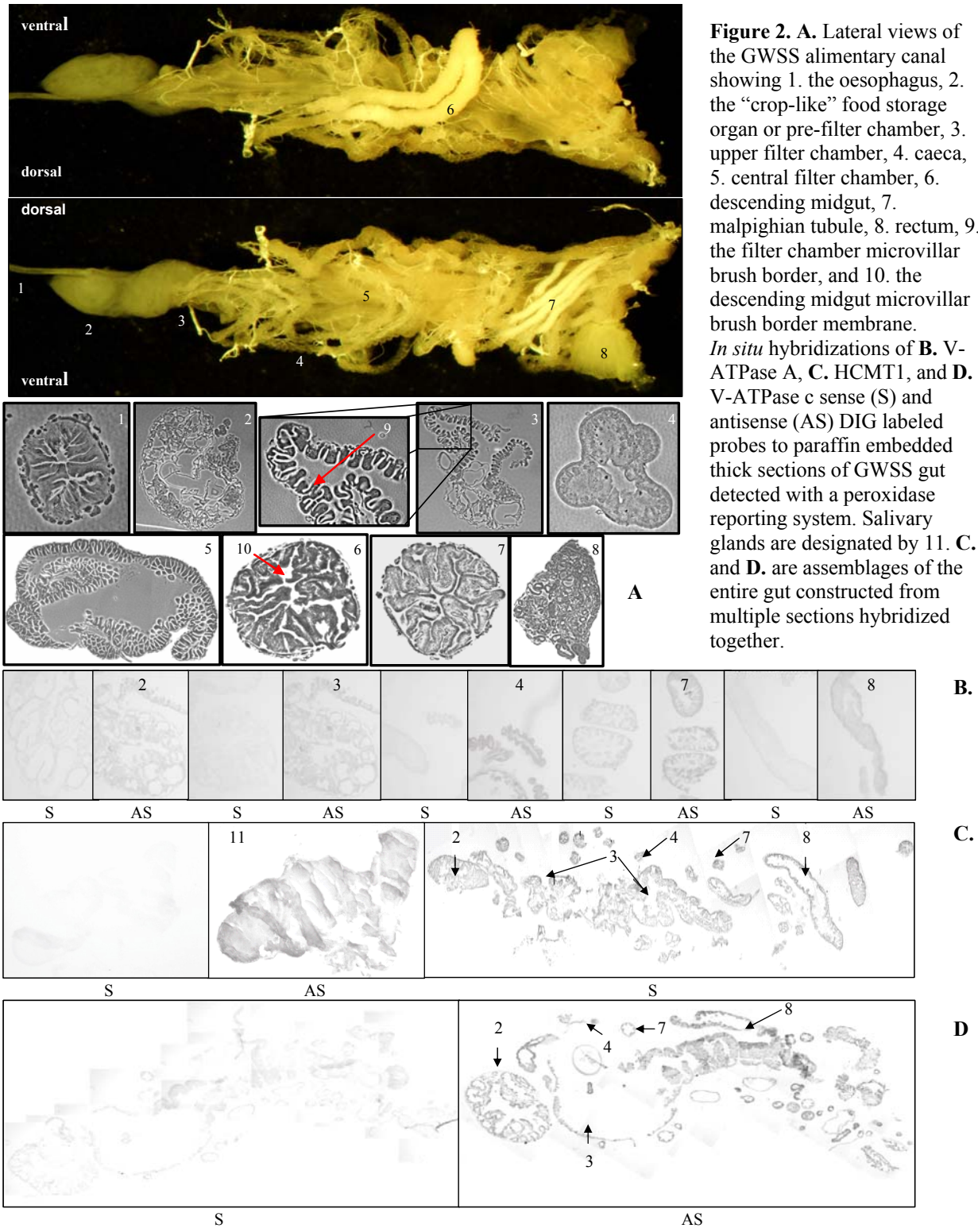

Figure 2. A. Lateral views of the GWSS alimentary canal showing 1. the oesophagus, 2. the "crop-like" food storage organ or pre-filter chamber, 3. upper filter chamber, 4. caeca, 5. central filter chamber, 6. descending midgut, 7. malpighian tubule, 8. rectum, 9. the filter chamber microvillar brush border, and 10. the descending midgut microvillar brush border membrane. *In situ* hybridizations of **B.** V-ATPase A, **C.** HCMT1, and **D.** V-ATPase c sense (S) and antisense (AS) DIG labeled probes to paraffin embedded thick sections of GWSS gut detected with a peroxidase reporting system. Salivary glands are designated by 11. **C.** and **D.** are assemblages of the entire gut constructed from multiple sections hybridized together.

Transcript sizes for each of the genes partially cloned have been determined by RNA blot hybridization (Figure 3). The transcript sizes were determined as: ~1,900 bp for V-ATPase A, which corresponds well with that determined from the cDNA sequence of 1,849 bp, ~1,200bp for V-ATPase c, and ~875 bp for HCMT1 and the trypsin-like gene. These values

RESULTS

We have had a normalized cDNA library constructed by Evrogen JSC from total RNA isolated from whole GWSS of both sexes and all life stages, as well as from GWSS that have fed on grape infected with *X. fastidiosa*. We've had 10,752 clones isolated, glycerol stocks prepared, and PCR products of all inserts amplified and purified for microarray spotting. This August three members of our laboratory were trained at the Custom Microarray Facility at the University of Arizona and we are currently repeating the results obtained there at the Core Instrumentation Facility in the Institute for Integrative Genome Biology on the Riverside campus. A subset of 1,536 clones was spotted in duplicate (side by side spots) and the entire array duplicated on the same slide. These arrays were hybridized to Cy3 labeled control cDNA and Cy5 labeled cDNA reverse transcribed and amplified from total RNA isolated from GWSS treated with a sub-lethal dose or an LD50 dose of esfenvalerate. Dye swap experiments were performed. These experiments are part of a collaborative related project funded by CDFA with Frank Byrne as Project Leader. Our results are presented in his report for the project entitled "Evaluation of resistance potential in the glassy-winged sharpshooter (GWSS) using toxicological, biochemical and genomics approaches." The arrays detected obvious differences in gene expression levels between the two treatments. These experiments were chosen for our test study because it is known that several genes encoding cytochrome P450 proteins are up-regulated dramatically in response to pesticide treatment. We have succeeded in cloning the entire GWSS V-ATPase A gene (Figure 1) by RLM-RACE. Differences in both the 5'- and 3'- sequences between the clones obtained indicate more than one copy of the V-ATPase A gene exists in the GWSS genome

```
   1  ctcgtgatatcagctggtgactgggtgaggtagctgcttcgtctgatcattacagtaagggatagctgaagatgcctttgcagaagattaaagatgag
                                                                                M  P  L  Q  K  I  K  D  E

  99 gacaaggagtccaagtttggatatgtgtacgctgtatccggaccggtcgttacggccgagaagatgtccgggtcagctatgtacgagttggtgcgtgtc
     D  K  E  S  K  F  G  Y  V  Y  A  V  S  G  P  V  V  T  A  E  K  M  S  G  S  A  M  Y  E  L  V  R  V

 198 ggctactttgagctggtaggagagatcattcgtctggaaggtgacatggcaactattcaggtatacgaagagacgtcaggtgtgacggttggtgaccct
     G  Y  F  E  L  V  G  E  I  I  R  L  E  G  D  M  A  T  I  Q  V  Y  E  E  T  S  G  V  T  V  G  D  P

 297 gtattgcggactggcaagcctctgtctgtggaactcggtcccgggatcctgggcgctatttttgatggtatccagcgacccttgaaggacatcaatgaa
                                              t  t  a     aag   c     c
     V  L  R  T  G  K  P  L  S  V  E  L  G  P  G  I  L  G  A  I  F  D  G  I  Q  R  P  L  K  D  I  N  E
                                                 M     S

 396 ctgtctcacagcatctacatccctaagggagtcaacgttcccgcactctccagaacagccacttgggagtttaacccccttaacatcaagactggaagc
                                                    t                                 c
     L  S  H  S  I  Y  I  P  K  G  V  N  V  P  A  L  S  R  T  A  T  W  E  F  N  P  L  N  I  K  T  G  S

 495 cacattaccggaggagatatttatggaatcgtccacgagaataccctggtgaaacacaagatgctgctgcctcctcgagctaagggaacagtgacgtac
                                                                                       g
     H  I  T  G  G  D  I  Y  G  I  V  H  E  N  T  L  V  K  H  K  M  L  L  P  P  R  A  K  G  T  V  T  Y

 594 atcgcatcccctggcaactacactgttgatgatgttgttctggaaacggagtttgacggtgagaagtctaagtatacaatgttgcaagtgtggcctgta
     I  A  S  P  G  N  Y  T  V  D  D  V  V  L  E  T  E  F  D  G  E  K  S  K  Y  T  M  L  Q  V  W  P  V

 693 cgtcagcccagacctgtgaccgagaagctgccagccaatcatccactgctcactggtcaacgtgtgctcgactctctattcccttgtgtgcaaggtggt
     R  Q  P  R  P  V  T  E  K  L  P  A  N  H  P  L  L  T  G  Q  R  V  L  D  S  L  F  P  C  V  Q  G  G

 792 accacggccatccctggagccttcggttgcggtaaaactgtcatctctcaggccctgtccaaatactccaactcggatgtcatcatttacgtaggatgt
     T  T  A  I  P  G  A  F  G  C  G  K  T  V  I  S  Q  A  L  S  K  Y  S  N  S  D  V  I  I  Y  V  G  C

 891 ggagagcgaggaaatgaaatgtctgaggtattgcaagacttccctgagctgtctgtagagattgatggagtcacggagagtataatgaagcgaactgct
     G  E  R  G  N  E  M  S  E  V  L  Q  D  F  P  E  L  S  V  E  I  D  G  V  T  E  S  I  M  K  R  T  A

 990 ctggtagccaacacctccaacatgcctgtcgccgcccgagaagcttccatttatacaggaatcacactgtctgaatatttccgagacatgggctacaat
     L  V  A  N  T  S  N  M  P  V  A  A  R  E  A  S  I  Y  T  G  I  T  L  S  E  Y  F  R  D  M  G  Y  N

1089 gtttctatgatggctgactctacatcccgttgggccgaagccctgagagaaatttctggacgattggcagaaatgcctgctgacagtggttaccccgca
     V  S  M  M  A  D  S  T  S  R  W  A  E  A  L  R  E  I  S  G  R  L  A  E  M  P  A  D  S  G  Y  P  A

1188 tacctgggtgcccgtctagcctccttctacgagagagccggtcgagtcaagtgcctgggaaatcccgatcgtgaaggctctgtcagtattgtcggtgct
     Y  L  G  C  P  S  S  L  L  L  R  X  S  R  S  S  Q  V  P  G  N  P  D  R  E  G  S  V  S  I  V  G  A

1287 gtgtcacctcctggtggtgacttctcagatcccgtcacctccgccacccttggtatcgtacaggtcttctggggtcttgacaagaaattggcacaaagg
     V  S  P  P  G  G  D  F  S  D  P  V  T  S  A  T  L  G  I  V  Q  V  F  W  G  L  D  K  K  L  A  Q  R

1386 aaacactttccctccattaactggctcatatcatacagtaaatacatgagagcactagatgacttctatgacaagagcttcccagagtttgtacccctg
     K  H  F  P  S  I  N  W  L  I  S  Y  S  K  Y  M  R  A  L  D  D  F  Y  D  K  S  F  P  E  F  V  P  L

1485 aggaccaaggtaaaggagattctgcaggaggaagaagatttgtcagaaattgtacaactggttggcaaagcatcacttgccgaaactgacaaaatcacc
                                                                                          gagtcc
     R  T  K  V  K  E  I  L  Q  E  E  E  D  L  S  E  I  V  Q  L  V  G  K  A  S  L  A  E  T  D  K  I  T
                                                                                             S

1584 ctcgaggtcaataggctactgaaagaagattttctgcaacagaacagctactctccgtatgatcgtttctgtcccttctacaagacagtgggcatgctc
     L  E  V  A  R  L  L  K  E  D  F  L  Q  Q  N  S  Y  S  P  Y  D  R  F  C  P  F  Y  K  T  V  G  M  L

1683 cgcaacatgattgcattctttgacatgtcaaggcatgccgtcgagtcgacagcacaaagtgaaaacaagatcacctggagtgtcatcaaggacggcatg
     R  N  M  I  A  F  F  D  M  S  R  H  A  V  E  S  T  A  Q  S  E  N  K  I  T  W  S  V  I  K  D  G  M

1782 ggcaacattctgtaccaactgtcgtcaatgaaattcaaggatcccgtgaaagatgaaaaaaaaaaaa
     G  N  I  L  Y  Q  L  S  S  M  K  F  K  D  P  V  K  D  E
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Figure 1. The complete cDNA and translated protein of GWSS V-ATPase A. The atg indicates the translational start site. Nucleic acid and protein sequence variations are indicated in bold. Sequence variations were determined from both sense and antisense sequences.

THE ALIMENTARY TRACK OF GLASSY-WINGED SHARPSHOOTER AS A TARGET FOR CONTROL OF PIERCE'S DISEASE, AND DEVELOPMENT OF MIMETIC INSECTICIDAL PEPTIDES FOR GLASSY-WINGED SHARPSHOOTER CONTROL

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Reporting Period: The results reported here are from work conducted from December 2, 2003 to October 15, 2004.

ABSTRACT
Transgenic insecticidal crops expressing *Bacillus thuringiensis* (*Bt*) toxins have been successfully developed to control major chewing insect pests of agriculture, such as caterpillars and beetles. The same *Bt* toxin technology also has been used with *Bacillus sphaericus* for the control of mosquito species such as *Aedes aegypti* and *Culex quinquefasciatus*, important vectors of human diseases. However, this transgenic technology has not yet been applied to economically important xylem-feeding sucking insect pests such as the glassy-winged sharpshooter, *Homalodisca coagulata* (GWSS). Our goal is to use a genomics approach to develop novel, highly specific mimetic insecticidal proteins derived from the variable binding domains of immunoglobulin molecules. "Mimetic" peptides mimic the normal substrates of key components of essential processes to block the activities of these proteins. Our research is targeting the exposed active domains of transport proteins on the surface of the GWSS midgut microvillar membrane and enzymes found in GWSS saliva. Degenerate PCR amplification of genes characterized in other insect species encoding proteins involved in gut transport and saliva activity and screening a cDNA microarray to identify novel gut and saliva protein encoding genes are the approaches being used to identify GWSS target proteins. Due to the target specificity, mimetic peptide technology can provide an environmentally sound approach to the control of vasculature feeding insect pests and could thereby provide a means of controlling Pierce's disease and crop losses due to GWSS feeding.

INTRODUCTION
Mimetic technology is new to agriculture, but has been used extensively and successfully in medicine (Clemens, 1996). Examples of medical uses include the inactivation of disease-related enzymes (Burke et al., 2001), blockage of metabolic receptors important to disease (Berezov et al., 2000), and the use of antibodies developed against disease constituents (Moe et al., 1999). Human cancers (Monzayi-Karbassi and Keiber-Emmons, 2001), diabetes (Deghenghi, 1998), and heart disease (Lincoff et al., 2000) all have been treated successfully through these applications of mimetic technology. In spite of lacking a history of application of mimetics to agriculture problems, its development should be straight forward. Antibody proteins have been synthesized successfully in plants for the production of antibodies to be used in medical applications (Larrick et al. 2001; Stoger et al., 2002), and the production of transformed lines of crop plants in which promoters that have been isolated by other researchers (Shi et al., 1994; Springer, 2000), which direct expression to the cell wall and vascular structures of plants, will assure that our antibody peptides are synthesized in a tissue-specific manner. Last year we succeeded in isolating portions of five GWSS genes by degenerate PCR: the A and c V-ATPase subunits, genes encoding trypsin-like and maltase-like saliva proteins, and a membrane transporter. This year we have added another membrane transporter gene clone, most closely related to the potassium coupled amino acid transporter isolated from Manduca sexta, KAAT1 (Castagna et al., 1998). These clones and others isolated from our normalized cDNA are being analyzed using bioinformatics tools to identify functional domains which will be effective and specific targets. The identified target peptides will be synthesized in a *Baculovirus* expression system. Peptides produced will be used as antigens for polyclonal antibody production, the products of which will be cloned into phage display libraries. Screening the phage display antibody libraries will identify the mimetic peptides that bind most efficiently to the targeted GWSS proteins. Ultimately these peptides will be used in feeding studies to identify those which are the best candidates for GWSS control.

OBJECTIVES
1. Determine the structure and cell types in the midgut epithelium and salivary glands of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*;
2. Prepare a normalized cDNA microarray of GWSS using pooled cDNAs isolated from each developmental stage.
3. Screen the microarray using cDNA probes derived from midgut and salivary gland tissue-specific probes to determine the tissue-specific expression of key midgut microvillar and saliva proteins;
4. Clone and sequence genes encoding one or more key midgut microvillar and saliva proteins and determine their suitability as targets for a molecular biological approach to GWSS and Pierce's disease control.
5. Predict functional domains of key GWSS midgut epithelium- and salivary gland-specific proteins based on sequences of genes using bioinformatics;
6. Express functional domain peptides for antibody production;
7. Clone single-chain fragment variable antibody genes into recombinant phage libraries and screen the libraries;
8. Conduct feeding studies to identify efficacious mimetic peptides effective in killing or deterring GWSS.

both GWSS (Figure 4A) and STSS (Figure 4B) DNA individually. The amount of DNA was varied from 0.05 to 0.80 ng. These experiments show the sensitivity limits with both GWSS and STSS DNA to be at 50 pg. The SCAR (6/9) marker set was tested with predators (Lacewings L1-12) that fed on GWSS eggs (Figure 5). At least 7 of the 12 specimens tested positive with this marker set. The assay system was tested for competition or interference of predator DNA with both Qiagen preps and crude DNA extracts. The DNA crude extract procedure was developed as a rapid method to assay hundreds of samples more efficiently. The results show that predator DNA does not compete or interfere with the SCAR-PCR assays.

Homalodisca and GWSS-specific Mitochondrial COII primers

Mitochondrial DNA is present in hundreds or multiple copies within each cell (Chen *et al*. 2000; Symondson 2002). In order to increase the sensitivity of our diagnostic assays, the mtCOII genes of both GWSS and STSS were sequenced and both *Homalodisca*- and GWSS-specific primers were designed. Figure 6 demonstrates that both GWSS- (Figure 6A) and *Homalodisca*- (Figure 6B) specific primers were successful without amplifying any other sharpshooters or predators.

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FUNDING AGENCIES

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

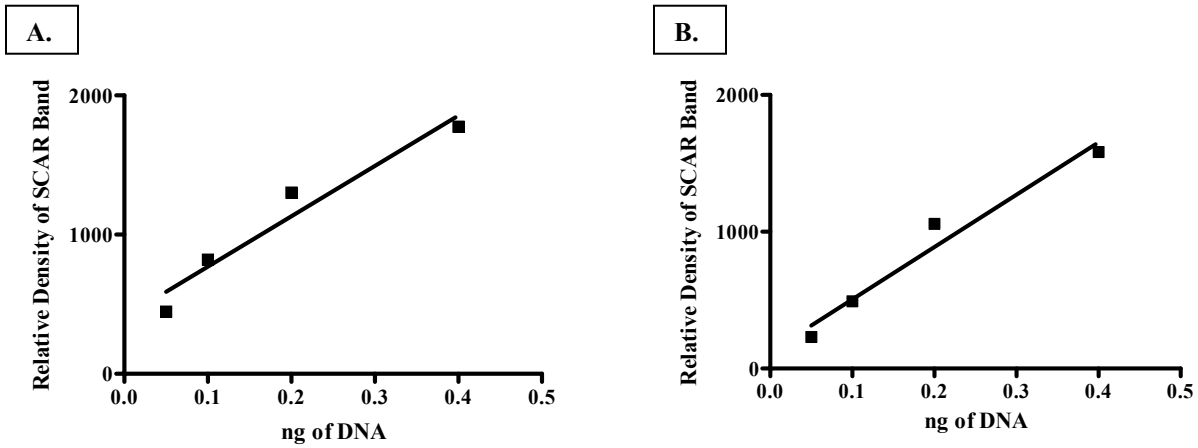

Figure 4. SCAR 6/9 sensitivity assays with GWSS DNA (A) and STSS DNA (B). DNA ranged from 0.05 to 0.80 ng with each point in triplicate. The three determinations per point were averaged and plotted vs relative density of the SCAR bands. The highest amount of DNA (0.80 ng) was not in the linear portion of the curve (saturated), so it was eliminated from the analysis.

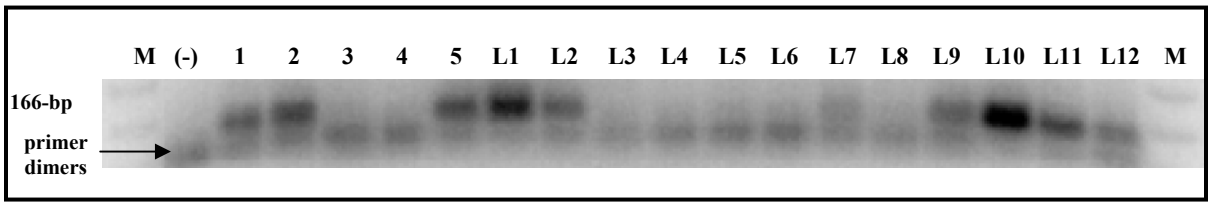

Figure 5. SCAR-PCR (6/9) assays with predators (Lacewing, L1-10) that fed on GWSS eggs. Lanes: 1, Qiagen prep control plus GWSS DNA; 2, crude extract control plus GWSS DNA; 3, crude extract negative control (not fed); 4, Qiagen prep negative control (not fed); 5, GWSS DNA positive control.

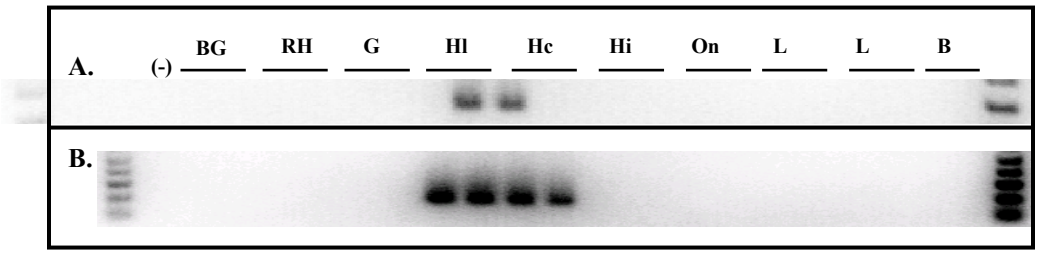

Figure 6 (below). California *Homalodisca* mitochondrial COII-specific primers. The mitochondrial COII genes of both GWSS and STSS were sequenced and both *Homalodisca*- and GWSS-specific primers were designed. Refer to fig. 1 for assignments.

Homalodisca (GWSS/STSS)-Specific SCAR (6/9) Markers

Figure 3 shows the specificity of the *Homalodisca* markers, as seen only GWSS and STSS DNA is amplified with this marker set and no other sharpshooters or predators amplified. The sensitivity of this SCAR (6/9) marker set was tested with

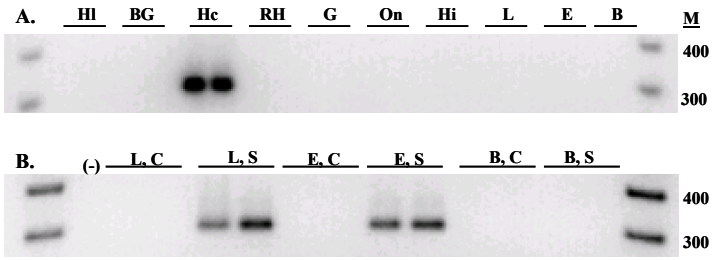

Figure 1. RAPD-PCR DNA fingerprinting was performed with the following sharpshooters: *Homalodisca liturata* (**Hl**); *Graphocephala atropuncta* [blue-green (**BG**)]; *H. coagulata* (**Hc**); *Carneocephala fulgida* [red-headed (**RH**)]; *Draeculacephala minerva* [green (**G**)]; *Oncometopia nigricans* (**On**); and *H. insolita* (**Hi**). Amplification products/bands unique to GWSS were excised, sequenced, and primers (SCAR markers) were designed to amplify a 302-bp fragment. **A).** Specificity of GWSS-specific SCAR-5/7 markers. **L**, lacewing larvae (*Chrysoperla carnea*); **E**, earwig (*Forficula auricularia*); and **B**, ground beetle (*Calosoma sp*.). **B).** Detection of GWSS in predator gut contents by SCAR-PCR assays. (-), negative control (no template); C, control (not fed on GWSS); S, sample (fed on GWSS). Lacewing and earwig fed on GWSS eggs and ground beetle fed on a GWSS adult.

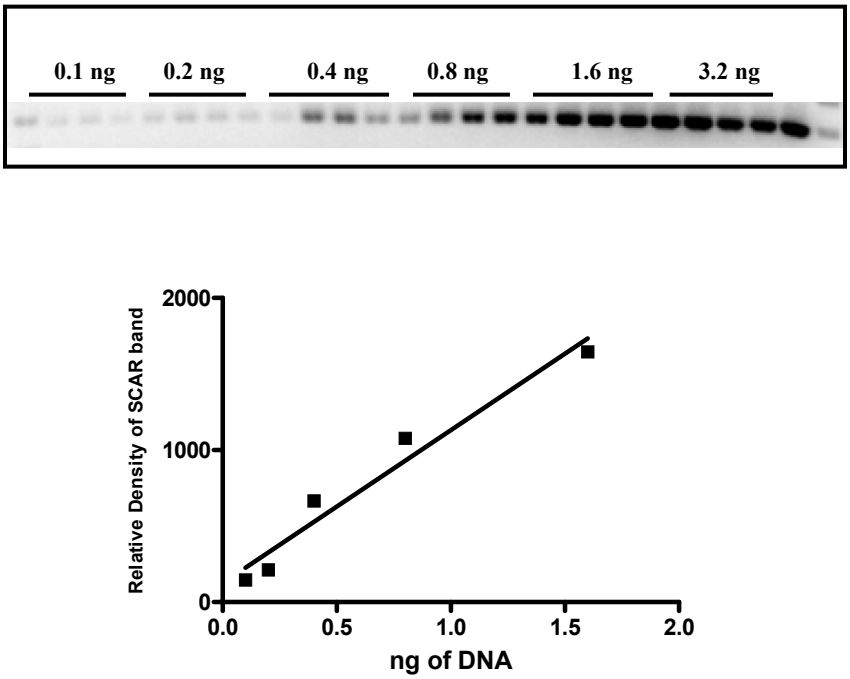

Figure 2. Sensitivity assay with GWSS-specific SCAR 5/7. GWSS DNA was varied from 0.1 to 3.2 ng, each point in quadruplicate (inset). The four determinations per point were averaged and plotted vs relative density of the SCAR bands. Since the highest amount of DNA (3.2 ng) did not fall within the linear portion of the curve (saturated) it was eliminated.

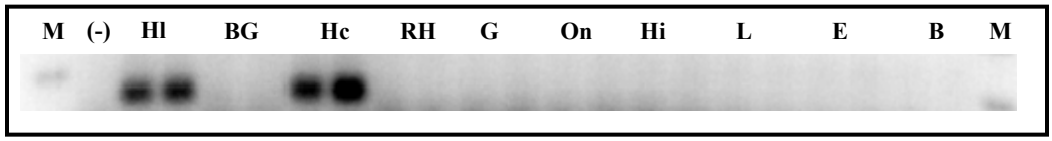

Figure 3. California *Homalodisca* (GWSS/STSS)-specific SCAR 6/9 specificity assay. California *Homalodisca*-specific primers were designed toward a RAPD-PCR fragment. Refer to Figure 1 for assignments.

DEVELOPMENT OF MOLECULAR DIAGNOSTIC MARKERS FOR *HOMALODISCA* SHARPSHOOTERS PRESENT IN CALIFORNIA TO AID IN THE IDENTIFICATION OF KEY PREDATORS

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

ABSTRACT

The aim of the present study was to develop molecular diagnostic markers to identify key predators of *Homalodisca* sharpshooter species present in California, *H. coagulata* (Glassy-winged Sharpshooter, GWSS) and *H. liturata* (Smoke-tree Sharpshooter, STSS). RAPD-PCR DNA fingerprinting of several sharpshooter species identified specific bands that were excised, sequenced, and SCAR (Sequenced Characterized Amplified Region) markers were designed. The results demonstrated that both GWSS- and *Homalodisca*-specific markers were specific toward their targets. The GWSS-specific markers amplified only GWSS and the *Homalodisca*-specific markers amplified only GWSS and STSS. The sensitivity limits for both marker sets was at 50 pg of DNA. The mitochondrial cytochrome oxidase subunit gene II (COII)-specific markers that were developed were each specific for GWSS and *Homalodisca* sharpshooters. The development of diagnostic markers designed toward *Homadisca* sharpshooters present in California should aid in finding key predators and therefore enhance biological control efforts against these sharpshooters.

INTRODUCTION

The Glassy-winged Sharpshooter, *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), is a large xylem feeding leafhopper that is a serious pest because it vectors a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* and *V. labrusca*) (Hopkins and Mollenbauer 1973). A biological control program is currently in progress in California against *H. coagulata*. Effective control of GWSS will require an area-wide pest management approach. A major component of such an approach 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 is known about GWSS natural enemies, this is especially true for their predators (Triapitsyn *et al*. 1998). Direct visual field observations of predation are difficult to obtain and historically, the study of insect predation has relied mainly on inexact and indirect techniques for measurement and analysis. Presently, Hagler and Naranjo (1997) and Hagler *et al*. (1991) have had success in developing monoclonal antibodies and detecting prey in predator gut contents by enzyme linked immunoassays (ELISA). Recently, other methods have been developed that allow for the detection of prey in predator gut contents. These molecular methods include, Sequence Characterized Amplified Region (SCAR), where RAPD-PCR species-specific bands are excised from gels, sequenced, and primers are designed toward those DNA fragments (Agusti *et al*. 1999; Agusti *et al*. 2000) and targeting genes that are present in the cell in high copy number, such as, mitochondrial genes (COI and COII) and Internal Transcribed Spacer regions (ITS1) (Agusti *et al*. 2003; Chen *et al*. 2000; Symondson 2002).

OBJECTIVE

Develop molecular diagnostic markers for *Homalodisca* sharpshooter species (GWSS and STSS) found in California in order to identify key predators.

RESULTS AND CONCLUSIONS

GWSS-specific SCAR (5/7) Markers

RAPD-PCR DNA fingerprinting was performed with several sharpshooter species and *Homalodisca*-specific bands were excised, sequenced, and primers designed (SCAR markers). Figure 1A demonstrates that GWSS-specific SCAR (5/7) markers were highly specific with no amplification of any other sharpshooter species or predators. The GWSS-specific markers were also able to detect GWSS eggs in predator gut contents (Figure 1B). The sensitivity of the SCAR marker set was tested by varying the amount GWSS DNA (0.1 to 3.2 ng) (Figure 2). In this experiment, the limit of sensitivity was at 100 pg, but later experiments showed the detection limit at 50 pg (not shown).

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FUNDING AGENCIES
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Table 3. Pairwise sequence distances (range) of ITS-2 rDNA fragments from geographic populations of *G. morrilli* showing percentage divergence. The alignment program ClutstalW (Thomas *et al.* 1994) from DNAStar was utilized for this analysis. To account for intra- and inter-populational variation, several individuals (2-7) were included. WTX, Weslaco, TX (two populations from Hidalgo Co; 7 total individuals); QFL, Quincy, Florida (2 individuals); CA, California (two populations, Orange Co. and San Diego Co; 5 total individuals) Ga, *G. ashmeadi* (outgroup) (4 individuals).

Pop	WTX	QFL	CA	Ga
WTX	0.0-1.70			
QFL	0.0-1.40	0.0-0.30		
CA	6.2-10.7	6.3-7.70	0.0-0.60	
Ga	7.9-13.3	8.4-12.4	7.8-12.0	0.5-0.9

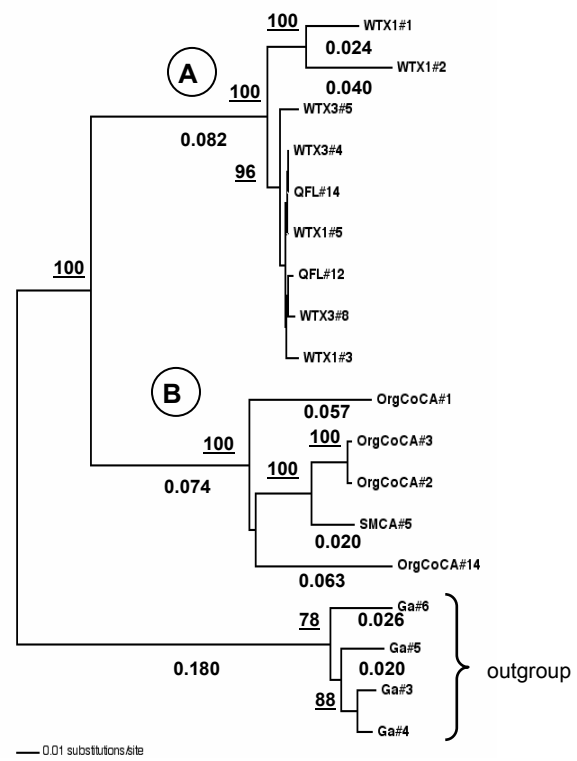

Figure 3. Phenogram of ITS2 rDNA sequence fragment from geographic populations of *G. morrilli*. Analysis was performed with the alignment program ClustalX (Thompson *et. al.* 1997) and the Nieghbor-Joining tree was created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *G. ashmeadi* are included as an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches underlined), as percentage of 1000 replications. To account for intra- and inter-populational variation, several randomly chosen individuals (2-7) were included. SMCA, San Marcos, CA; OrgCo CA; Orange county, California.

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Sequence divergence in ITS rDNA fragment in G. morrilli geographic populations. The percentage sequence divergence (%D) for ITS2 is shown on Table 3. The %D between WTX and QFL is 0.0-1.40%, this falls within the intra-populational range of both populations and therefore shows that these populations are closely related. In contrast, the %D between WTX and CA is 6.2-10.7%, falling within the range (7.9-13.3%) of the outgroup (*G. ashmeadi*). The Nieghbor-Joining distance tree in Fig. 3 demonstrates that the CA and WTX and QFL populations cluster into two distinctive clades (A and B). These clades are supported by very strong bootstrap values (100%).

Table 1 (COI) and Table 2 (COII). Pairwise sequence distances (range) of mitochondrial COI and II genes from geographic populations of *G. morrili* showing percentage divergence. The alignment program ClutstalW (Thomas *et al.* 1994) from DNAStar was utilized for these analyses. To account for intra- and inter-populational variation, several individuals (3-6) were included. WTX, Weslaco, TX (two populations from Hidalgo Co; 5-6 total individuals); QFL, Quincy, FL (3 individuals); CA, California (two populations, Orange Co. and San Diego Co.; 6 total individuals); Ga, *G. ashmeadi* (outgroup) (3 individuals).

Table 1. COI.

Pop	WTX	QFL	CA	Ga
WTX	0.0-0.6			
QFL	*0.0-4.8*	*2.0-4.8*		
CA	*5.4-5.6*	*5.4-8.6*	*0.0-0.2*	
Ga	5.4-6.9	5.4-10.8	6.7-7.1	0.0-0.2

Table 2. COII.

Pop	WTX	QFL	CA	Ga
WTX	0.3-4.50			
QFL	*0.3-4.70*	*0.2-0.6*		
CA	*7.4-11.1*	*7.6-8.9*	*0.0-3.2*	
Ga	7.4-10.5	7.1-7.8	6.9-8.0	0.0-0.2

Figure 1. OI

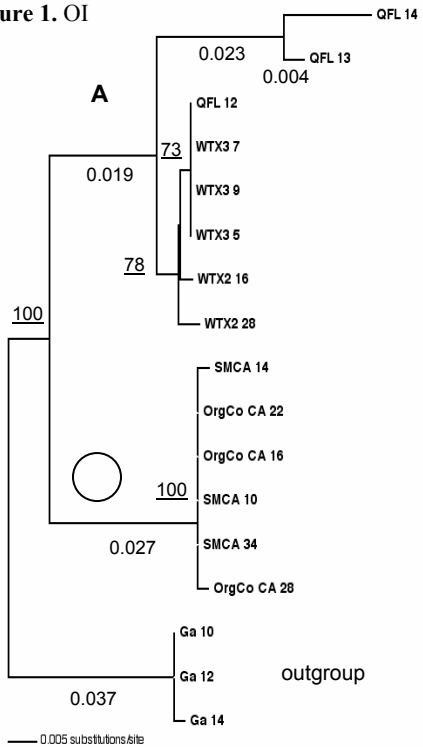

Figure 2 COII

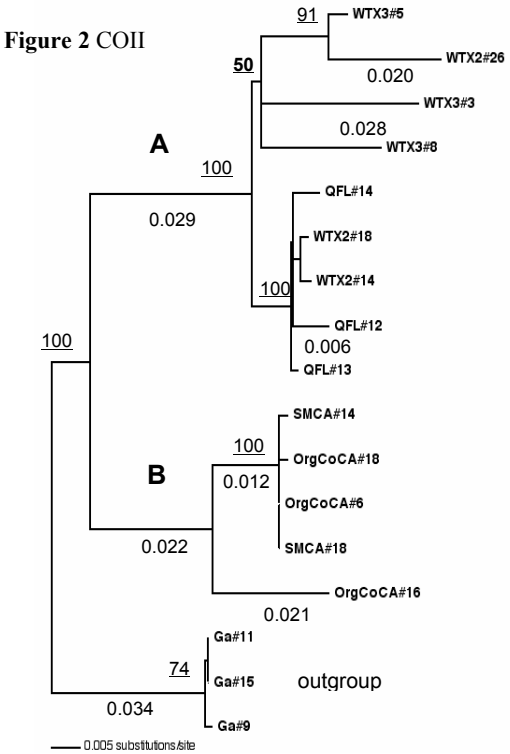

Figure 1 (COI) and Figure 2 (COII). Phenograms of mitochondrial COI and COII genes from geographic populations of *G. morrilli*. Analyses were performed with the alignment program ClustalX (Thompson *et. al.* 1997) and the Nieghbor-Joining trees were created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *G. ashmeadi* are included as an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches underlined), as percentage of 1000 replications. To account for intra- and inter-populational variation, several randomly chosen individuals (3-6) were included. SMCA, San Marcos, CA; OrgCo CA; Orange county, California.

SEQUENCE DIVERGENCE IN TWO MITOCHONDRIAL GENES (COI AND COII) AND IN THE ITS2 RDNA FRAGMENT IN GEOGRAPHIC POPULATIONS OF *GONATOCERUS MORRILLI*, A PRIMARY EGG PARASITIOD OF THE GLASSY-WINGED SHARPSHOOTER

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

ABSTRACT

The aim of the present study was to resolve the genetic relationships of geographic populations of *Gonatocerus morrilli*, a primary egg parasitoid of the Glassy-winged Sharpshooter. A phylogenetic approach was implemented by sequencing two mitochondrial genes (COI and COII) and the Internal Transcribed Spacer-2 (ITS2) region of several individuals per population. Two populations from Weslaco, TX (WTX) (collected at different times), one from Quincy, FL (QFL), two from California (CA) (Orange and San Diego counties), and an outgroup (*G. ashmeadi*) were analyzed. For all three sequence fragments, percentage sequence divergence (%D) (as measured by genetic distance), the results demonstrated that both the WTX and QFL populations were closely related; in constrast, the %D between WTX and CA fell within the range of the outgroup, *G. ashmeadi*. For all three sequence fragments, Nieghbor-Joining distance trees separated the CA and WTX and QFL populations into two distinctive clades (A and B). The topology of the clades in each case was supported by very strong bootstrap values, 100% in the three sequence fragments (COI, COII, and ITS2). The present molecular phylogenetics results provide strong evidence that *G. morrilli* from California may be a different species. The findings of the present study are important to the Glassy-winged Sharpshooter/Pierce's disease biological control program in California.

INTRODUCTION

Gonatocerus morrilli (Howard) (Hymenoptera: Mymaridae) is an egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the Glassy-winged Sharpshooter (Turner and Pollard 1959; Triapitsyn *et al*. 1998). This primary egg parasitoid species is common in the southern United States and Mexico (Huber 1988). A biological control program is currently in progress in California against *H. coagulata*, a xylem feeding leafhopper, which is a serious economic pest that transmits a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* L. and *V. labrusca* L.) (Hopkins and Mollenhauer 1973). Accurate identification of natural enemies is critical to the success of classical biological control programs. Lack of proper identification procedures has affected the early stages of several projects (Messing and Aliniazee 1988; Löhr *et al*. 1990).

OBJECTIVES

Determine the phylogenetic relationships of geographic populations of *G. morrilli* by sequencing two mitochondrial genes (COI and COII) and one rDNA spacer region (ITS2).

RESULTS AND CONCLUSIONS

Sequence divergence in the mitochondrial COI gene in G. morrilli geographic populations. Levels of genetic divergence in the mtCOI gene among populations were determined by calculating the pairwise estimates for genetic distance. Recently, we determined that populations of *G. morrilli* from California and Texas shared no ISSR-PCR banding patterns, indicating that these populations were reproductively isolated. In addition, we demonstrated that the ITS2 rDNA fragments varied in size between these geographic populations (de León *et al*. 2004). The percentage sequence divergence (%D) for mtCOI is shown on Table 1. In general, the intra-populational variation (0.0-0.6%) was small within each population and species, with the exception of the Quincy, FL population (QFL) (2.0-4.8%). The %D between Weslaco, TX (WTX) and QFL is 0.0-4.8%, which falls within the intra-populational variation of these populations; these results indicate that these geographic populations are genetically similar. In constrast, the %D of WTX and CA is 5.4-5.6%, falling within the range (5.4-6.9%) of the outgroup (*G. ashmeadi*). The Nieghbor-Joining distance tree in Fig. 1 demonstrates that the CA and WTX and QFL populations cluster into two distinctive clades (A and B). These clades are supported by very strong bootstrap values (100%).

Sequence divergence in the mitochondrial COII gene in G. morrilli geographic populations. The percentage sequence divergence (%D) for mtCOII is shown on Table 2. Intra-populational variation is seen in both the WTX (0.0-4.5%) and QFL (0.0-3.2%) populations. The %D between WTX and QFL is 0.3-4.7%, these values fall within the intra-populational variation range and therefore these populations would be considered closely related. On the other hand, the %D between WTX and CA is 7.4-11.1%, these values fall within the range (7.4-11.5%) of the outgroup (*G. ashmeadi*). The Nieghbor-Joining distance tree in Fig. 2 demonstrates that the CA and WTX and QFL populations cluster into two distinctive clades (A and B). These clades are supported by very strong bootstrap values (100%).

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FUNDING AGENCIES

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

These novel observations strongly suggest that *G. morrilli* may exist in nature as a species-complex. Results from our recent study with *H. coagulata* suggest that a subset of these insects have their origin in Texas (de León *et al*. 2004). Those results together with our present results with *G. morrilli* may suggest that this egg parasitoid from Texas may be a good candidate for the biological control efforts in California against *H. coagulata,* the causative agent of Pierce's disease.

Table 1. Nei's analysis of gene diversity in populations of *G. morrilli* from Texas and California. Ten individuals per population (40 total) were subjected to ISSR-PCR DNA fingerprinting. Genetic variation was analyzed using the POPGENE 3.2 genetic software program and the program Tools for Population Genetic Analyses (TPFGA). X^2, exact tests (simultaneous analysis) for population differentiation, df = degrees of freedom; Ht, total genetic diversity (SD), Hs, average genetic diversity within populations (SD); G_{ST} (mean), coefficient of gene differentiation; θ theta (analogous to F_{ST}), and Nm, gene flow. $^{***}P = 0000$.

X^2 (df)	Ht	Hs	G_{ST}	θ	Nm
400.8 (50)***	0.35	0.03 (0.04)	0.92 (0.00)	0.94	0.04 (0.02)

Table 2. Nei's unbiased (1978) genetic distance (below diagonal) and Reynolds et al. (1983) genetic distance (above) diagonal. Four geographic populations of *G. morrilli*, two from Texas (Hidalgo Co, Wes-2 and Wes-3) and two from California (OrCo, Orange county and SDCo, San Diego county).

Pop	OrCo	SDCo	Wes-2	Wes-3
OrCo	***	undef	3.40	2.88
SDCo	0.00	***	3.40	2.88
Wes-2	1.07	1.07	***	1.40
Wes-3	0.89	0.89	0.20	***

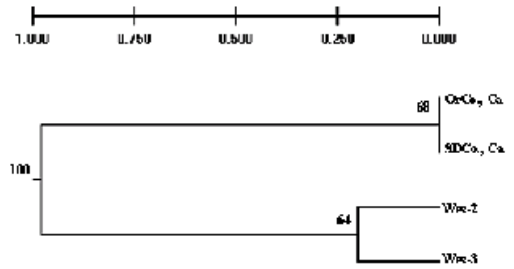

Figure 2. Dendrogram based on Nei's genetic distance (1978) by the method of UPGMA. Relationships among the four geographic populations of *G. morrilli* performed by ISSR-PCR DNA fingerprinting. Genetic distances are indicated above the dendrograms and bootstrap support values are indicated at the nodes.

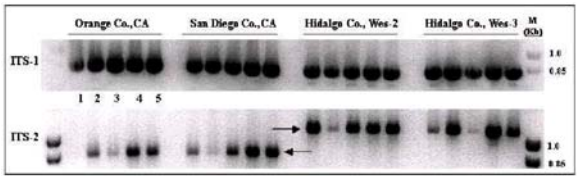

Figure 3. Amplification of the Internal Transcribed Spacer regions (ITS). The ITS-1 and –2 regions were amplified with standard ITS-specific primers with genomic DNA from five separate individuals from each geographic population. Arrows indicate different ITS fragment sizes. M: 1.0 Kb Plus DNA Ladder.

ISSR-PCR Differentiation Among Four G. morrilli Populations.
Exact tests (simultaneous analysis) for population differentiation indicated that highly significant differences in marker frequencies existed among the *G. morrilli* populations (Table 1). Total genetic diversity (Ht) was high (35%), whereas the average genetic diversity within populations was low (3%). Table 1 also shows a comparison of other genetic differentiation estimates, G_{ST} and θ, which evaluate the degree of genetic subdivision among populations. Excellent agreement was seen between G_{ST} and θ values, 0.92 and 0.94, respectively. Theses values indicate that about 92 to 94% of the variance is distributed among populations. The indirect estimate of gene flow, Nm base on G_{ST}, demonstrated a low value (0.04) among the geographic populations; this value indicates highly restrictive gene flow. Overall, genetic differentiation measurements (exact tests, G_{ST}, θ, and Nm) indicate profound genetic divergence/structuring between *G. morrilli* populations from Texas and California.

Genetic Relatedness Among G. morrilli Populations.
Levels of genetic divergence among populations were also determined by calculating pairwise estimates for genetic distance by the procedures of Nei (1978) and Reynolds *et al*. (1983) (Table 2). Average genetic divergence (D) among populations was extremely high [Nei = 0.82 (0.89-1.07) and Reynolds = 2.79 (1.4-3.4)]. A dendrogram based on Nei's genetic distance is shown on Fig. 2 with all *G. morrilli* geographic populations. Two clades are identified on the dendrogram with the California and Texas populations appearing on separate clusters. These two clusters are supported by strong bootstrap support values, 68 and 64%, respectively for the California and Texas populations.

Amplification of the ITS-1 and –2 regions in G. morrilli Geographic Populations.
Monomorphic patterns were demonstrated with amplification of the ITS-1 region in all of the populations from California and Texas (~850 bp) (Fig. 3); whereas, polymorphic or different DNA fragment sizes were detected within the ITS-2 region. The California populations were observed with an ITS-2 fragment size of about 865 base pairs and the Texas populations with a size of about 1099 base pairs.

Good agreement is seen between the two molecular methods and they both suggest that cryptic species may exist. The results with ISSR-PCR demonstrating distinct banding patterns (no band sharing) between geographic populations typically is not found unless the populations are reproductively isolated. Similar results were obtained by Hoy *et al*. (2000) with two populations of *Ageniaspis citrocola* performed by RAPD-PCR. The following genetic differentiation parameters, extract test, G_{ST}, θ, genetic distances, and gene flow (Nm) lend support to this observation. The extremely low value for gene flow between the populations from California and Texas lend support that these populations are isolated reproductively. Restricted gene flow usually leads to increased differentiation among populations as seen from the G_{ST} and θ values (92 to 94% of the variance is seen among populations). In addition, the divergence (D) between these populations is also high.

Methods incorporating SSR appear to be sensitive at detecting DNA polymorphisms in natural populations. Previously, we utilized ISSR-PCR to distinguish three species of *Homalodisca* sharpshooters (*H. coagulata*, *H. liturata*, and *H. insolita*) (de León and Jones 1994). Even though this method is sensitive, there are not many reports in the literature utilizing ISSR-PCR to study insect population genetics and phylogenetics. We have also had success determining the population genetic structure of *H. coagulata* representing 19 populations from through the U. S. (de León *et al*. 2004). The Internal Transcribed Spacer regions (ITS-1 and –2) have been used extensively to examine the taxonomic status of species and for diagnostic purposes, and success with this approach has been reviewed by Collins and Paskewitz (1996). Stouthamer *et al*. (1999) used ITS-2 DNA fragment sizes as taxonomic characters to develop a precise identification key for sibling species of the genus *Trichogramma*. In cases where species were observed with similar sized ITS fragments these authors suggested amplification, sequencing, and restriction digestion.

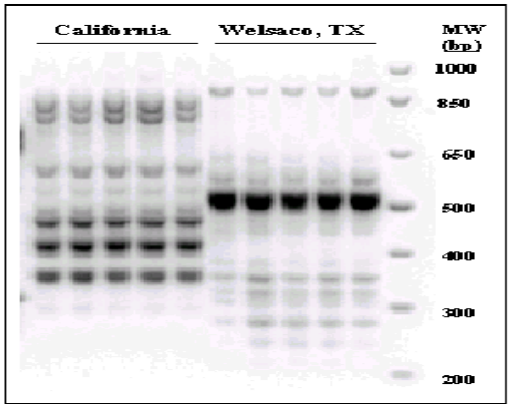

Figure 1. Representative example of ISSR-PCR DNA fingerprinting of *G. morrilli* populations from California and Texas. Reactions were performed with genomic DNA from separate individuals and the 5'-anchored ISSR primer HVH$(TG)_7$T (Zietkiewicz *et al*. 1994) as describe in the Materials and Methods. M: 1.0 Kb Plus DNA Ladder.

MOLECULAR DISTINCTION BETWEEN POPULATIONS OF *GONATOCERUS MORRILLI*, EGG PARASITOIDS OF THE GLASSY-WINGED SHARPSHOOTER, FROM TEXAS AND CALIFORNIA

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

ABSTRACT
Two molecular methods were utilized to distinguish geographic populations of *Gonatocerus morrilli* (Howard) from Texas and California and to test the possibility that this species could exist as a species-complex. Inter-Simple Sequence Repeat-Polymerase Chain Reaction (ISSR-PCR) was performed with a 5'-anchored ISSR primer. Twenty-five markers were generated with four populations (40 individuals) of *G. morrilli*, 23 were polymorphic and percentage of polymorphic loci was 92%. Most markers could be considered diagnostic since there was no band sharing between the Texas and California populations. Such differences typically are not found unless the populations are reproductively isolated. Exact tests for population differentiation indicated significant differences in markers frequencies among the populations. Comparison of other genetic differentiation estimates, which evaluate the degree of genetic subdivision, demonstrated excellent agreement between G_{ST} and θ values, 0.92 and 0.94, respectively; indicating that about 92 to 94% of the variance was distributed among populations. Average genetic divergence (D), as measured by genetic distance, was extremely high (Nei = 0.82 and Reynolds = 2.79). A dendrogram based on Nei's genetic distance, separated the Texas and California populations into two clusters, respectively. Amplification of the Internal Transcribed Spacer-1 (ITS-1) region showed no size differences, whereas the ITS-2 DNA fragments varied in size between the two geographic populations. The ITS-2 fragment sizes were about 865 and 1099 base pairs for the California and Texas populations, respectively. The present study using the two molecular methods provides novel data critical to the glassy-winged sharpshooter/Pierce's disease biological control program in California.

INTRODUCTION
Gonatocerus morrilli (Howard) (Hymenoptera: Mymaridae) is an egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the Glassy-winged Sharpshooter (Turner and Pollard 1959; Triapitsyn *et al*. 1998). This primary egg parasitoid species is common in the southern United States and Mexico (Huber 1988). A biological control program is currently in progress in California against *H. coagulata*, a xylem feeding leafhopper, which is a serious economic pest that transmits a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* L. and *V. labrusca* L.) (Hopkins and Mollenhauer 1973). Accurate identification of natural enemies is critical to the success of classical biological control programs. Lack of proper identification procedures has affected the early stages of several projects (Messing and Aliniazee 1988; Löhr *et al*. 1990). There is a need for molecular markers for natural enemies to provide new characters for studies of phylogenetic relatedness, for identification of cryptic species and biotypes, and for the assessment of heritable variation for population genetics and ecological investigations (Unruh and Woolley 1999). Studies of allele or marker frequencies in naturally occurring parasitoid populations are important, not only for identifying genetic variation of potential benefit, but also for the detection of genetic markers indicative of specific biological traits or geographic origins. Furthermore, the recognition of intraspecific variation can be as crucial for the success of biological control programs as is sound species determination (Powell and Walton 1989; Narang *et al*. 1993; Unruh and Woolley 1999).

OBJECTIVES
1. Survey molecular methods useful in egg parasitoid identification and discrimination
2. Investigate the possibility that *G. morrilli* could exist as a species-complex in nature

RESULTS AND CONCLUSIONS
ISSR-PCR DNA Fingerprinting.
Figure 1 shows an example of ISSR-PCR DNA fingerprinting demonstrating the banding pattern differences between the geographic populations of *G. morrilli* from California (OrCo) and Texas (Wes-2) performed with a 5'-anchored ISSR primer. Markers ranged in size from about 200 to 900 base pairs. Overall, a total of 25 markers were generated among all four populations with a total of 40 individuals. Twenty-three were polymorphic and percentage of polymorphic loci was 92%. Within individual populations, no diversity was seen within the California populations and only slight diversity was observed in the Texas populations. For the Texas populations, Wes-2 and Wes-3, 5 polymorphic markers each were generated and 20% of the markers were polymorphic. Most markers are geographic-specific and can therefore be considered diagnostic since there is no band sharing between the Texas and California populations.

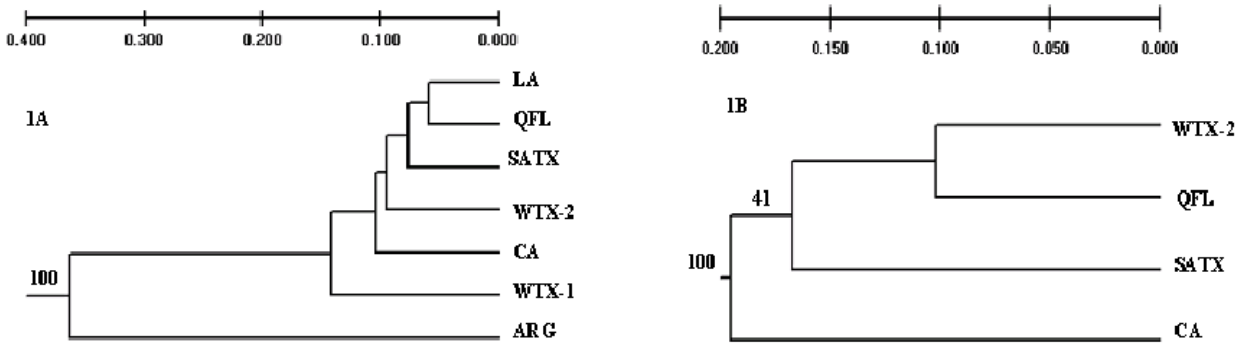

Figure 1: Dendrograms based on Nei's genetic distance by the method of UPGMA. Relationships (A) showing the six US geographic populations of *G. ashmeadi* and a population classified as near *G. ashmeadi* (M2012) from Argentina performed by ISSR-PCR DNA fingerprinting. Field collected populations were also analyzed separately (B). Genetic distances are indicated above the dendrograms and bootstrap support values are indicated at the nodes.

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FUNDING AGENCIES

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

Table 1. Single-populations descriptive statistics for *G. ashmeadi* from the U. S. and genetic variation statistics for all loci. Genetic variation was analyzed using the POPGENE 3.2 genetic software program and the program Tools for Population Genetic Analyses (TPFGA). No. M, number of monomorphic markers; No. P., number of polymorphic markers; %P, percentage of polymorphic loci; Poym. ratio, number of polymorphic markers per number of insects; *h*, gene diversity (SD). One-tailed unpaired *t* test performed for *h* values.

Pop.	No. Insects	No. M	No. P	Total# markers	%P	Polym. ratio	*h* (SD)
CA	30	5	16	21	39.2	0.53	0.1329 (0.182)[a]
WTX-1	30	7	12	19	29.3	0.40	0.0290 (0.158)
WTX-2	30	6	13	19	31.7	0.43	0.0901 (0.160)
SATX	30	5	16	21	39.0	0.53	0.1123 (0.170)[a]
LA	30	5	17	22	41.5	0.57	0.1252 (0.182)[a]
QFL	13	1	20	21	58.8	1.54	0.1431 (0.199)[a]
Fc	103	0	34	34	100.0	0.33	0.2300 (0.184)
All	163	0	41	41	100.0	0.25	0.2082 (0.187)
ARG	30	11	8	19	16.7	0.27	0.0434 (0.127)

[a]Significantly different from WTX-1, $P < 0.05$; df = 58

Table 2. Nei's analysis of gene diversity in populations of *G. ashmeadi* from the US (fc, field collected; Ht, total genetic diversity (SD); Hs, average genetic diversity within populations (SD); G_{ST} (mean), coefficient of gene differentiation; θ (mean), theta (SD) is analogous to F_{ST}; and Nm, gene fow).

Ht	Hs	G_{ST}	θ	Nm
fc				
0.2312	0.1442	0.3761	0.4957	0.8295
(0.032)	(0.016)		(0.077)	
All				
0.2087	0.1161	0.4438	0.4927	0.6267
(0.034)	(0.013)		(0.057)	

Table 3. Nei's unbiased (1987) genetic distance (below diagonal) and Reynolds et al. (1983) genetic distance (above diagonal). Six populations *of G. ashmeadi* from the US field populations were also analyzed separately (bottom portion of table).

Pop.	CA	WTX-1	WTX-2	SATX	LA	QFL
CA	*****	0.8682	0.6818	0.6441	0.6275	0.4227
WTX-1	0.2024	*****	0.8080	0.8703	0.6871	0.8890
WTX-2	0.1341	0.1391	*****	0.7213	0.6663	0.5322
SATX	0.1384	0.1789	0.1286	*****	0.4842	0.4956
LA	0.1422	0.1335	0.1233	0.0890	*****	0.3705
QFL	0.0896	0.2020	0.0890	0.0951	0.0715	*****

Pop.	CA	WTX-2	SATX	QFL
CA	*****	0.8138	0.8075	0.4559
WTX-2	0.2215	*****	0.7741	0.4069
SATX	0.2230	0.2015	*****	0.4666
QFL	0.1308	0.1021	0.1328	*****

except the WTX-2, were associated with population-specific markers (data not shown). Within populations, gene diversity values (h) were observed ranging from 2.9 to 14.3% with WTX-1 having the lowest and QFL having the highest value (Table 1). In general, the two Weslaco populations (WTX-1 and -2) were found to have the lowest h values. No significant differences in h were seen between the two Weslaco populations ($t = 1.49$, $df = 58$, $P > 0.05$), but significant differences ($P < 0.05$) were observed between WTX-1 and the rest of the U. S. populations. Interestingly, no significant differences in h were observed between the reared LA and the rest of the field populations. The fact that QFL was associated with an h value of 14.3% was surprising since this population was from a single egg mass. Overall, the field populations and all the U. S. G. ashmeadi populations together had an h value of 23.0 and 20.8%, respectively. The number of polymorphic markers ranged from 12 to 20 with WTX-1 and -2 having the lowest and QFL the highest. Percentage of polymorphic markers (%P) ranged from 29.3 to 58.8%, but overall, 100% of the ISSR-PCR markers were polymorphic, including the field populations analyzed separately. The two Weslaco populations were associated with the lowest %P and QFL with the highest. It is interesting to note that even though both LA and WTX-1 were reared, WTX-1 is presented with a significantly ($P < 0.05$) lower h value. These results may indicate a real genetic difference between the two Weslaco populations, including the possibility of sympatric strains.

ISSR-PCR Differentiation Among US G. ashmeadi Populations

Table 2 presents the results from the different approaches used to apportion variation into within- and among-populations levels. Simultaneous exact tests for population differentiation indicated that highly significant differences in marker frequencies exist among the six U.S. populations (All: $\chi^2 = 676.2$; $df = 82$; $P = 0.0000$, and fc: $\chi^2 = 485.2$; $df = 68$; $P = 0.0000$). These statistically significant tests suggest that discrete subpopulations exist. The average genetic diversity within populations (Hs) value for the field populations is 14.4%. Table 2 also shows a comparison of other genetic differentiation estimates, G_{ST} and θ. Good agreement was seen between G_{ST} and θ values, respectively for field and for all populations. The G_{ST} values for field and all populations indicate that about 38 and 44% of the variance is distributed among populations, and 62 and 56% is distributed within populations, respectively. The θ values show that about 50% of the variance is seen among populations in both field and all populations. The indirect estimate of gene flow, Nm base on G_{ST}, demonstrated low values for both field and all U. S. populations. These values indicate restricted gene flow among the populations.

Genetic Relatedness among G. ashmeadi Populations from the US

Average genetic divergence (D) among both field [Nei = 0.1702 (0.1021-0.2230); Reynolds = 0.6208 (0.4069-0.8138)] and all populations [Nei = 0.1304 (0.0715-0.2024); Reynolds = 0.6512 (0.3705-0.8890)] was high (Table 3). We compared the level of genetic divergence between the field populations and the WTX-1 and LA reared populations and found mean D values of 0.1806 (Nei) and 0.8589 (Reynolds) and 0.1065 (Nei) and 0.5371 (Reynolds), respectively. These results indicate that WTX-1 is more diverged than LA. A comparison of Nei's genetic distance within the Texas populations, WTX-2 vs WTX-1 (0.1391) and WTX-2 *vs* SATX (0.1286), showed that divergence is slightly higher between the Weslaco populations. Sympatric species tend to have higher levels of genetic differentiation; more work is needed to confirm this possibility. The divergence between ARG and all U. S. *G. ashmeadi* populations was very high, 0.3633 (Nei) and 1.6093 (Reynolds), respectively. These results support the taxonomic data that ARG is another species. Dendrograms based on Nei's genetic distance are shown on Fig. 1 with all populations including ARG (Fig. 1A) and the field populations analyzed separately (Fig. 1B). At least two main clusters are identified on the dendrogram with ARG clustered as an outlier (Fig. 1A). Within a second cluster or all *G. ashmeadi* from the U. S., WTX-1 appears to be the most differentiated (Fig. 1A). The CA population appears to form a second subcluster and the two southeastern populations, LA and QFL form a single cluster. The WTX-1 and –2 populations are distributed in different clusters. Also within Texas (Fig. 1B), WTX-2 and SATX show divergence as they appear on a separate cluster. It is interesting to note that this same pattern of differentiation is seen with *H. coagulata* within Texas (de León *et al*. 2004).

In summary, the major observations of this study were that 1) among *G. ashmeadi* populations, based on genetic differentiation measurements (exact test, G_{ST}, θ), extensive genetic structure was identified; 2) the mean expected gene diversity value for LA did not differ from field populations, whereas WTX-1 was observed with a significantly lower mean expected gene diversity value as compared to field populations (except WTX-2); 3) QFL generated the most polymorphic markers (20) with only 13 individuals, even though they were all siblings or from one egg mass. This is an interesting result since it may be assumed that siblings are not associated with high variability or have isofemale line characteristics. These results indicate that *G. ashmeadi* parasitoid siblings somehow manage to maintain their genetic diversity. Further studies are required to confirm this observation in this species and other *Gonatocerus* species. Variation within 10 male individuals (*Anaphes* sp.nov.) was demonstrated with RAPD markers by Landry *et al*. (1993), but they were not from the same egg mass; 4) based on genetic distance or average divergence, WTX-1 appeared to be the most differentiated population. Within Texas, field populations WTX-2 and SATX appeared on separate clusters, indicating that these populations are differentiated even though they are within the same state; and 5) The ARG population is confirmed to be a different species. More research is required to confirm these results, sequencing of standard genes [e. g., mitochrondia cytochrome oxidase (COI)] and ITS-2 fragments are in progress.

GENETIC DIFFERENTIATION AMONG GEOGRAPHIC POPULATIONS OF *GONATOCERUS ASHMEADI*, A PRIMARY EGG PARASITOID OF THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT

The aim of genetically comparing different populations of the same species of natural enemies is to identify the strain that is most adapted to the environment where it will be released. In the present study, Inter-Simple Sequence Repeat-Polymerase Chain Reaction (ISSR-PCR) was utilized to estimate the population genetic structure of *Gonatocerus ashmeadi*. Six populations from throughout the U. S. and a population from Argentina identified as near *G. ashmeadi* were analyzed. Four populations [California (CA), San Antonio, TX (SATX), Weslaco, TX (WTX-2), and Quincy, Florida (QFL)] were field collected and two [Louisiana (LA) and Weslaco, TX (WTX-1)] were reared. Three ISSR-PCR reactions were pooled to generate 41 polymorphic markers among the six U. S. populations. Nei's expected heterozygosity values (*h*), including the reared population from Louisiana were high (9.0-14.3%) for all populations, except for a reared population from WTX-1 (2.9%). The total genetic diversity value (Ht) for the field populations was high (23%). Interestingly, the Florida population that was collected from one egg mass generated the greatest number of polymorphic markers (20) and was observed with the highest gene diversity value (14.3%). All populations, except WTX-2 generated population-specific markers. Comparison of genetic differentiation estimates, which evaluate the degree of genetic subdivision, demonstrated good agreement between G_{ST} and θ values, 0.38 and 0.50, respectively for field populations, and 0.44 and 0.50, respectively for all populations. Average genetic divergence (D) indicated that the WTX-1 population was the most differentiated. Average D results from the Argentina population support the taxonomic data that it is a different species. The present results estimate the population genetic structure of *G. ashmeadi*, demonstrating extensive genetic divergence and restricted gene flow (Nm = 0.83) among populations. These results are of interest to the Pierce's Disease/Glassy-winged Sharpshooter biological control program because the key to successful biological control may not be in another species, but instead in different geographic races or biotypes.

INTRODUCTION

Gonatocerus ashmeadi (Girault) (Hymenoptera: Mymaridae) is a primary egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the glassy-winged sharpshooter (Huber 1998). A biological control program is currently in progress in California against *H. coagulata* because this xylem feeding sharpshooter is a serious economic pest that vectors a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's Disease in grapevines. Studies of allele or marker frequencies in naturally occurring parasitoid populations are important, not only for identifying genetic variation of potential benefit in the selection and screening of biological control organisms, but also for the detection of genetic markers indicative of specific biological traits or geographic origins. In addition, the recognition of intraspecific variation can be as crucial for the success of biological control programs as is sound species determination. Populations of parasitoids from distinct geographical regions may differ in relevant biological characteristics of importance to biological control (Powell and Walton 1989; Narang *et al*. 1993; Unruh and Woolley 1999). An aim of genetically comparing different populations of the same species of natural enemies is to identify the strain that is most adapted to the environment where it will be released (Messenger and van den Bosch 1971); in other words, the key to successful biological control may not be in another species, but instead in different geographic races or biotypes (Diehl and Bush 1984). Reliable methods are needed for distinguishing various exotic strains of these biological control agents from those indigenous to the U. S., including parasitoids from different states within the U. S. Release of unidentified and uncharacterized strains can make it difficult to document their establishment and dispersal. Therefore, genetic typing of strains prior to their release in the field is highly desirable (Narang *et al*. 1993).

OBJECTIVES

1. Estimate genetic variation or gene diversity within and among populations.
2. Estimate the population genetic structure.
3. Determine whether ISSR-PCR was sensitive enough to identify diagnostic markers in geographic populations.
4. Confirm the species identification of a population of egg parasitoids from Argentina identified as near *G. ashmeadi*.

RESULTS AND CONCLUSIONS

ISSR-PCR Marker Heterozygosity and Genetic Diversity

A total of 41 polymorphic markers were generated in the six populations of G. ashmeadi (163 individuals) from the U. S. with three pooled ISSR-PCR reactions. G^2-contingency tests indicated significant heterogeneity of marker frequency across all U. S. populations for 31 of 41 markers and for 25 of 34 markers for the field populations (not shown). All populations,

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FUNDING AGENCIES

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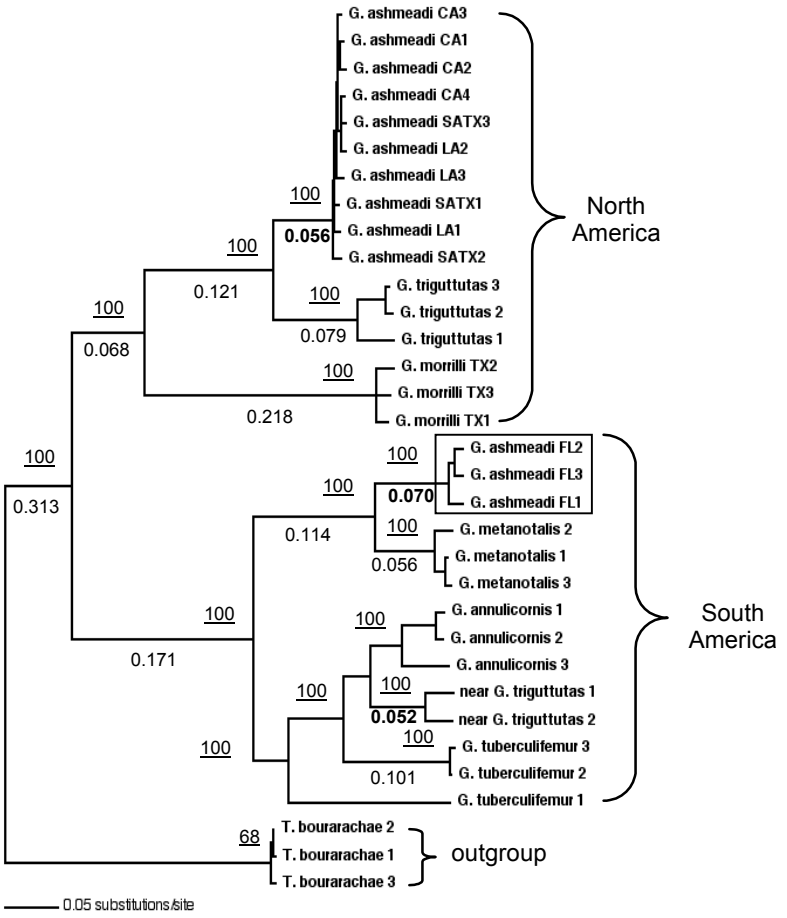

Figure 2. Phenograms of ITS2 rDNA sequence fragments from *Gonatocerus* egg parasitoid species, including candidate species from South America (Argentina). Analysis was performed with the alignment program ClustalX (Thompson *et. al.* 1997) and the Nieghbor-Joining trees were created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *Trichogramma bourarachae* (1, AF043624; 2, AF043625; 3, AF043626) are included are an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches underlined), as percentage of 1000 replications. To account for intra- and inter-specific variation, several randomly chosen individuals (2-4) were included.

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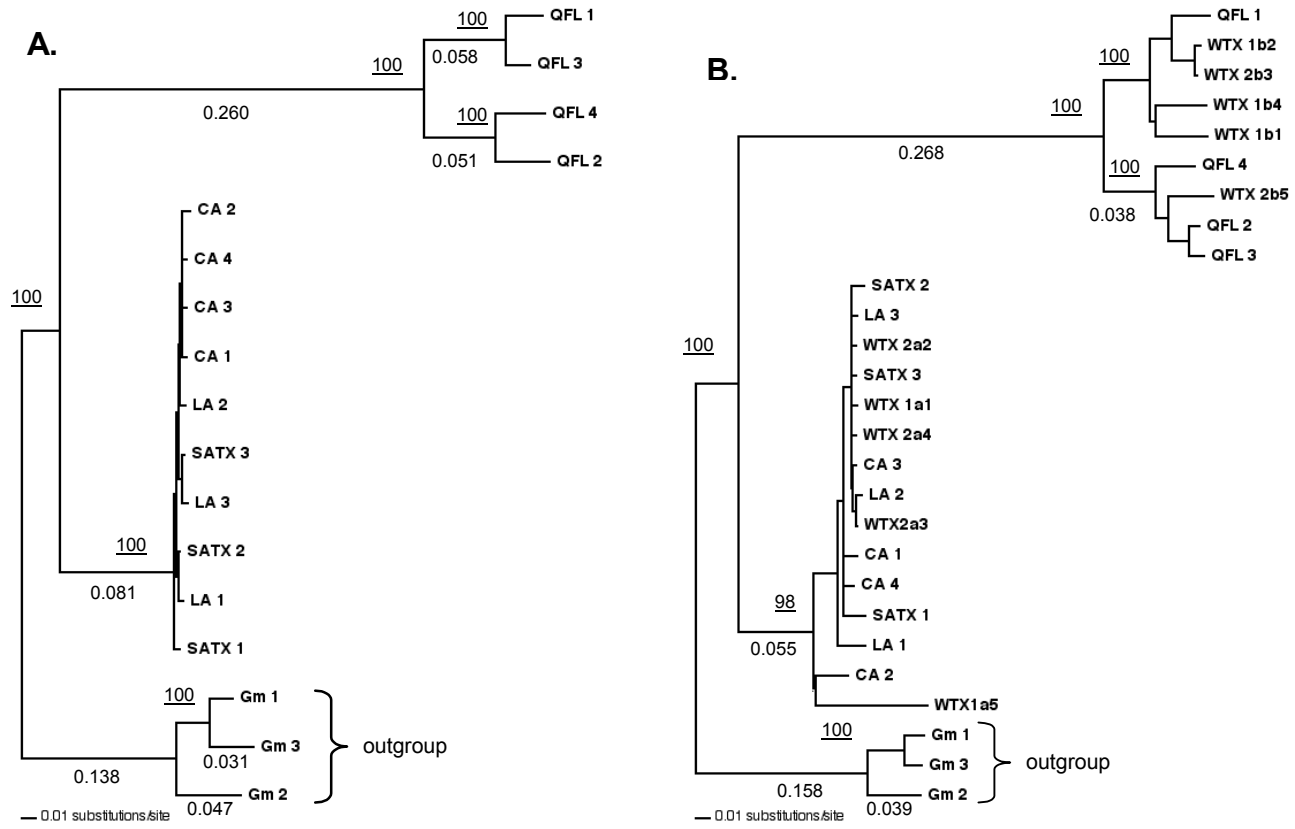

Figure 1. Phenograms of ITS2 rDNA sequence fragments from geographic populations of *G. ashmeadi*. Analyses were performed with the alignment program ClustalX (Thompson *et. al.* 1997) and the Nieghbor-Joining trees were created with the phylogenetic program PAUP 4.0 (Swofford 2002). In the genetic distance trees *G. morrilli* are included as an outgroup, displaying branch lengths (below branches) and bootstrap values (above branches underlined), as percentage of 1000 replications. Trees are presented both without Weslaco, TX populations **(A)** and with Weslaco, TX populations **(B)**. To account for intra- and inter-populational variation, several randomly chosen individuals (3-4) were included.

Table 2. Pairwise sequence distances (range) of ITS-2 rDNA fragments from *Gonatocerus* species showing percentage divergence. The alignment program ClutstalW (Thomas *et al.* 1994) from DNAStar was utilized for this analysis. To account for intra- and inter-specific variation, several individuals (2-3) were included. Ga*, *G. ashmeadi* (California, San Antonio, TX, and Louisiana were pooled for a total of 10 individuals); Gt, *G. triguttutas* (TX); Gm, *G. morrilli* (TX); and candidate South American (Argentina) species: Gann, *G. annulicornis*; nGt, near *G. triguttutas*; Gtub, *G. tuberculifermur*; Ga(FL), *G. ashmeadi* (Quincy, FL USA); Gmet, *G. metanotalis*; and Tb, *Trichogramma bourarachae* (outgroup).

G species	***Ga****	***Gt***	***Gm***	***Gann***	***nGt***	***Gtub***	***Ga(FL)***	***Gmet***	***Tb***
Ga*	0.10-0.90								
Gt	15.8-17.9	0.10-0.20							
Gm	35.0-38.9	41.7-45.5	1.80-1.80						
Gann	82.4-87.2	97.5-101	87.0-88.1	0.00-0.10					
nGt	80.0-83.5	94.8-97.3	82.7-84.2	3.40-3.60	0.10-0.10				
Gtub	*78.0-82.0*	*90.8-92.0*	*81.4-84.1*	*11.5-12.1*	*11.6-11.8*	*0.10-0.50*			
Ga(FL)	75.4-79.8	88.4-90.2	84.3-87.0	37.7-39.3	36.7-38.1	35.9-36.4	0.10-1.00		
Gmet	76.2-80.4	87.6-89.4	85.5-88.2	35.4-36.4	34.7-35.3	34.9-36.1	8.30-9.00	0.10-.040	
Tb	84.8-92.5	87.2-91.5	88.4-90.4	66.1-67.6	69.0-70.3	68.5-70.5	77.3-79.6	74.2-76.2	0.20-0.90

and WTXb is very low (0.00-0.40%) and falls within the range of the inra-populational variation. In contrast, the %D between WTXb and the rest of the populations falls within the same range that the QFL population (65.9-69.8%) fell in. The phylogenetic analysis of all populations (Fig. 1B), including the two Weslaco populations (WTXa and WTXb) demonstrated that these two populations fell on separate clades, confirming the existence of sympatric strains in Weslaco. WTXb clustered with QFL and WTXa clustered with the rest of the *G. ashmeadi* populations. Again, the distance tree is supported by extremely high bootstrap support values (100%). The very high %D values indicate that the QFL and WTXb complex diverged some time ago. The earliest record of *G. ashmeadi* in California was from 1979 (Vickerman et al. 2004) and recently, we showed that a subset of glassy-winged sharpshooters in California had their origin in central Texas (de León et al. 2004). The present results lend support to the idea that *G. ashmeadi* may have its origins in central Texas (SATX) (including the very closely located Louisiana). So it is possible that *G. ashmeadi* was transported to California along with the Glassy-winged Sharpshooter from central Texas.

Phylogenetic Relationships Among Gonatocerus Species

Resolution of relationships requires information about variability not only at the level of populations within a species but also between species (Narang et al. 1993; Unruh and Woolley 1999); therefore, a molecular systematic approach was undertaken with various *Gonatocerus* species, including candidates from South America (Argentina). For the pairwise sequence distance analyses, the *G. ashmeadi* populations (LA, SATX, WTXa, and CA) that formed one clade in fig. 1 were pooled (Ga*, Table 2) and compared to the rest of the *Gonatocerus* species. The %D values among these populations were very low (0.10-0.90%), falling within the range of the intra-specific variation seen within each individual species. The %D of *G. triguttutas* (Gt) and *G. morrilli* (Gm) vs Ga* is 15.8-17.9 and 35.0-38.9%, respectively. In contrast, the %D of *G. ashmeadi* from Florida [Ga(FL)] vs Ga* is 75.4-79.8%, these values fall within the %D range of all South American species (Table 2). This is demonstrated visually on the phenogram in Fig. 2 with very strong bootstrap values supporting the topology of the Nieghbor-Joining distance tree. As seen from the phenogram, the North and South American *Gonatocerus* species are separated into their perspective clades. It is interesting to note that Ga(FL) is more closely related to *G. metanotalis* (Gmet) (8.30-9.00%), a South American species than it is to any North America species (Fig. 2). The *Gonatocerus* species more closely related to Ga* is Gt (15.8-17.9%). The present results showing extensive sequence divergence at the ITS2 rDNA fragment in a population of *G. ashmeadi* from Florida lends strong support to the fact that these individuals may actually be another species or rather *G. ashmeadi* exists in nature as a species-complex. Our results are in contrast with those of Vickerman et al. (2004). In our studies we performed a phylogenetic analyses of the ITS2 rDNA sequences. In addition, Vickerman et al. (2004) demonstrated that populations of *G. ashmeadi* from Florida vs other geographic regions were able to hybridize. We have not yet performed these types of studies, but it may be necessary to extend these crossing studies to the F2 generation to seen a negative effect or as demonstrated by Wu et al. (2004) a negative effect was not seen until backcrosses were performed. The findings of the present study are important to the Glassy-winged Sharpshooter/Pierce's Disease biological control program in California.

Table 1. Pairwise sequence distances (range) of ITS-2 rDNA fragments from geographic populations of *G. ashmeadi* showing percentage divergence. The alignment program ClutstalW (Thomas *et al.* 1994) from DNAStar was utilized for this analysis. To account for intra- and inter-populational variation, several individuals (3-4) were included. QFL, Quincy, Florida; WTXb, Weslaco, TX; LA, Louisiana; SATX, San Antonio, TX; WTXa, Weslaco, TX; CA, California; Gm, *G. morrilli* (outgroup). Relate to figure 1B.

Pop	QFL	WTXb	LA	SATX	WTXa	CA	Gm
QFL	0.10-0.40						
WTXb	0.00-0.40	0.00-0.10					
LA	68.0-69.8	68.1-70.4	0.60-0.90				
SATX	68.2-69.8	67.9-70.8	0.30-0.80	0.20-0.90			
WTXa	67.1-69.5	66.6-70.1	0.20-0.70	0.20-0.90	0.10-0.90		
CA	65.9-67.6	66.0-67.9	0.80-1.00	0.60-1.10	0.30-1.00	0.20-0.80	
Gm	77.8-81.2	77.6-82.3	32.3-36.3	31.4-37.0	31.8-40.6	36.3-36.7	0.00-0.30

EXTENSIVE SEQUENCE DIVERGENCE IN THE ITS2 RDNA FRAGMENT IN A POPULATION OF *GONATOCERUS ASHMEADI* FROM FLORIDA: PHYLOGENETIC RELATIONSHIPS OF *GONATOCERUS* SPECIES

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ABSTRACT

The aim of the present study was to resolve the genetic relationships of geographic populations of *Gonatocerus ashmeadi*, a primary egg parasitoid of the glassy-winged sharpshooter. A phylogenetic approach was implemented by sequencing the Internal Transcribed Spacer-2 (ITS2) region of several individuals per population. In addition, the phylogenetic relationships of several *Gonatocerus* species were also determined. Six geographic populations of *G. ashmeadi* were analyszed: Quincy, FL (QFL), two populations from Weslaco, TX (WTXa and WTXb), Louisiana (LA), San Antonio, TX (SATX), and California (CA). The percentage divergence (%D) of the ITS2 sequences, as measured by genetic distance, was small among LA, SATX, and CA (0.10-1.10%); whereas, the %D for QFL vs these populations was extremely high (65.9-69.8%). A Nieghbor-Joining distance tree separated the QFL population into a separate clade supported by very high bootstrap values (100%). When the Weslaco populations were included in the anaylsis, they clustered into two distinctive clades, WTXb clustered with QFL and WTXa clusterd with the rest of the populations; again very high bootstrap values (100%) supported the topology of the distance tree. These results indicate the present of sympatric strains in Weslaco. The phylogenetic analysis of several *Gonatocerus* species clustered the respective species into North and South American clades. The %D of the QFL population fell within the range (75.4-87.2%) of the South American *Gonatocerus* species and clustered within the South American clade. The present molecular phylogenetics results provide strong evidence that *G. ashmeadi* from Florida may be a different species. In addition, the data is suggestive that the origin of *G. ashmeadi* in California is the Texas region, including the closely located Louisiana. The findings of the present study are important to the Glassy-winged Sharpshooter/Pierce's Disease biological control program in California.

INTRODUCTION

Gonatocerus ashmeadi (Girault) (Hymenoptera: Mymaridae) is a primary egg parasitoid of *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae), the Glassy-winged Sharpshooter (Huber 1998). A biological control program is currently in progress in California against *H. coagulata* because this xylem feeding leafhopper is a serious economic pest that vectors a strain of *Xylella fastidiosa* (Wells), a bacterium that causes Pierce's Disease in grapevines. Accurate identification of natural enemies is critical to the success of classical biological control programs. Lack of proper identification procedures has affected the early stages of several projects (Messing and Aliniazee 1988; Löhr *et al*. 1990). The Internal Transcribed Spacer regions (ITS-1 and –2) have been used extensively to examine the taxonomic status of species and for diagnostic purposes, and success with this approach has been reviewed by Collins and Paskewitz (1996).

OBJECTIVES

1. Determine the phylogenetic relationships of geographic populations of *G. ashmeadi*.
2. Determine the phylogenetic relationships of several *Gonatocerus* species, including candidate species from South America (Argentina).

RESULTS AND CONCLUSIONS

Genetic Relatedness Among Geographic Populations Of G. ashmeadi

Levels of genetic divergence in the ITS2 rDNA fragment among populations were determined by calculating the pairwise estimates for genetic distance (Table 1). Recently, we determined by ISSR-PCR DNA fingerprinting that *G. ashmeadi* geographic populations were highly differentiated (de León and Jones 2004). The data demonstrated that the Quincy, FL (QFL) population had the highest gene diversity value. In addition, the data indicated that two Welsaco, TX populations collected at different times of the year were divergence or differentiated from each other and gave a first clue as to the presence of sympatric strains in Weslaco. As seen on Table 1, the sequence percentage divergence (%D) between the QFL population and the rest of the *G. ashmeadi* geographic populations (LA, SATX, WTXa, and CA) was extremely high, ranging from 65.9 to 69.8%. The %D between QFL and the outgroup population (*G. morrilli*) ranged from 77.8-81.2%, whereas LA, SATX, WTXa, and CA ranged from 31.4 to 37.0% compared to the outgroup. The %D among LA, SATX, WTXa,,and CA populations was extremely low, 0.10 to 1.10%, indicating the very close genetic similarity among these geographic populations. This range is within the intra-populational variation found within each of these populations. A phylogenetic anaylsis (Fig. 1A) demonstrated that the QFL and the LA, SATX, WTXa, and CA populations formed two distinct clades supported by extremely high bootstrap support values; in most case they were at 100%. Our second goal was to confirm whether sympatric strains of *G. ashmeadi* indeed existed in Weslaco. Table 1 shows that the %D between QLF

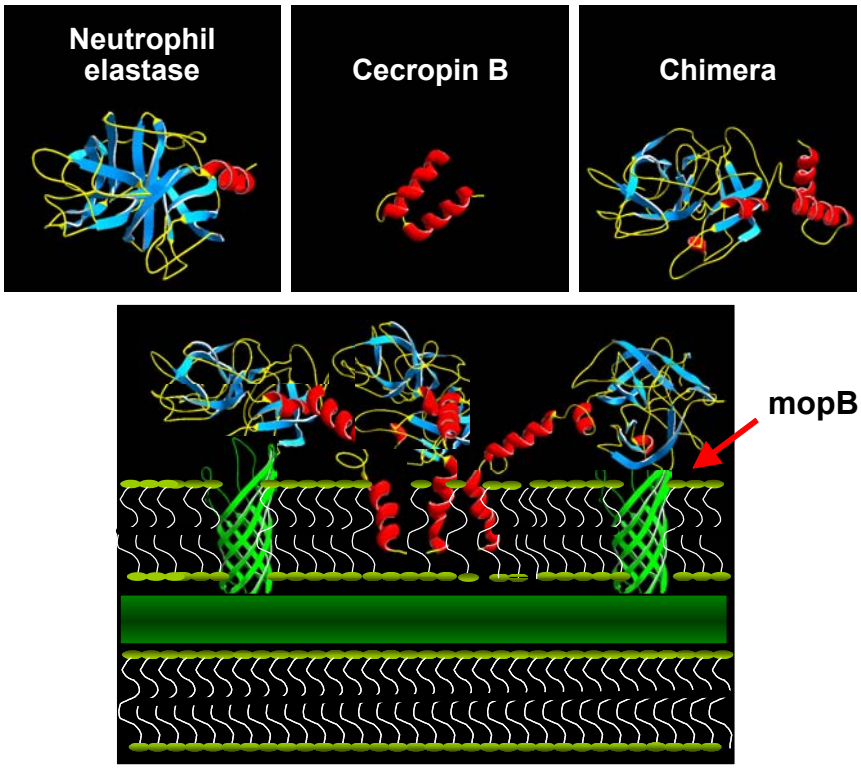

Figure 2. Design and mechanism of chimeric protein targeted to *X. fastidiosa.* The top panel shows the two domains of the chimera in separate planes: neutrophil elastase (1HNE from PDB) is on the left. A homology model of ceropin B is shown in the middle. The right plane shows the energy minimized model of the elastase-cecropin B chimera. The bottom panel is a schematic of the hypothetic mechanism of the chimeric protein. Elastase binds to and cleaves a specific loop on the *X. fastidiosa* outer membrane protein mopB. This action brings cecropin B in close contact with the membrane, where is associates with other cecropin molecules and disrupts the membrane by forming a pore, thereby disabling the bacterium.

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FUNDING AGENCIES

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

OBJECTIVES

Objective 1: a) Utilize literature data and computer modeling to identify an SRD that specifically targets mopB (Elastase)
b) Utilize literature data and computer modeling to identify a useful Cecropin (i.e., Cecropin B)
c) *In vitro* testing of anti-*Xylella* activity of the mopB-specific SRD (Elastase) and *Xyllela*-specific Cecropin B and demonstration of synergistic killing effect due to the combined use of Elastase and Cecropin B.

Objective 2: a) Design and construction of synthetic gene encoding Elastase-Linker-Cecropin B Chimeric protein
b) Expression Elastase-Linker-Cecropin B in insect and plant cells and testing activity *in vitro*.

Objective 3: a) Expression in transgenic plants
b) Testing for anti-*Xylella* activity *in planta* and testing for graft transmissibility.

RESULTS AND CONCLUSION

Human Neutrophil Elastase (HNE) (6) was chosen as our first SRD. Neutrophils contain a variety of proteins that enable the cells to migrate toward and eliminate microbial pathogens (7). Until 1991, no specific antibacterial activity had been ascribed to HNE (8). However recent research has established that HNE is the only human neutrophil protein, which is capable of individually killing *Borrelia burgdorferi*, the causative agent of Lyme disease (9, 10). Furthermore, it is known that HNE can augment the cidal properties of other active proteins (11). Sequence-structure analysis of mopB revealed that it contained an specific cleavage site for HNE that is exposed on the surface. We have studied the efficacy of HNE in combination with the antibacterial peptide Cecropin B, that inserts preferentially into the lipid bilayer of gram-negative bacteria, in killing *Xf*. Measuring the number of colony forming units remaining after the bacterium was exposed to HNE, Cecropin B and the combination of both, we found that HNE greatly stimulates the lysis induced by Cecropin B. In addition, we found that Mop B was partially digested by HNE after incubating either purified Mop B or *Xf* cells with HNE for an hour. Based on these preliminary results, we have designed a chimeric protein of Cecropin B and HNE; in order to stabilize the Cecropin B peptide and enhance the overall affinity of the ligands for the bacterial surface. The covalent attachment of Cecropin B to HNE is proposed to increase the stability of the peptide by lowering the conformational entropy of its unfolded state and to increase the overall affinity for the bacterial surface by minimizing the degrees of motion at the binding site, thereby increasing binding between the ligands and the surface.

Our strategy began with the generation of a 3-D model of the chimera. The modeling was based on published protein data bank (PDB) structures of HNE and nuclear magnetic resonance structures of peptides homologous to Cecropin B. A short G-S-T-A peptide linker was inserted between the C-terminus of HNE and the N-terminus of Cecropin B to allow both functional domains to make contact with the bacterial surface simultaneously without steric interference. Energy minimization and molecular dynamics analysis using the AMBER 7.0 force field indicated that the chimera forms a stable structure. The HNE-GSTA-Cecropin B chimera gene was synthesized and is currently being cloned into a baculovirus vector for overexpression in insect cells. The chimera will be purified from insect cells and tested for its activity against *Xf in vitro*. The chimera will be also cloned into a plant vector for transformation of grape embryogenic callus growing in a CELLline 350 bioreactor where they will be analyzed for the production and anti-*Xf* activity of the secreted protein. We will choose the most promising embryogenic lines for plant regeneration. The plant expression vector will have necessary regulatory sequences to facilitate transcription and extracellular delivery of the protein product. Currently we are investigating grapevine embryogenic callus for the extracellular production of the pear polygalacturonase inhibiting protein (pPGIP). This protein has been found in the xylem exudate of transgenic grapes expressing the pPGIP gene and will be used to modify delivery of the chimeric protein to grapevine xylem tissues.

IVGGRRARPHAWPFMVSLQLRGGHFCGATLIAPNFVMSAAHCVANVNVRAVRVVLGAHNLSRREPTR
QVFAVQRIFEDGYDPVNLLNDIVILQLNGSATINANVQVAQLPAQGRRLGNGVQCLAMGWGLLGRNRG
IASVLQELNVTVVTSLCRRSNVCTLVRGRQAGVCFGDSGSPLVCNGLIHGIASFVRGGCASGLYPDAFAP
VAQFVNWIDSIIQ<u>GSTA</u>**KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL**

Figure 1. HNE-cecropin B chimeric amino acid sequence. HNE is attached to cecropin B (shown in bold) by the GSTA linker, which is underlined.

DESIGN OF CHIMERIC ANTI-MICROBIAL PROTEINS FOR RAPID CLEARANCE OF *XYLELLA*

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ABSTRACT

Xylella fastidiosa (*Xf*), is a gram-negative xylem-limited bacterium and causative agent of Pierce's disease (PD) in California grapevines. During very early stages of *Xf* infection, specific carbohydrates/lipids/proteins on the outer membrane of *Xf* interact with plant cells and are important for virulence (3). Design of a protein inhibitor that interrupts this step of the plant-*Xf* interaction will be useful in anti-microbial therapy and controlling PD. Traditionally, antibiotics are prescribed as a preferred therapy; however, a pathogen often develops antibiotic resistance and escapes their anti-microbial action (4). In this UC/LANL project, we propose a novel protein-based therapy that circumvents the shortcomings of an antibiotic. We have designed a chimeric anti-microbial protein with two functional domains. One domain (called the surface recognition domain or SRD) will specifically target the bacterium outer-membrane whereas the other will lyse the membrane and kill *Xf*. In this chimera, Elastase is the SRD that recognizes mopB, the newly discovered *Xf* outer membrane protein (5). The second domain is Cecropin B, a lytic peptide that targets and lyses gram-negative bacteria. We have successfully tested each of these components individually and demonstrated that they each (Elastase and Cecropin B) display activity against *Xf*, which is increased when both proteins are combined. We have tested Elastase against purified mopB and intact *Xf* cells and found that mopB is degraded in both cases, suggesting that it is potentially a target for Elastase. The HNE-GSTA-Cecropin B chimera gene has been synthesized and is currently being cloned into vectors for overexpression in insect and grapevine cells in order to test its activity against *Xf in vitro*. We have also initiated transgenic grapevine cultures expressing a pear polygalacturonase inhibiting protein that is secreted into the medium using a CELLline 350 bioreactor. In the future, we plan to use this system to test secretion and anti-*Xf* of the chimeric protein.

INTRODUCTION

Globally, one-fifth of potential crop yields are lost due to plant diseases primarily of bacterial origin. *Xylella fastidiosa* (*Xf*) is a devastating bacterial pathogen that causes PD in grapevines, citrus variegated chlorosis (CVC) in citrus, and leaf scorch disease in numerous other agriculturally significant plants including almonds in California (http://danr.ucop.edu/news/speeches). Since the glassy-winged sharpshooter (an insect vector) efficiently transmits PD, a great deal of effort has been focused on using insecticides to localize and eliminate the spread of this disease. However, the availability of the whole genome sequences of PD and CVC strains of *Xf* offer new avenues to directly target and inactivate the pathogen. In this project, we propose a structure-based approach to develop chimeric anti-microbial proteins for rapid destruction of *Xf*. The strategy is based upon the fundamental principle of innate immunity that plants recognize and clear pathogens in rapid manner (1-2). Pathogen clearance by innate immunity occurs in three sequential steps: pathogen recognition, activation of anti-microbial processes, and finally pathogen destruction by anti-microbial processes. Different sets of plant factors are involved in different steps of innate immunity. Our strategy of combining a pathogen recognition element and a pathogen killing element in the chimeric molecule is a novel concept and has several short and long term impacts.

Efforts to develop an artificial diet capable of supporting larval and pupal development will initially focus on testing established diets formulated for the *in vitro* rearing of other egg parasitoids, e.g., those used for rearing lepidopteran egg parasitoids including several *Trichogramma* spp. (Hoffman et al., 1975; Li-Ying 1992; Consoli and Parra, 1997; Xie et al., 1997; Grenier et al., 1998; Qin, Beijing Univ. pers. comm.;), *Telenomus heliothidis* (Strand et al., 1988), and *Ooencyrtus* spp. (Masutti et al., 1994; Lee and Lee, 1994); a coleopteran egg parasitoid, *Edovum puttleri* (Hu et al., 1999; Hu et al., 2001) , and a pentatomid egg parasitoid, *Trissolcus basalis* (Volkoff et al., 1992*)*. For studies on the development of an artificial ovipositional substrate, membranes that will be derived from a variety of sources will be tested, such as: oxygen-permeable films used for mass rearing *Trichogramma* spp. (Qin, Beijing University, pers. comm.), parafilm (Wittmeyer et al., 2001; Cooperband and Vinson, 2001), and polycarbonate, polyvinylchloride, polyethylene, and/or polypropylene membranes (Masutti et al., 1994; Morrison et al., 1983; Consoli and Parra 1999).

OBJECTIVES

1. Formulate an artificial diet capable of supporting the development and reproduction of *Gonatocerus* spp. parasitoids of the eggs of glassy-winged sharpshooter, *Homalodisca coagulata.*
2. Screen, modify, and evaluate existing materials for their suitability as ovipositional substrates for these egg parasitoids.
3. Develop and optimize an *in vitro* rearing unit, consisting of an artificial diet and ovipositional substrate, that can be utilized for *Gonatocerus* spp. oviposition, parasitoid development, and release.

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 have been conducted in collaboration with Leopold at ARS in Fargo that indicate cold-storage processes should offer suitable method(s) to preserve the natural host of the parasitoid for these studies.

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DEVELOPMENT OF AN ARTIFICIAL DIET AND EVALUATION OF ARTIFICIAL OVIPOSITIONAL SUBSTRATES FOR THE *IN VITRO* REARING OF *GONATOCERUS* SPP. PARASITOIDS OF THE EGGS OF 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 *in vitro* rearing system for one or more of the three mymarid species of *Gonatocerus* currently being reared and released in California to control GWSS. A complete *in vitro* rearing system will include both a growth-enhancing artificial diet for larval and pupal development as well as a suitable oviposition substrate, or "artificial egg". Initial studies will formulate artificial diets based on those developed previously for hymenopteran parasitoids, with an emphasis being placed on diets for other egg parasitoids. To accomplish this, *Gonatocerus spp.* eggs and/or larvae will be dissected from host eggs and placed in cell culture plates containing selected diets. Comparisons will be made between the development of parasitoids on these artificial diets, and those developing on the natural host. Developmental parameters measured will include extent of development, developmental time per stage, and weight. Once a promising diet is formulated, the reproductive rate and reproductive fitness of adults reared from these diets will be compared by using ovarian scoring and by assessing differences in fecundity and egg viability from crosses of diet-reared and host-reared adult wasps (Wittmeyer et al., 2001; Wittmeyer and Coudron, 2001). Refinement of the diet will be performed by modifying the diet based on its ability to meet the nutritional, phagostimulatory, and endocrine requirements of the parasitoid, and may include the additional of undefined components such as insect or cell-culture derived components. The suitability of artificial eggs, composed of different combinations of membranes and cupule sizes, will be evaluated statistically using pairwise comparisons of the proportion of "artificial eggs" and natural host eggs successfully parasitized by the same number of female *Gonatocerus* parasitoids (SAS, 2002).

INTRODUCTION
Surveys of potential biological control agents in Texas (where GWSS is endemic and under natural control) and California revealed that *Gonatocerus* spp. parasitoids are the predominant natural enemy of GWSS in the field, parasitizing between 75-90% of GWSS egg masses (Phillips, 2000; Jones, 2002; Hoddle 2003a). In California, over 90% of the eggs laid by the second generation of GWSS in late summer and early fall are parasitized by *Gonatocerus* spp., however, only 10 – 50% of the eggs laid by the first generation in the early spring are parasitized (Phillips et al., 2004; Hoddle 2003b). This suggests that survival of overwintering adult parasitoids is low, or that the current cohort of species of *Gonatocerus* are not effective in parasitizing GWSS eggs early in the season (Hoddle, 2003b; Jones, pers. comm.). However, augmentation of *Gonatocerus* spp. populations in early spring may be able to significantly reduce the population of GWSS that vector the disease later in the season and could be used to reduce pesticide use thereby aiding in the development of a classical biological control program. The current list of species being considered for biocontrol of GWSS in CA include the solitary egg parasitoids *Gonatocerus ashmeadi* (which accounts for 80-95% observed GWSS egg parasitization in California) and *G. triguttatus* (the primary GWSS egg parasitoid in Texas), as well as the gregarious egg parasitoid *G. fasciatus* (which may have a greater host finding efficiency than the other two) (Hoddle 2003a).

The implementation of current classical and augmentative biological control programs against GWSS has been complicated by a number of factors. Currently, no artificial diet exists for GWSS, and high costs are associated with rearing the sharpshooters in sufficient numbers to provide the necessary quantity of host eggs (Lauziere et al., 2002; Jones, pers. comm.). Long-term stockpiling of host eggs is not feasible at this time because host acceptance declines after refrigeration for 20 days at 13^{o}C, and parasitized eggs only remain viable for 7 days at 2^{o}C (Leopold, 2003). Consequently, augmentation of *Gonatocerus* spp. in many areas of California relies on the labor-intensive process of rearing the parasitoid on host eggs collected from the field (Jones, pers. comm.). Thus, the development of an artificial diet and ovipositional substrate as part of an *in vitro* mass rearing system for *Gonatocerus* spp. has a number of potential advantages over current rearing techniques. Additionally, *in vitro* rearing would also be more easily automated, reducing labor costs (Li-Ying, 1992; Qin, Beijing Univ., pers. comm.) and would provide an easier means for studying the reproductive and nutritional physiology of *Gonatocerus* spp.

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FUNDING AGENCIES

Funding for this project was provided by the USDA Animal and Plant Health Inspection Service and the University of California Agricultural Experiment Station.

RESULTS

During the reporting period, we have screened an additional 90 antimicrobial peptides derived from a combinatorial library for activity on 11 *X. fastidiosa* and 3 *Alcaligenes* strains. *Axd* was isolated from the mouthpart of wild captured GWSS by Carol Lauzon. We found that 44 AMPs showed potent antimicrobial toxicity against all strains studied. Six AMPs were found with activity toward *X. fastidiosa* and non-toxic to *Alcaligenes*. These 6 peptides (along with 4 these screened last year) were more extensive examined for effective inhibitory concentration to *Xylella* and toxicity to *Alcaligenes* and *E. coli* as a target organism (Table 1). Blake Bextine studied the ability of GWSS to transmit *X. fastidiosa* to naive grapevine seedlings by oral delivery one of several antimicrobial peptide - indolicidin at 2 concentration: 100 μg/ml and 500 μg/ml. *X. fastidiosa* transmission rates were reduced from 50% in the control group, to 35% with the 100 μg/ml concentration and 7% with the 500 μg/ml concentration when GWSS were exposed to indolicidin prior to inoculation access. Therefore, indolicidin was chosen to be the first candidate for the development of gene-cassette. Artificial gene(s) to code indolicidin were designed and constructed for expression in *E. coli*. cDNA-encoding this peptide was amplified by PCR with incorporation of a *Sal*1 restriction site and/or *BamH*1 and *EcoR*1 restriction sites. We are using the Glutathione s-transferase gene fusion system (GST) (Pharmacia Biotech. Inc) and *trc* expression system (Invitrogene Co.) to express individual peptides. The GST gene fusion system is an integrated system for the expression, purification and detection of fusion proteins produced in *E. coli*. A pTrcHisTOPO expression kit provides a highly efficient, rapid cloning strategy for direct insertion of *Taq* polymerase-amplified PCR product into a plasmid vector for expression in *E. coli*. No ligase, post-PCR procedures, or PCR primers containing specific sequences were required. We transformed competent cells of *E. coli* DH5λ and TOPO by pGEX and pTrcHisTOPO vectors containing indolicidin gene. Several transformants were selected using LB medium containing ampicillin at 50 μg/ml (Sigma) and currently are being examined for production of indolicidin with and without IPTG.

Table 1. Toxicity of antimicrobial peptides to X. fastidiosa, Alcaligenes, and E. coli strains

Peptide	Range of MICs (μg/ml) to *X. fastidiosa*[a]	*Alcaligenes sp.*[b]	*E. coli*[c]	Source
1. Indolicidin	16-64	-	-	APS[d]
2. PA2	32-128	-	-	NCSU[e]
3. PA6	32-64	-	-	NCSU
4. PA7	32-64	-	-	NCSU
5. DCR1	16-32	-	-	TPIMS[f]
6. DCR2	8-16	-	-	TPIMS
7. DCR3	32-64	-	-	TPIMS
8. DCR4	16-32	-	-	TPIMS
9. DCR5	16-32	-	-	TPIMS
10.DCR6	8-16	-	-	TPIMS

[a] *– MICs of the antimicrobial peptides to eleven* X. fastidiosa *strains studied*
[b] *– Activity of AMPS to* Alcaligenes xylosoxidans denitrificans *134, 135, and 136 is negative*
[c] *– Activity of AMPs to* E. coli *DH5λ and TOPO is negative*
[d] *– American Peptide Company, Sunnyvale, CA*
[e] *– North Carolina State University, Raleigh, NC*
[f] *– Torrey Pines Institute for Molecular Studies, San Diego, CA*

CONCLUSIONS

The 10 antimicrobial peptides were found with toxicity to 11 *X. fastidiosa* strains isolated from grape, oleander and almond, but not against the glassy-winged sharpshooter gut bacterium *Alcaligenes xylosoxidans denitrificans*. We consider these AMPs as a candidates for use as reagents in delivery vehicle for paratransgenesis: Indolicidin, a 13-residue peptide-amide, isolated from the cytoplasmic granules of bovine neutrophils (Selsted 1992); 3 pescidins, isolated from the mast cells of aquacultured fish (Silphaduang and Noga 2001); and 6 peptides derived from a combinatorial peptide library (Blonde and Lohner 2000) (Table 1). *Alcaligenes* will be engineered to produce a peptide(s) toxic substance that would inhibit *X. fastidiosa* and reduce disease transmission. To develop a transformation system to express peptide(s) in *E. coli* first, we are using the Glutathione s-transferase gene fusion and *trc* expression systems. We got several ampicillin resistant transformants which are being studied for production of indolicidin. Artificial genes of other peptides are being designed for expression and secretion by *E. coli* and *Alcaligenes* as well.

PARATRANSGENESIS TO CONTROL PIERCE'S DISEASE: TOXIC PEPTIDES AGAINST *XYLELLA*

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

ABSTRACT
The use of symbiotic bacteria in insects to disrupt pathogen transmission is a new approach to disease control. *Alcaligenes xylosoxidans denitrificans* bacterium was isolated from the mouthparts of wild glassy-winged sharpshooter and was chosen to be the first candidate for delivery products that inhibit *X. fastidiosa.* To find an appropriate agent for control of Pierce's disease, 90 antimicrobial peptides (AMPs) derived from a combinatorial peptide library (in addition to 59 screened previously from different sources) were tested for activity on 11 *X. fastidiosa* and 3 *Alcaligenes* strains. Forty four peptides showed potent antimicrobial activity against all strains studied. Six antimicrobial peptides (in addition to 4 found last year) were selected with toxicity to *X. fastidiosa* but not against *Alcaligenes* as a candidates for engineering of the sharpshooter's symbiont. More detailed studies of minimum inhibitory concentrations of these peptides were conducted. The Glutathione s-transferase gene fusion and *trc* expression systems are being developed to express individual AMPs *in vitro.*

INTRODUCTION
Xylella fastidiosa causes of Pierce's disease (PD), an important disease of grapevines in the United States. Because of the mobility and vector capacity of glassy-winged sharpshooter (GWSS), PD has become a great concern to grape production in California. One promising method for long-term *X. fastidiosa* control is limiting pathogen spread by rendering GWSS vector-incompetent. Paratransgenesis (Beard et al. 2001), which is the genetic alteration of bacteria carried by insect is currently being developed to deliver pathogen toxic substances that would inhibit *X. fastidiosa* and reduce disease transmission.

Traditional antibiotics are natural or chemically synthesized small molecules that can selectively kill or stop growth of bacteria. A second type of antibiotics called antimicrobial peptides (AMPs) are produced by organisms including bacteria, plants, insects, birds, amphibians, and mammals (Cammue et al. 1992, Casteells et al. 1993, Nayler et al. 1989, Schroder 1999). These compounds interact directly with target bacterial membranes, but can do so with a receptor-like specificity, and can act via both membrane ion pore formation and by preventing cell wall formation (Maloy and Kari 1995). Because AMPs are "gene-based", they can be produced directly at the location where they are needed and their synthesis can potentially be regulated by using appropriate gene promoters. For example, the antimicrobial peptide MSI-99, an analog of Magainin 2, was expressed via the chloroplast genome to provide inhibition of growth against *Pseudomonas syringae* pv *tabaci*, a major plant pathogen (DeGray 2001). A combinatorial libraries represent a vast new source of molecular diversity for the identification of potential lead antimicrobial and antifungal compounds (Blonde and Lohner 2000, Jing et al. 2003). A combinatorial peptides are significantly shorter than other AMPs isolated from various biological sources. An amphipathic structure may allow this peptide to penetrate deeper into the interfacial region of membranes, leading to local membrane destabilization (Jing et al. 2003).

Use of symbiotic bacteria to deliver gene-based product is a new strategy of disease control. We demonstrated previously the expression of *Bacillus thuringiensis* toxin Cyt1A in the symbiotic bacterium *Enterobacter gergoviae* isolated from the gut of the pink bollworm (Kuzina et al. 2002). Bextine et al. (2004) used the expression of a red fluorescent protein (dsRed) by *Alcaligenes* (*Axd*) to study the colonization of the cibarial region of the GWSS. Genetically transformed symbiotic bacteria have been used to control the pathogen that caused Chagas disease (Beard et al. 1992, Beard et al. 2001, Durvasula et al. 1997).

OBJECTIVES
The overall goal of this project is to genetically transform symbiotic bacterium of the glassy-winged sharpshooter to produce toxic substances that would inhibit or kill *X. fastidiosa* and reduce disease transmission.
1. Identify toxic peptides effective against *X. fastidiosa* but non-toxic to *Alcaligenes*, selected symbiotic bacterium.
2. Design and construct genes encoding indolicidin and other peptides.
3. Develop a transformation system for expression of indolocidin .
4. Construct a transport cassette for secretion of indolicidin into *Alcaligenes.*

Figure 1. Oleander 'White' after 1 year of inoculation with *X. fastidiosa* strain Texas.

Figure 2. Oleander 'White' after 1 year of co-inoculation with *X. fastidiosa* strain Texas and GX123.

Figure 3. Oleander 'White' after 1 year of inoculation with GX123.

Sequential Inoculation of the Xylella Gum-degrader Endophyte and X. fastidiosa in Oleander Plants

To examine the effect of different strategies to introduce the *Xylella* gum-degrader endophyte to control *Xf* in plants, GX123 was inoculated in oleander plants (cultivar white) prior to *Xf*. Sequential inoculation of *Xf* was done 20 days after GX123 was inoculated in the same point when the titers of GX123 were already around 10^4-10^5 cfu/g of plant tissue. This experiment is still ongoing and symptoms have not developed yet, consequently the effect on disease expression is still unknown.

CONCLUSIONS

The *Xylella* gum-degrader endophyte *Acinetobacter johnsonii* GX123 colonized plants and delayed symptoms of infected oleander plants in preliminary experiments. It is a potential candidate as a biocontrol agent for *Xylella fastidiosa,* and therefore a promising tool to fight Pierce's disease.

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FUNDING AGENCIES

Funding for this project was provided by the USDA Animal and Plant Health Inspection Service and the University of California Agricultural Experiment Station.

OBJECTIVES

1. Characterize xanthan-degrading enzymes from endophytic bacteria isolated from grape
2. Explore applications of naturally-occurring endophytic bacteria that produce xanthan-degrading enzymes for reduction of Pierce's disease and insect transmission
3. Clone and characterize genes encoding xanthan-degrading enzymes for enzyme overproduction and construction of transgenic endophytes and plants

RESULTS

Co-inoculation of the Xylella Gum-degrader Endophyte and X. fastidiosa in Oleander Plants

GX123 was co-inoculated with *Xf* strain Texas in 3 different cultivars of oleander in the green house: White, Single Red and Betty. At the same time, controls were inoculated with GX123 alone, *Xf* alone or PBS buffer. Four plants were used per inoculation condition and per cultivar, totaling 48 plants obtained commercially. The appearance of symptoms was checked at approximately monthly intervals. Chlorotic mottling along the edges of leaves (Purcell et al, 1999) started to appear approximately in the eighth month after the inoculations, slowly developing into generalized chlorotic mottling and dried tissue (Table 1). The oleander cultivars White and Single Red were the first ones to show symptoms, while the cultivar Betty started to show symptoms 12 months after the inoculations. For all the cultivars, symptoms appeared in both plants inoculated with *Xf* and plants co-inoculated with the endophyte. However, the severity of the symptoms was less for the plants co-inoculated with the endophyte than for the plants not co-inoculated (Figures 1-3). Symptoms were more severe and appeared earlier in plants inoculated with *Xf* than in those co-inoculated with GX123 (Table 1 and 2). One year after being inoculated with *Xf* alone all the plants infected by *Xf* (positive result in ELISA test) showed symptoms, while one year after co-inoculations only 75% of the plants infected by *Xf* showed symptoms (Table 3). On the other hand, one year after inoculations *Xf* was detected in infected plants (10^5-10^6 ufc/g of plant tissue), while GX123 was not detected, showing a probable need for re-inoculation of the endophyte for a long term survival or a different strategy of introducing the biocontrol endophyte.

Table 1. Severity of the symptoms in oleander plants, regardless of the cultivar, inoculated with *X. fastidiosa* strain Texas alone or co-inoculated with GX123; 12 plants total per inoculation condition per month sampling.

	X. fastidiosa strain Texas				*X. fastidiosa* strain Texas/GX123			
Months	8	10	12	14	8	10	12	14
(+)	2	0	2	3	3	1	2	3
+	3	1	0	0	2	2	3	0
++	2	3	4	1	0	4	4	2
+++	0	3	3	4	0	0	0	5
AD	0	0	0	1	0	0	0	0
D	0	0	0	2	0	0	0	0

(+) chlorotic mottling along the edges of a few leaves; + chlorotic mottling along the edges of many leaves evolving into a uniform chlorotic mottling; ++ chlorotic mottling of many leaves, starting to wrinkle and dry; +++ chlorotic mottling of many leaves and zones of dead tissue (dried, straw color), smaller leaves; AD many dried leaves, plant almost dead; D plant dead.

Table 2. Number of symptomatic plants after inoculation with *X. fastidiosa* strain Texas alone, co-inoculated with GX123, GX123 alone or PBS buffer; 12 plants total per inoculation condition per month sampling.

Months	*X. fastidiosa* strain Texas	*X. fastidiosa* strain Texas/GX123	GX123	PBS
8	7	5	0	0
10	7	7	0	0
12	9	9	0	1
14	11	10	0	1

Table 3. Symptomatic plants and ELISA results after 1 year of inoculation; 12 plants total per inoculation condition.

Inoculations	*X. fastidiosa* strain Texas	*X. fastidiosa* strain Texas/GX123
Symptomatic plants	9	9
Positive ELISA for *X. fastidiosa*	9	12

CONTROL OF PIERCE'S DISEASE THROUGH DEGRADATION OF XANTHAN GUM

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

ABSTRACT
Acinetobacter johnsonii GX123, a *Xylella* gum-degrading endophyte was co-inoculated with *Xylella fastidiosa* strain Texas in oleander plants to determine its efficacy as a biocontrol agent in preliminary experiments. Symptoms appeared in both plants inoculated with *X. fastidiosa* alone and plants co-inoculated with the endophyte. However, symptoms were more severe and appeared earlier in plants inoculated with *X. fastidiosa* than in those co-inoculated with the endophyte. *A. johnsonii* GX123 seems to be a promising candidate to control *X. fastidiosa*. Experiments using a sequential strategy of inoculating the *Xylella* gum-degrader endophyte prior to *X. fastidiosa* are ongoing and its effects on symptom expression are still under investigation.

INTRODUCTION
Pierce's disease (PD) of grapevine and other leaf scorch diseases caused by *Xylella fastidiosa* (*Xf*) are associated with aggregation of bacteria in xylem vessels, formation of a gummy matrix, and subsequent blockage of water uptake. In the closely-related pathogen, *Xanthomonas campestris* (Xc), xanthan gum is known to be an important virulence factor (Katzen et al, 1998), probably contributing to bacterial adhesion, aggregation, and plugging of xylem. The published genome sequence of *Xf* (Simpson et al, 2000; Bhattacharyya et al, 2002; Van Sluys et al, 2003) revealed that this pathogen also has genes for producing an exopolysaccharide with a very similar structure to that of xanthan gum. In PD, this *Xylella* gum is likely to contribute to plugging of the grapevine xylem (Keen et al, 2000) and possibly to the aggregation of the bacterium in the mouthparts of the glassy-winged sharpshooter. Because of its importance as an industrial thickener and emulsifier, xanthan gum synthesis and degradation have been extensively studied (Becker et al, 1998). Bacteria that produce xanthan-degrading enzymes have been isolated from soils using enrichment techniques with xanthan gum as the sole carbon source (Sutherland 1987; Ruijssenaars et al, 2000).

The purpose of this project is to identify bacteria that produce xanthan-degrading enzymes to target this specific virulence factor of *Xf*. This approach has the potential to significantly reduce the damage caused by PD in grapes and potentially in other hosts of *Xf* such as almond and oleander. If the gum is important in the aggregation of the pathogen in the insect vector, then our approach may also reduce the efficiency of transmission of PD. Our first approach will be to develop endophytic bacteria that produce these enzymes in the xylem of grapevines, but another approach is to engineer grape plants to produce these enzymes. Through the cloning and characterization of genes encoding xanthanases and xanthan lyases we will facilitate possible efforts to transform grapevines to produce these enzymes.

Previously, we used modified xanthan gum that mimics *Xylella* gum from a Xc mutant as the sole carbon source for enrichment culture from infected grapevines and oleanders. The *Xylella* gum biosynthetic operon in the *Xf* genome is different than the one in Xc from which the commercial xanthan gum is obtained. Since it is not feasible to produce *Xylella* gum for our studies from the slow-growing *Xf*, we genetically modified a strain of Xc to produce a modified xanthan gum that is predicted to have the same chemical structure as that from *Xf*. This was accomplished by deleting the *gumI* gene from the biosynthetic operon. Over 100 bacterial strains were initially recovered from enrichment experiments, and 11 were subsequently confirmed to effectively degrade *Xylella* gum. These strains were then tested for cellulase activity. Degradation of the cellulosic backbone of the gum polymer would be desirable, but we do not want enzymes that recognize and degrade plant cellulose. One particular strain (GX123) with high gum-degrading activity but no cellulase activity isolated from oleander was identified as *Acinetobacter johnsonii* (Aj), and characterized in more detail. In vitro, growth and biofilm production by GX123 were enhanced by *Xylella* gum as a substrate and by cells of *Xf* added to a minimal medium. The gum was degraded rapidly during log-phase growth of this endophyte, and viscosity was reduced almost to non-detectable levels. GX123 colonized stems and leaves of oleander systemically (10^4-10^5 cfu/g of plant tissue 20 days after inoculation), and systemic colonization was enhanced by co-inoculation with *Xf*. The effect of using GX123 as an endophyte to reduce the ability of *Xf* to produce disease symptoms in oleander was studied.

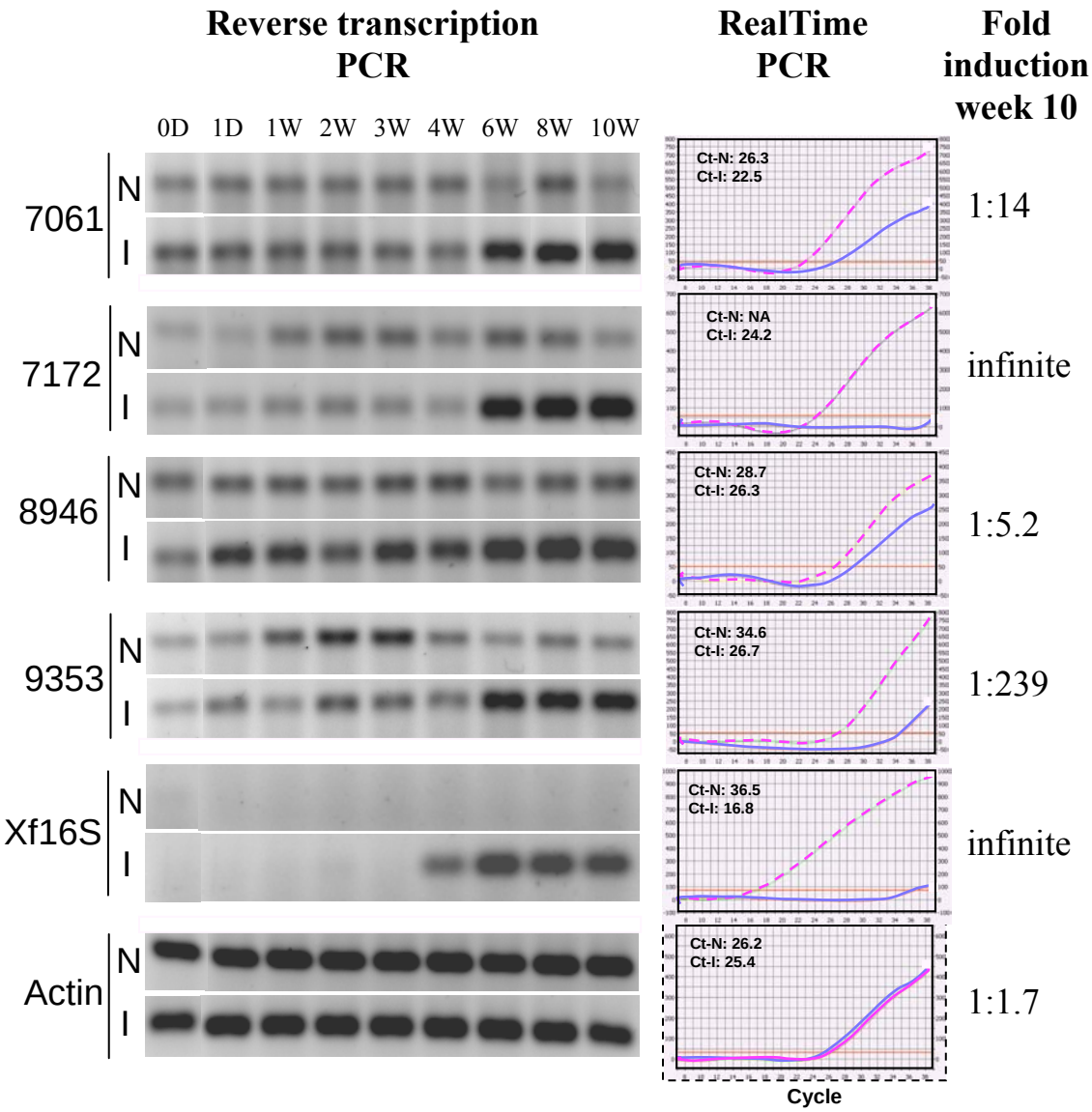

Figure 1. **Monitoring of PD-induced genes using conventional reverse transcriptase-PCR and Real Time PCR.** Leaf tissue was sampled from growth chamber-grown plants at nine time points (0, 1d, 1w, 2w, 3w, 4w, 6w, 8w, 10w: d-day, w-week) after inoculation. *Xylella* up-regulated genes identified from in silico analysis are 7061, 7172, 8946, and 9353. Actin serves as a constitutively expressed control. *Xf*16S = *Xylella fastidiosa* 16S gene. N; Non-inoculated, I; Inoculated with *X. fastidiosa*.

Based on the *in silico* analysis, described above, four *Xylella*-induced genes, a constitutively expressed control *Vitis* gene, and a bacterial gene, were selected to develop a multiplex PCR assay. This "dual-diagnosis" system may have potential as a tool for disease diagnosis.

Isolation of Pathogen-induced Promoters

DNA probes were developed based on the *Xylella*-induced genes and used to screen high-density filters of *Vitis vinifera* genomic DNA libraries. Clones were isolated, fingerprinted to confirm relatedness, and analyzed by PCR and sequencing to verify that they contained the genes of interest. A shotgun sequencing strategy is being used to obtain the complete sequence of each clone and promoter constructs are being made to test in transient and stable transformation assays. Gene fusions will include reporter proteins to monitor temporal and spatial patterns of transcription (e.g., green fluorescent protein and ß-glucuronidase) and candidate pathogen resistance proteins that may protect grapes against *Xylella* infection.

CONCLUSIONS

To date we have identified several genes of *Vitis vinifera* that are up-regulated in response to *Xylella* infection. Ongoing research will identify larger sets of grape genes expressed in response to this pathogen and provide the basis for biotechnological approaches to dealing with Pierce's disease.

How will these technologies help in solving Pierce's disease? *In the short term* they will (1) yield improved genetic tools for breeding resistance to Pierce's disease (for example single nucleotide polymorphism "SNP" and simple sequence repeat "SSR" genetic markers currently available from our web site "http://cgf.ucdavis.edu), (2) provide gene-promoters that are an essential, but currently unavailable, tool for effective genetic engineering in grapes, and (3) potentially provide the basis for more reliable detection of the pathogen based on Real Time PCR using a "biomarker" strategy. (4) *In the long term*, transcriptional profiling will identify candidate genes and gene pathways that may confer resistance to the pathogen (*Xylella fastidiosa*) and/or to the insect vector (Sharpshooter leaf hopper) and it will allow testing of long-standing hypotheses such as the relationship between host response to drought and host response to *Xylella*. Other strategies, such as reverse genetics and analysis of natural genetic variation for host responses, will be required to establish a causal role for candidate genes.

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FUNDING AGENCIES

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

Three co-lateral benefits from the identification of pathogen-induced genes are: (1) the promoters for such genes are candidates to control the expression of transgenes for resistance to Pierce's disease, (2) the protein products of induced genes may have roles in disease resistance, and (3) knowledge of host gene expression can be used to develop improved diagnostic assays for disease. In the first case, we are currently characterizing pathogen-responsive promoters, which would allow us to test candidate genes (the second case) for resistance phenotypes. In the third case, gene expression patterns can be used to develop so-called "molecular signatures" or "biomarkers" [MacNeil 2004] that are diagnostic of an organism's physiological status. Biomarkers are finding application in clinical medicine, where data on gene expression patterns are useful for characterizing disease states and improving clinical outcome [Alizadeh et al., 2001; Van't Veer et al., 2002; Ramaswamy et al., 2003]. In the case of Pierce's disease, the identification of early genes (i.e., genes expressed prior to the appearance of visible symptoms), and/or genes that are induced systemically in response to local infection, would greatly increase the reliability of disease diagnosis, which is currently prone to false negatives due to mis-sampling of locally-infected asymptomatic vines. At the same time, the identification of disease-related gene expression profiles would provide a novel measure of host response, and thus provide tools for basic Pierce's disease research applications.

OBJECTIVES AND PRODUCTS OF THE RESEARCH

Completed objectives

1. The public release of 61,203 EST sequences to the National Center for Biotechnology Information.
2. Development of a public, on-line relational database for analysis of the grape genome (http://cgf.ucdavis.edu).
3. Production of a public Affymetrix microarray, in collaboration with international researchers, available May 2004.

Ongoing Objectives

4. Identify genes and gene pathways in susceptible *Vitis vinifera* correlated with *Xylella* infection: (a) identify *Xylella*-responsive genes in *V. vinifera*, (b) distinguish early from late gene expression, and (c) determine the correlation between drought stress and Pierce's disease.
5. Determine host genotype affects on gene expression in response to *Xylella* infection: (a) susceptible *Vitis vinifera* compared to resistant genotypes of *Vitis arizonica* and *Vitis aestivalis*, (b) comparison of pathogen-induced gene expression with gene expression triggered by salicylic acid and ethylene, and (c) analysis of gene expression in resistant and susceptible bulked segregants of *Vitis arizonica* X *Vitis rupestris*.
6. Development of Real Time PCR assay for routine monitoring of *Xylella*-induced genes under field, greenhouse and laboratory settings.
7. Isolation and characterization of *Xylella*-responsive plant promoters.

RESULTS

Analysis of the Grape Transcriptional Response to Pathogen Challenge

The results described below are based on the analysis of combined data sets generated under this project and that of our collaborators at the University of Nevada-Reno, and other members of the grape genomics community. In total, 40% of the 135K *V. vinifera* ESTs and 100% of the sequencing focused on Pierce's disease originated from this project.

In silico Identification of Xylella-induced Genes in Vitis vinifera

We have identified 31 genes that appear to be up-regulated in response to infection by *Xylella fastidiosa*. The analysis, which involved construction of a correlation matrix and 2-dimensional hierarchical clustering, was based on EST frequency in various tissues with or without *Xylella* infection. The most abundant contig (7061) shares homology with a stress-related RNA from Arabidopsis, although the function is unknown in any system. Interestingly, this gene is up-regulated in infected plants, prior to symptom development, making it a top candidate for an early and sensitive marker of Pierce's disease. Other genes in the list have homology to proteins implicated in signaling during disease resistance, while others have been identified as pathogen responsive, or have been implicated in plant-insect interactions. After confirmation of the *Xylella*-specific transcription of such contigs (see Real Time PCR assays, below) we initiated the isolation of the promoters from these genes from genomic DNA libraries. The potential application of such promoters to drive *Xylella*-induced and/or tissue specific expression of transgenes is planned as a topic of a future grant proposal.

Development of Real-Time PCR for Gene Expression Analyses and Disease Diagnosis

Detailed analysis of transcriptional responses will require methodical analysis by means of microarray gene expression studies, which we initiated in July 2004 under a one-year renewal to this project. At the same time, the current list of putatively *Xylella*-induced genes may provide leads for further analysis by means of Real Time PCR.

Real Time PCR has three primary uses for Pierce's disease research: (1) It can be used as an alternative to pathogen-based assays for disease diagnosis. For example, the identification of host genes that are expressed early and systemically could provide a significantly more reliable test for PD infection. This "biomarker" strategy is gaining increasing use for human medicine. (2) Real Time PCR assays offer a useful point of comparison for data from *in silico* analysis of gene expression (i.e., from statistical analysis of EST data) and for confirming results for key genes identified in Affymetrix microarray experiments. (3) Real Time PCR of differentially expressed host genes can provide a convenient research tool for investigators in need of a sensitive measure of host response.

FUNCTIONAL GENOMICS OF THE GRAPE-*XYLELLA* INTERACTION: TOWARDS THE IDENTIFICATION OF HOST RESISTANCE DETERMINANTS

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ABSTRACT
We have used *in silico* mining of EST data and Real Time PCR to identify a set of *Xylella*-induced grape genes. Controlled time course analyses demonstrate that the genes are induced prior to symptom development, in coincidence with pathogen colonization. Analysis of field samples from grapes under a variety of biotic and abiotic stresses demonstrate that these genes are up-regulated in response to *Xylella* but not in response to the other pathogens assayed, including common viral, nematode and fungal pathogens, or by *Phylloxera* infestation or herbicide damage. By contrast, transcriptional responses similar to those observed in *Xylella*-infected tissues were observed in grapes under severe drought stress (in excess of normal field drought) and in plants where the vascular system had been blocked by damage from the grape cane girdler insect. These results are consistent with transcriptional regulation in response to insult within the vascular tissue of grape, but not to pathogen infection generally.

INTRODUCTION
All organisms adapt to external stressors by activating the expression of genes that confer adaptation to the particular stress. For example, when exposed to conditions of heat or drought, genes for adaptation to heat and drought stress are up-regulated. Similarly, when a plant is exposed to a pathogen, numerous genes are induced including those that encode proteins involved in disease resistance. In the case of Pierce's disease, such genes are likely to include those coding for resistance to *Xylella* or to the insect vector.

Genomics technology offers an opportunity to monitor gene expression changes on a massive scale (so-called "transcriptional profiling"), with the parallel analysis of thousands of host genes conducted in a single experiment. In the case of Pierce's disease of grapes, the resulting data can reveal aspects of the host response that are inaccessible by other experimental strategies. Prior to carrying out transcriptional profiling, it is first necessary to (1) catalog the gene content of grapes by means of sequencing and bioinformatic analyses, and (2) develop gene-based arrays that allow the simultaneous monitoring of gene expression for >10,000 genes. Our research to date has contributed significantly in each of these areas. In May of 2004, the first Affymetrix gene chip was made available for public use, with ~15,000 *Vitis* genes represented. This gene chip has been developed based primarily on a collaboration between the Cook laboratory and researchers at the University of Nevada-Reno. With the arrival of the Affymetrix gene chip, we are poised to make a quantum leap in the identification of host gene expression in response to *Xylella fastidiosa*.

In addition to enumerating differences between susceptible and resistant genotypes of *Vitis*, the ongoing research will test a long-standing but largely untested hypothesis that pathogen-induced drought stress is one of the fundamental triggers of PD symptom development. The utility of this type of data will be to inform the PD research community about the genes and corresponding protein products that are produced in susceptible, tolerant and resistant interactions. Differences in the transcriptional profiles between these situations are expected to include host resistance and susceptibility genes, and thus provide the basis for new lines of experimental inquiry focused on testing the efficacy of specific host genes for PD resistance. It should be possible, for example, to determine the extent to which resistance responses in grapes are related to well-characterized defense responses in other plant species [e.g., Maleck et al., 2002; Tao et al., 2003; de Torres et al., 2003]. In addition to identifying candidate effectors of disease resistance, such knowledge would aid the development of testable hypotheses regarding susceptibility and resistance to *Xylella fastidiosa* in grapes.

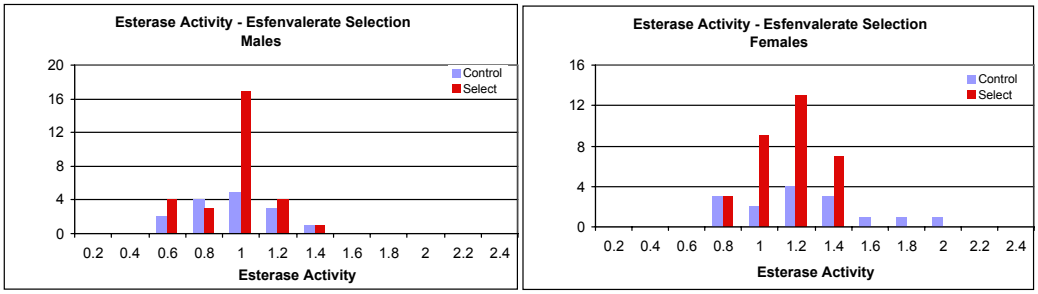

Figure 1. Distributions of esterase activity in adult male and female glassy-winged sharpshooters rom a Riverside citrus orchard. Insects were treated topically with either acetone (Control) or 0.75ng esfenvalerate (Select), and esterase activity measured in survivors.

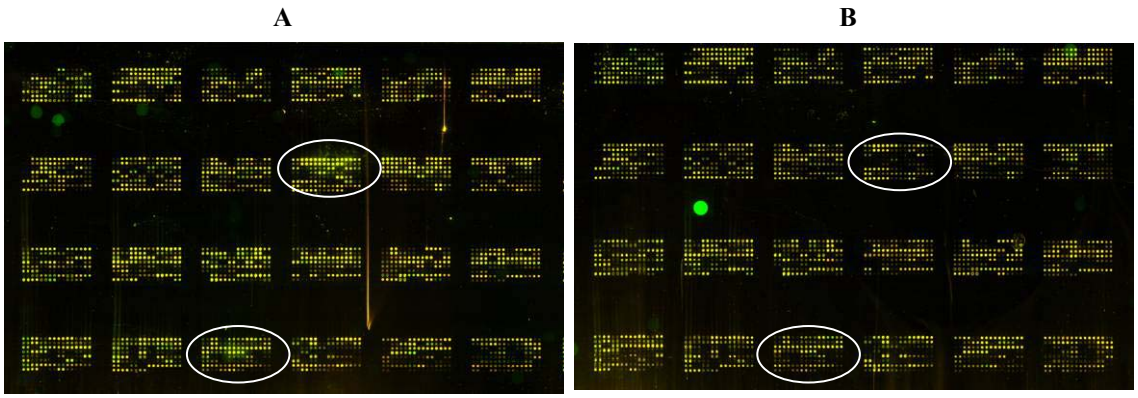

Figure 2. Scan data of microarrays hybridized to Cy3 labeled control target (green) and Cy5 labeled sub-lethal target (**A**) or LD50 target (**B**) (red). Circled results show obvious gene expression differences.

CONCLUSIONS

In this study, we tested populations of GWSS from Riverside citrus orchards with 0.75ng esfenvalerate. This dose of esfenvalerate is the LD50 for the Riverside population when topically applied to the insect abdomen. Distributions of esterase activity revealed that there were no differences between the untreated insects and the treated survivors. These results suggest that esterases do not contribute directly to the toxicological differences between these populations. In addition, many and different gene expression changes occur in GWSS in response to sub-lethal and LD50 doses of esfenvalerate.

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FUNDING AGENCIES

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EVALUATION OF RESISTANCE POTENTIAL IN THE GLASSY-WINGED SHARPSHOOTER USING TOXICOLOGICAL, BIOCHEMICAL, AND GENOMICS APPROACHES

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

ABSTRACT

Geographically distinct populations of GWSS differ in their toxicological responses to pyrethroid insecticides. We have shown that these different responses are unlikely to be caused by an esterase-mediated mechanism. The distributions of esterase activity in insects tested from Riverside and Redlands citrus orchards remained unchanged after selection with an LD50 dose of esfenvalerate.

INTRODUCTION

We are using a multi-disciplinary approach to understand the biological and genetic mechanisms contributing to the toxicological differences between GWSS populations. This will allow us to determine whether the basis for decreased tolerance is due to target site changes or due to the selection of detoxification mechanisms. Whereas target-site modifications will only impact the pyrethroid class of insecticides, the selection of detoxification mechanisms are more critical due to their potential to confer cross-resistance to chemical classes that differ in their modes of action. In this first report, we describe selection experiments designed to test the potential involvement of esterases in conferring pyrethroid tolerance (Objective 2).

OBJECTIVES

1. Monitor toxicological responses of geographically distinct populations of GWSS to pyrethroid insecticides
2. Measure biochemical activity of putative resistance-causing enzymes in these populations.
3. Clone and sequence the sodium-channel genes in GWSS populations differing in susceptibility to insecticides.
4. Perform microarray gene expression profiles in GWSS populations differing in susceptibility to insecticides to isolate novel genes involved in resistance.

RESULTS

Bioassays

Topical application bioassays (Byrne et al., 2003) have been conducted on Riverside GWSS adults to determine an LD50 for esfenvalerate. The LD50 was determined to be 0.75ng esfenvalerate per insect.

Selections

For selection experiments, insects were collected from the UC Agricultural Operations orchard in Riverside. Adults were treated with 0.75ng esfenvalerate by topical application. Esterase activity was measured in a subsample of insects taken before the bioassay, and in the survivors (at 48 hours) from the bioassay (Figure 1). Although there were differences in activities between males and females, there were no differences in activities attributable to selection by esfenvalerate.

In additional selection experiments, insects from Redlands and Riverside orchards were treated with 0 (controls), 0.075ng (sub-lethal) and 0.75ng (LD50) esfenvalerate per insect. Control and survivors at each treatment were used to prepare target RNA for gene expression profiling studies.

Microarrays

PCR amplified inserts from 1,536 normalized library clones were spotted onto amino-silane coated glass slides. Each clone was spotted in side by side duplicate spots and the entire array was duplicated on each slide. Total RNA was isolated from two individual insects from each treatment for target preparation. Each total RNA was reverse transcribed and PCR amplified separately with Cy3- and Cy5-tagged dUTP. Slides were hybridized for 16 hours at 42^{o}C on a Genomics Solutions GEN TAC® hybridization station and washed twice at medium stringency for 40 seconds. Each hybridization was repeated as a target dye swap. Slides were scanned on an Applied Precision Array Worx fluorescence scanner. Data is being evaluated using the Silicon Genetics GeneSpring program.

approximately 3-fold more toxic than thiamethoxam, and the dose-response was steeper as indicated by the higher slope. It was evident during these bioassays that the toxic effects of thiamethoxam were delayed compared with the other insecticides, suggesting that thiamethoxam may require activation to a toxic derivative within the GWSS.

Table 1. Toxicity of neonicotinoids to the GWSS in topical application bioassays.

Compound	LD50 (ng a.i. per insect)	95% FL	Slope	No. of insects
Imidacloprid	4.8	2-8	1.5 ±0.4	100
Thiamethoxam	2.6	2.0-3.3	1.4 ±0.3	200
Clothianidin	0.7	0.6-0.9	5.2±0.9	125
Acetamiprid	0.7	0.6-0.9	3.7±0.6	125

CONCLUSIONS

In this study, we tested four neonicotinoids against the GWSS. Although there were differences in LD50s, all compounds were highly toxic. These results confirm that the newer neonicotinoids could have a place in GWSS management programs. We are currently investigating the fate of these chemicals in both citrus trees and grapevines. Establishing the potential for conversion of thiamethoxam into clothianidin is of particular importance if these chemicals are to be incorporated into management strategies.

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Byrne, F.J., and N.C. Toscano. 2003. Characterization of plant metabolites of imidacloprid in citrus trees and grapevines, and evaluation of their efficacy against the glassy-winged sharpshooter, *Homalodisca coagulata*.

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FUNDING AGENCIES

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CHARACTERIZATION OF NEONICOTINOIDS AND THEIR PLANT METABOLITES IN CITRUS TREES AND GRAPEVINES, AND EVALUATION OF THEIR EFFICACY AGAINST THE GLASSY-WINGED SHARPSHOOTER

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

ABSTRACT
The toxicities of established and new members of the neonicotinoid insecticide class were assessed against the glassy-winged sharpshooter in topical application bioassays. All compounds were highly toxic to the insect. Clothianidin elicited its toxic response more rapidly than thiamethoxam and was 3-fold more toxic overall at the LD50 level. Clothianidin has been proposed as an active derivative of thiamethoxam, so it is important to establish the fate of these chemicals within plant systems that are likely to be treated for GWSS control.

INTRODUCTION
The primary means of controlling the spread of Pierce's disease (PD) in California vineyards is through the elimination of its vector using insecticides. The glassy-winged sharpshooter (GWSS) *Homalodisca coagulata* feeds directly from the plant xylem system and, therefore, systemic insecticides are currently being evaluated on both citrus and grapes. Of the various classes of insecticide under consideration, the neonicotinoids, especially imidacloprid, have proven to be the most effective at suppressing GWSS populations. Imidacloprid (1-[(6-chloro-3-pyridinyl)methyl]-4,5-dihydro-N-nitro-1H-imidazol-2-amine) is a nicotinic acetylcholine receptor agonist that combines high potency with low mammalian toxicity and favorable persistence. As a systemic, seed, soil or foliar treatment, it has proved to be especially effective against a wide range of homopterous insect pests, including the GWSS. The success of imidacloprid in controlling GWSS is due largely to its excellent systemic properties. Systemic applications exploit the xylophagous feeding behavior of the insect, and thereby disrupt the transmission of PD and other *X. fastidiosa*-related diseases.

This project is an extension of a one-year project that was funded by the UC Pierce's Disease Research Grant Program. It will focus on the fate of imidacloprid and other neonicotinoid insecticides in citrus and grapevines, and the impact of these chemicals on GWSS. In a previous study, imidacloprid and two of its derivatives were shown to be highly toxic to GWSS adults (Byrne and Toscano, 2003).

The aims of this study are to determine the extent to which metabolites of neonicotinoids are formed in citrus trees and grapevines, and to determine their toxicological significance towards GWSS. The presence of insecticidal metabolites in xylem sap could contribute to the excellent persistence of imidacloprid treatments against sharpshooters. As well as maintaining the toxic pressure of the initial application, the metabolism of neonicotinoids to yield equally or more toxic metabolites may also account for the stability of this chemical class to resistance.

Of particular interest to us are thiamethoxam and clothianidin, which are being evaluated for use against citrus and grape pests. During the past year, it has been established that thiamethoxam is converted into clothianidin by insects and cotton plants (Nauen et al., 2003). This is an important finding, as it could have ramifications for the use of these products on grapes and citrus. When several products from the same class become available for pest management, it is important that their use be carefully monitored in order to circumvent potential resistance problems. The possibility that thiamethoxam is converted into clothianidin is, therefore, of concern when formulating management strategies based around the neonicotinoids. Receptor binding studies have suggested that thiamethoxam does not bind to the same receptor site as imidacloprid and so it has been proposed as a suitable product for alternation with imidacloprid because of the reduced resistance risk (Weisner and Kayser, 2000). Now that thiamethoxam has been shown to be a potential pro-insecticide, and clothianidin has been shown to bind to the same receptors as imidacloprid, new issues are raised about its suitability as a product for rotation with other neonicotinoids. This is an important reason for determining the fate of thiamethoxam in citrus and grapes.

OBJECTIVES
1. Determine the metabolic fate of neonicotinoids within citrus trees and grapevines.
2. Determine the relative toxicities of neonicotinoids and their metabolites to the adult and egg stages of the GWSS.

RESULTS
The toxicity of four neonicotinoid insecticides has been assessed for GWSS adults using a topical application bioassay (Table 1). Thiamethoxam, clothianidin and acetamiprid were all more toxic than imidacloprid. Clothianidin was

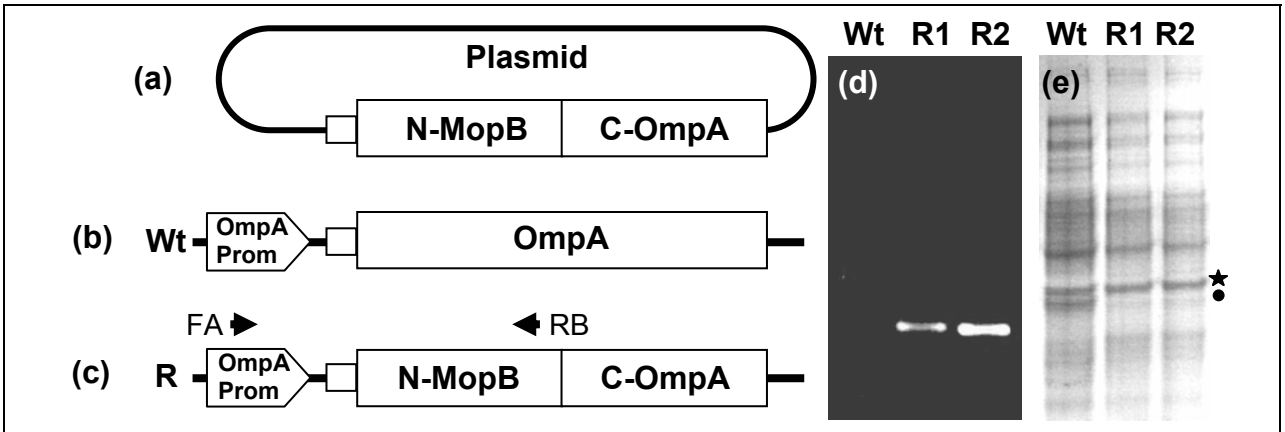

Figure 3. *E. coli* strains with the *E. coli* OmpA gene replaced by a chimeric *MopB-OmpA* gene. (**a**)A low copy number plasmid was prepared with an insert composed of the 5'UTR and leader peptide (small rectangle) of OmpA fused to codons 1-171 of MopB (N-MopB), which in turn is fused to codons 172-325 of OmpA (C-OmpA). (**b**)Representation of the wildtype chromosomal *OmpA* gene (Wt). (**c**)Desired recombinant between the plasmid and the chromosomal *OmpA* gene to give a chromosomal, chimeric *MopB-OmpA* gene in place of *OmpA*. (**d**)Analysis of a polymerase chain reaction (PCR) 916bp product expected to be amplified, by forward (FA) and reverse (RB) primers designed as indicated in part (c), only from the recombinant sequence. Lanes received PCR incubation mixtures from Wt *E. coli* and two candidate recombinant strains, R1 and R2. (**e**)Gel electro-phoresis (SDS-PAGE) of protein extracts from *E. coli* lines Wt, R1 and R2. Unfortunately, the loading for the Wt lane is substantially greater than the loading for lane R1, which is more heavily loaded than lane R2. Dot indicates a band that is lost in R1 and R2 compared to Wt. The star marks a band of enhanced intensity, relative to other bands in the same lane, in R1 and R2 compared to Wt.

E. coli transformants displaying MopB sequences were selected using magnetic beads covalently coupled to anti-MopB IgG. Beads were plated on agar medium to recover colonies growing up from bead-selected cells. Pooled colonies were cultured, and the cells were exposed to the OmpA-specific bacteriophage K3 at a multiplicity of infection of 15 to deplete the population in cells still bearing OmpA. Fig. 3 provides evidence for the occurrence of the expected recombination events and for the production of the chimeric MopB-OmpA protein in amounts visible on a coomassie brilliant blue-stained gel [Fig. 3(d) and (e)]. The cells derived by these approaches agglutinate beads displaying anti-MopB IgG, providing evidence that some part of the MopB portion of the chimera, presumably the MopB outer loops, is displayed on the exterior of the *E. coli* cell.

CONCLUSIONS

Based on results reported here and in previous progress reports, MopB is a highly suitable target for strategies designed to interfere with the ability of *Xf* to initiate infections leading to development of Pierce's disease. Our overall strategy for creating grape plants resistant to *Xf* is revealed by the four new objectives stated above in the Objectives section. Experimental steps (i), (ii) and (iii) outlined at the end of the Introduction reveal how we intend to satisfy new Objective 1. Results in Fig. 3 suggest that we have completed experimental step (i) and that we are ready to proceed to the selection of variant gp38 proteins capable of high affinity binding to MopB on the surface of *Xf* cells, i.e., experimental steps (ii) and (iii).

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Funding

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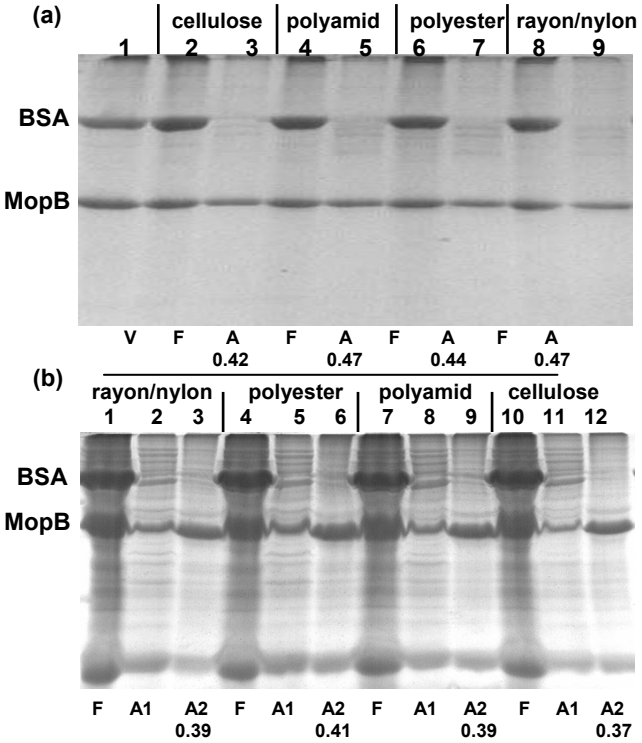

Figure 2. Polymer disk accumulation of *Xf* MopB from protein mixture and *Xf* cells. **(a)**. A solution of SP fraction MopB and BSA was dispersed in 1x SCP, 1mg/mL NP-40. 8mm diameter disks were prepared from filter paper (2 disks, 19mg), polyamid (3 disks, 21mg), polyester (5 disks, 20mg), and 30% nylon, 70% rayon (3 disks, 19mg). 0.25mL of the BSA-MopB dispersion was dispensed into an empty vial (lane 1, V) and into vials containing polymer disks as indicated. The vials were incubated at room temperature for 2hr with orbital shaking at 100rpm. Free, unassociated material rinsed off with SCP: lanes 2, 4, 6 and 8 (F below lanes). Material eluted from polymer disks with alkaline hot SDS-mercaptoethanol solution: lanes 3, 5, 7 and 9 (A below lanes). **(b)** *Xf* cells were dispersed into 1xSCP, 1mg/mL NP-40 containing a great excess of BSA (150µg/mL). 0.25mL of the suspension was dispensed to vials containing polymer disks as indicated. Elution was in two stages: A1, SDS in SCP at 30˚C and A2, hot SDS-mercaptoethanol at alkaline pH. Numbers under lanes indicate fraction of MopB band material in A and A2 fractions.

<u>*E. coli* displaying MopB outer peptide loops</u>. Attempted cloning and expression of the full *Xf mopB* gene in *E. coli*, including the *Xf MopB* promoter, were not successful. However, a system that included an inducible bacteriophage T7 RNA polymerase and T7 promoter driving the MopB-encoding sequence was adapted to create *E. coli* cultures generating low levels of MopB when induced with the gratuitous inducer IPTG. Intact *Xf* MopB accumulation may sicken *E. coli*, accounting for the low level accumulation. The Introduction describes in outline a strategy for creating a MopB-binding, anti-*Xf* protein. This strategy requires substitution of *E. coli* OmpA by a new outer membrane protein that portrays the characteristics of MopB on the surface of *Xf* cells. To this end, we created a chimeric MopB-OmpA construction in *E. coli* and subjected the cells to conditions designed to select cells in which recombination events resulted in the *E. coli* OmpA gene being replaced by the MopB-OmpA chimera (Fig. 3).

The predominant conformation of the OmpA protein as it resides in the outer membrane of *E. coli* probably has amino acid residues 1-171 inserted with 8 trans-membrane segments and four external loops (Singh et al., 2003).
MopB can be cast in a similar conformation based on the crystallographic structure of OmpA and computer predictions of folding for OmpA and MopB. Our design for the chimeric MopB-OmpA gene retains the OmpA promoter and replaces only the 1-171 residue region of OmpA with the corresponding MopB sequence. Our rationale is that retaining the OmpA leader peptide, which targets the molecule to the outer membrane, and the OmpA carboxy-terminal portion, which includes the trans-periplasmic space sequences and the sequence that is inserted into the peptidoglycan layer, will result in a molecule that is more compatible with *E. coli* that an intact *MopB* gene would be.

The low-copy-number plasmid construction indicated in Fig. 3(a) encodes the desired chimeric molecule and the associated OmpA 5'UTR and leader peptide but lacks the OmpA promoter, so the chimeric protein should be expressed at a very low level, at the most, in transformed *E. coli*. The robust, highly recombination competent *E. coli* strain ER2738 was transformed with the Fig. 3(a) plasmid under the expectation that recombination events would replace the chromosomal *OmpA* gene [Fig. 3(b)] with sequences encoding the MopB amino-half molecule flanked by the OmpA leader peptide and carboxy-half OmpA sequences, creating the desired structure diagrammed in Fig. 3(c).

OBJECTIVES

For period 15 Oct 2003 through 30 June 2004, previous project title "Roles of *Xylella fastidiosa* Proteins in Virulence"

1. To identify specific *Xylella fastidiosa* (*Xf*) protein(s) and determine their roles in virulence, particularly major outer membrane protein MopB
2. To develop strategies for interfering with *Xf* infection of grape and/or with development of Pierce's disease

For period 1 July 2004 through 11 October 2004, new project title "Exploiting *Xylella fastidiosa* Proteins for Pierce's Disease Control"

1. Discover or develop low molecular weight proteins with high affinity for portions of the MopB protein that are displayed on the *Xf* cell exterior.
2. Test MopB-binding proteins for their ability to coat *Xf* cells, for possible bactericidal activity, and for interference with disease initiation following inoculation of grape with *Xf*.
3. In collaboration with the Gupta laboratory, develop gene constructions for chimeric proteins designed to bind tightly to and inactivate *Xf* cells; express and test the chimeric proteins for their effects on *Xf* cells in culture.
4. In collaboration with the Dandekar laboratory, prepare transgenic grape expressing the candidate anti-*Xf* proteins; test the transgenics for resistance to infection by *Xf*

RESULTS

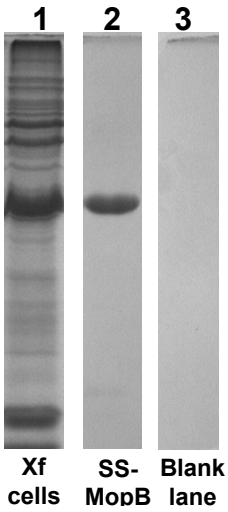

Figure 1. Purification of MopB protein from *Xf* cells. All samples were analyzed on a 12.5% polyacrylamide gel Lane 1, hot SDS extract of *Xf* cell suspension. Lane 2, MopB purified through a step of solubilization at pH8.8 in sodium perchlorate-SDS. Lane 3, no sample, for lane 2 comparison.

Purification of MopB from *Xf* cells. A dilute suspension of *Xf* cells scraped from plates is incubated at 30°C for 30 min in Tris-HCl-EDTA buffer pH 8.5 containing 8mg/mL SDS, 0.2µL/mL 2-mercaptoethanol. High speed centrifugation collects a precipitate (designated SP-MopB) that is highly enriched in MopB but includes substantial amounts of non-protein material from the *Xf* cells. The precipitate is dispersed into Tris-HCl-EDTA buffer, pH 8.8, containing 1.2M sodium perchlorate, 1mg/mL SDS, 10µL/mL 2-mercaptoethanol and is incubated at 30°C for 18hr. The supernatant after centrifugation at 50K rpm, 10°C for 20min is designated as the SS-MopB fraction. Sodium perchlorate reduces the solubilization of non-MopB proteins from SP-MopB preparations. The effective concentration of SDS is very low in SS-MopB due to the common ion effect with sodium perchlorate. SS-MopB, concentrated by centrifugal filtration, binds to porous polymer disks as described below.

Preponderance of MopB in the *Xf* outer membrane. *Xf* cells were washed with cold 1M perchloric acid to elute low molecular weight compounds. The cell suspension was assayed for DNA by the diphenylamine assay and for protein using the BCA reagent. The amount of DNA per stationary state cell is assumed to be 2.7 x 10^6 base pairs. MopB appears to be 10-15% of the *Xf* cell protein, based on analyses such as those in Fig. 1. From these results, *Xf* cells have at least 80,000 MopB molecules per cell. We assume that the packing volume of MopB is similar to the packing volume derived from x-ray crystallography for the amino-terminal domain (residues 1-171) for *E. coli* OmpA, which crystallized as a 2.6nm diameter cylinder (Pautsch and Schutz, 1998). The diameter of a *Xf* cell is about 400nm. 80,000 molecules of hexagonally packed MopB would form a cylinder 400nm in diameter and almost 400nm high, accounting for more than 10% of the surface area of the 1000 to 5000nm long *Xf* cell.

General association of MopB with porous substances. We reported previously on the spontaneous association of MopB from solution with balsa wood (composed largely of xylem) and cellulose disks (filter paper). Other proteins, mixed with the MopB, did not absorb to balsa wood or cellulose. Fig. 2 reports our extension of this work to other porous polymeric materials of diverse chemical character. Cellulose, polyamid, polyester, and a rayon-nylon blend provided in approximately the same mass, all became associated with MopB, whether the MopB was supplied as partially purified protein in solution or as MopB in the outer membrane of *Xf* cells. Quantitatively, there was little variation in the extent of association among the polymers, all of which were exposed to the same NP-40 (non-ionic detergent) solution. Bovine serum albumin (BSA) was not absorbed by any of the porous polymer disks. Elution of polymer disks exposed to *Xf* cells in the presence of excess BSA was carried out in two stages. A mild elution ("A1" under the lanes in Fig. 2B), with neutral-pH SDS solution at 30˚C, eluted most of the proteins not already removed from the polymer disks by the initial rinses with SCP buffer ("F" under lanes, Fig. 2B). Elution with hot, alkaline SDS-mercaptoethanol solution should remove all of the remaining proteins to the "A2" fractions. The A2 fractions contained about 40% of the MopB supplied to the disks in the initial incubation. However, only limited amounts of other *Xf* proteins remained after the A1 elution, i.e., to be eluted in the A2 fraction. We interpret these results as showing a tight association between MopB displayed on the outside of *Xf* cells and the polymers or a polymer-mediated precipitation of the MopB protein, which then could be released and/or solubilized only by exposure to hot, alkaline SDS solution. These results indicate no specificity of MopB for association with (or precipitation by) a specific polymer, so, unlike MopB itself, the polymer side of the MopB-polymer pair is not an attractive target for interfering with *Xf*-xylem interactions.

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

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Reporting Period: The results reported here are from work conducted from October 15, 2003 to October 11, 2004.

ABSTRACT
The *Xylella fastidiosa* (*Xf*) is the causal agent of Pierce's disease of grape. In previous work, we discovered, partially purified, and investigated the processing of the *Xf* protein MopB, which previously had been known only from the nucleotide sequence of its gene. The amino acid sequence of MopB, the uniform staining of *Xf* cells with fluorescent anti-MopB antibody and the abundance of MopB in total protein extracts of *Xf* cells suggest that MopB is the major outer membrane protein of *Xf*. As such, MopB is expected to participate in *Xf* colonization of grape xylem elements. We previously demonstrated that partially purified MopB binds to (xylem-rich) balsa wood or cellulose (filter paper) disks under conditions in which other proteins do not adhere. Here we report improvements in our MopB purification procedure and observations on adherence of MopB in *Xf* cells to cellulose disks under conditions that eluted other *Xf* proteins. A high (0.25mM) concentration of the cellulose fragment cellotetraose did not interfere with the binding of MopB to cellulose, suggesting that the binding reaction of MopB is not specific for cellulose. We exposed *Xf* cells or MopB to each of three fibrous polymer disks and to cellulose disks and observed similar adherence of MopB from both sources to all four polymer disk types. Thus, MopB appears to associate with porous materials generally when it is exposed to such materials in purified form or as *Xf* cells. The abundance and exterior exposure of MopB makes MopB an ideal target for Pierce's disease control strategies. We seek to develop soluble proteins with high affinity for MopB. We will apply, as an anti-*Xf* agent, a selected MopB-binding protein alone or as a chimera with a bacterial cell-inactivating peptide or protein. Our expectation is that expression of the anti-*Xf* protein, targeted to the xylem in grape rootstock, may result in the anti-*Xf* protein moving into and protecting the grafted scion. In this reporting period, experiments were initiated with the objective of creating a protein having high affinity for MopB. As a first step towards this objective, Project Scientist Paul Feldstein developed *E. coli* strains expressing surface elements of MopB protein, so that the experimentally compliant *E. coli* can be used to select proteins with high affinity for *Xf* MopB.

INTRODUCTION
We have been investigating an abundant protein of *Xf*, MopB. We showed that MopB is the major outer membrane protein of *Xf* and is partly exposed on the outside of the bacterial cell. We purified MopB, prepared antibodies against it, and demonstrated an apparent affinity of MopB for cellulose. This last observation and the abundance of MopB suggested that MopB may participate in the initial attachment of *Xf* to the inner surface of the xylem vascular elements or in some other critical event in the initiation of infection leading to the development of Pierce's disease. Regardless of whether MopB is critical in this process, its location and prevalence support our contention that MopB is an ideal target for a *Xf*-specific bactericide or for a reagent that would coat and thereby inactivate *Xf* cells. Our strategy for creating a high-affinity MopB-binding protein is to begin with a protein that has evolved to bind tightly to the major outer membrane protein of *E. coli*, OmpA, and to convert the specificity of that protein from OmpA-binding to MopB-binding. The T2-like *E. coli* bacteriophage K3 has OmpA as its receptor. The K3 tail fiber adhesion gp38 is responsible for binding of bacteriophage K3 to OmpA in a reaction whose rate and irreversibility suggest a high-affinity association. Mutational conversion of gp38 from its natural receptor OmpA to other *E. coli* surface proteins has been demonstrated in several publications (Drexler et al., 1991, and references cited therein). In outline, our planned experimental steps for creating an anti-*Xf* protein are (i) replace the OmpA protein of *E. coli* with a protein that has MopB sequences displayed on the cell exterior, (ii) select variants of bacteriophage K3 that can infect the modified *E. coli* and also can bind to *Xf* cells, (iii) isolate the variant bacteriophage K3 gene *gp38* (expected to encode a MopB-binding gp38 protein), and (iv) genetically modify the MopB-binding gp38 to confer solubility and (in collaboration with the Gupta laboratory) possibly fuse the gp38 to a bactericidal peptide-encoding sequence. Step (v) will be the expression of a xylem-targeted version of the gp38 or gp38 fusion protein in rootstock and will be performed in collaboration with the Dandekar laboratory.

1. Natural populations of GWSS are commonly found thriving on several citrus varieties.
2. *Axd* colonized and grew best in the citrus varieties tested.
3. *Axd* colonized the xylem vessels of test plants, the same tissue from which GWSS feed.
4. *Axd* passively moved through populations of GWSS.
5. *Axd* did **not** negatively affect GWSS.

Interestingly, *Axd* appears to mirror the host range of GWSS. Genetically marked *Axd* colonizes several host plants. This suggests that genetic modification does not interfere with the biology of *Axd*, which should enter into the insect-plant cycle and be transmitted along with the pathogenic bacteria target. While GWSS is the vector of greatest interest in California, two other native sharpshooters also transmit the vehicle bacterium, *Axd*, and several plants can serve as hosts.

In the laboratory, inhibition of *Xf*-transmission by GWSS was demonstrated using two different categories of reagents, a surface antibody fragment and an antibiotic peptide (Indolicidin). The antibody fragment was specific to *Xf*. In our trials the antibody fragment was being expressed in the coat of a phage, so the effects on transmission might be greater when the antibody fragment is expressed on the surface of *Axd*. Indolicidin inhibited *Xf* growth *in vitro*, but did not affect growth of *Axd*. Transformation of *Axd* to produce each/or both of these reagents is currently under way.

We concluded that *Axd* will be an effective delivery agent of a symbiont control strategy for combating *Xf*. GWSS readily acquired *Axd* from a plant source and this bacterium translocated and colonized a variety of plants tested. We have yet to determine the effect of the reagents on *Xf* in infected grapevines.

Previously, plant symptoms confirmed by ELISA or PCR detection were used to determine if transmission had occurred. Unfortunately, these systems require the bacterium to colonize and infect the host plant to determine transmission. If an infected plant is asymptomatic, important but less obvious transmission events may be missed. Our system removes the plant "unknowns" from the equation. However, we recognize the importance of actual plant infection as a measure of transmission importance, but suggest using the artificial disease cycle as an initial rapid measure of vector competence.

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cases, positive phloem samples were detected only when the corresponding xylem samples was positive, whereas, most xylem samples were positive when phloem samples were negative. This indicated that positive detection of *Axd* in the xylem was due to actual presence of the bacterium; detection in phloem may have been due to contamination. Of the samples that tested positive, xylem samples contained 10X more cells on average than phloem although these values were not significant at the p=0.05 level (Trial 1: F=0.911, 1df, p=0.368.Trail 2: F=3.123, 1df, *p*=0.092). All plant samples which tested positive by RT PCR were confirmed by culturing followed by visualization under fluorescent microscopy.

Movement of Axd into GWSS Populations

After being exposed to an artificial feeding system containing DsRed *Axd* for 48h [6], 2 GWSS were marked with paint and placed on an individually caged chrysanthemum with 10 naive GWSS for 2 weeks. At the end of this period, all GWSS were collected from the cage and analyzed for the presence of DsRed *Axd* by QRT PCR. In two trial, each with 10 replicates (10 individually caged plants), 81% of the test insects survived through the studies. In both trials, more than 57% of the surviving, previously "naïve", GWSS tested positive for the presence of *Axd* (Trial 1, 51.2%; Trial 2, 64.3%). Therefore, through passive delivery of the symbiont in a finite period of time, more then ½ of the insects acquired the bacterium

Effect of Axd or Xf on GWSS Biology

Colonies of GWSS which were orally inoculated with DsRed *Axd*, wild-type *Axd*, S1 *Axd* (bacterium expressing an antibody), *Xf*, or no introduced bacteria (control) were maintained under laboratory conditions. Feeding ability, natural mortality, and dry weight post-mortem were compared between groups to determine if the presence of bacterium influenced any of these biological factors. In preliminary studies, mean g consumed after 5 was not significantly different for any of the 5 groups (n=20, p<0.001). Mortality rates of GWSS maintained in the laboratory were plotted over an 18 day period (Figure 1). Decline of the colony was consistent over time and no significant differences in mortality rates were detected (p<0.001). Randomly, individual GWSS were collected from each colony, dried in a desiccating oven for 48 h, and weighed. The average weight of a dried GWSS was 0.01g and no significant differences in dry weight were revealed (*p*=0.7). In two trials, 50 wild GWSS that were collected on the UCR campus were microinjected with different amounts either, DsRed *Axd* or H_2O. Bacteria were detected in the hemocoel; however, based on Chi-square analysis there were no significant differences in the mortality rates between the two groups.

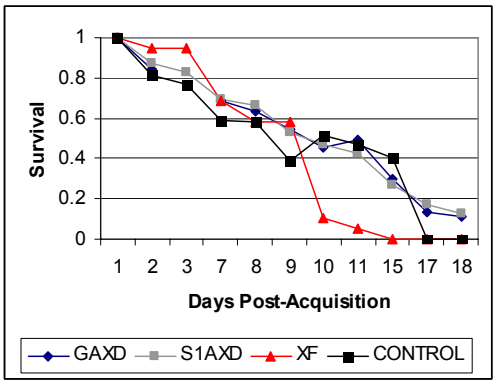

Figure 1. Mortality rate in four GWSS colonies maintained in the lab oratory.

Laboratory-Based Artificial Disease Cycle

A simple and efficient transmission cycle was developed for the study of *Xf* transmission by GWSS which allowed collection of sufficient transmission data in 1 wk. Specific numbers of cells were detected both in plant tissue and within the insect vector by QRT PCR. *Xf* cells were scraped from a PD3 plate and suspended in sterile ½ strength PBS (OD^{600}=2.0). Five cm sections of cut chrysanthemum stems were used for bacterial inoculations [6]. Five GWSS per 5 cm of stem were caged in snap cap vials for 48 h. After the acquisition access period (AAP), GWSS were placed on *Xf*-free chrysanthemums for 48 h, so that any detection of bacteria would be associated with transmission and not stylet contamination. Pairs of GWSS were transferred to sterile vials containing a fresh chrysanthemum stem cutting. The insects were exposed to a stem for an inoculation access period (IAP) of 48 or 96 h. DNA was extracted from the inoculation targets with the XNAR Extract-N-Amp kit (Sigma-Aldrich, St. Louis, MO) and PCR was run following a standard QRT-PCR protocol. Across 9 replicates using a 48h IAP, the mean transmission rate of *Xf* by GWSS was 0.508±0.122, while the mean rate when given a 96h IAP was 0.341±0.138. Using Chi-square analysis, these ratios were significantly different (χ^2=16.281, df=1, p≤0.001). The lower rate associated with the longer IAP is probably due to the non-hospitable environment of the test plant stems.

Interruption of PD Cycle

Transmission of *Xf* from infected grapevine to healthy grapevine by GWSS was blocked by feeding GWSS on the plant-based AFS containing an *Xf*-specific antibody fragment (scFV S1) expressed in the coat of a M13 bacteriophage, between a 5 d AAP and the 5 d inoculation access period (IAP). At two concentrations of phage/antibody (10^{14} and 10^{15}) transmission of *Xf* was 0% (n=10 and n=13, respectively), compared to 50% transmission in the control group (n=8). Transmission of *Xf* was reduced when GWSS were fed Indolicidin (American Peptide Company, Inc., Sunnyvale, CA) between the AAP and IAP from 50% in the control group to 35% (n=14) at 100µg/ml and 7% (n=14) at 500µg/mL. These experiments are currently being replicated. While the rate of *Xf* transmission was higher than previously reported [1, 2, 9], we feel this is a fair assessment of the insects' ability to transmit.

CONCLUSIONS

Several major biological associations were found which support the feasibility of symbiotic control to reduce transmission of *Xf* by GWSS:

PARATRANSGENESIS TO CONTROL PIERCE'S DISEASE: BIOLOGY OF ENDOPHYTIC BACTERIA IN GRAPE PLANTS AND BIOASSAY OF REAGENTS TO DISRUPT PIERCE'S DISEASE

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

ABSTRACT

Xylella fastidiosa (*Xf*), which causes Pierce's disease (PD) in grapevines, is transmitted by the glassy-winged sharpshooter (GWSS). Symbiotic control employs symbiotic bacteria to deliver anti-pathogen compounds to disrupt transmission of the pathogen to new host plants. *Alcaligenes xylosoxidans denitrificans* (*Axd*) was identified as a potential agent for paratransgenesis because it inhabits the foregut of GWSS and the xylem of plants, as does *Xf*. In this report, we describe the relationship between *Axd* (the symbiont), *Xf* (the plant pathogen), GWSS (the insect vector), and host plants to develop a delivery strategy for symbiotic control. Additionally, disruption of *Xf*-transmission by GWSS was demonstrated using two reagents, a single chained antibody fragment and an antibiotic peptide.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) is the principal vector of the xylem-limited bacterium *Xylella fastidiosa* (*Xf*), which causes Pierce's disease (PD) in grapevines. Limiting the spread of this pathogen by rendering GWSS incapable of pathogen transmission would control the disease.

Symbiotic control approaches have been developed to disrupt Triatomid transmission of *Trypanosoma cruzi* [3], to prevent colitis in mammals [4, 11], and to interfere with transmission of HIV [8]. Candidate microbes that live in close proximity to the pathogen in the vector insects and in host plant tissues would by ideal vehicles to control *Xf*.

Alcaligenes xylosoxidans denitrificans (Axd), originally isolated from the cibarium of GWSS, has been described as a non-pathogenic plant endophyte and a non-pathogenic soil-borne microbe [10, 12]. *Axd*, genetically marked with DsRed or EGFP protein, colonized the cibarium of GWSS for up to 35 days, the longest period tested [6]. *Axd* readily colonized the xylem vessels of several plants with citrus being the most hospitable to the bacterium. [5]

Two categories of anti-pathogen reagents, single-chained antibodies (scFV) and antibiotic peptides, were tested for activity against *Xf*. Screening of scFV uncovered an antibody fragment that was specific to *Xf* and may be specific to the PD-causing strain of *Xf*. Four toxic peptides were identified that inhibited the growth of *Xf*, but did not inhibit the growth of *Axd*.

OBJECTIVES

1. Identify relationships between *Axd* (the symbiont), *Xf* (the plant pathogen), GWSS (the insect vector), and host plants to develop a delivery strategy for symbiotic control.
2. Test the ability of anti-pathogens to disrupt *Xf* disease cycle.

RESULTS

Axd Movement and Colonization within Host Plants

In two trials, chrysanthemums (n=20) were needle inoculated with DsRed *Axd,* which contains a kanamycin-resistance gene. One week later phloem and xylem fluid samples were collected independent of one another using a Scholander pressure bomb [7]. The collection resulted in about 20-50 µl of phloem fluid and 100-150 µl of xylem fluid per stem. DNA was extracted from the remaining half of each phloem and xylem fluid sample from each plant using the Extract-N-Amp™ Plant kit (Sigma Aldrich, Steinheim, Germany). Presence of *Axd* was then determined using QRT PCR. The other half of each phloem and xylem fluid sample was inoculated into LB broth containing kanamycin and incubated for 48 h at 37ºC. After the incubation period, bacteria were screened for red fluorescence using a MZ12 fluorescent microscope (Leica Microsystems Inc., Heerbrugg, Switzerland). Positive samples were confirmed by QRT PCR.

A higher proportion of xylem fluid samples tested positive for the presence of *Axd* than phloem samples in both trials: in trial 1 xylem 8/20, phloem 2/20 (χ^2=4.8, 1df, p=0.0284); in trial 2 xylem 15/20, phloem 8/20 (χ^2=5.013, 1df, p=0.025). In all

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Table 1. Detection of *RAxd* in grapevines from three field sites (2003).

	Application Method	*RAxd* positive samples[1]						
		Weeks post-inoculation					During grapevine removal[2]	
		0	2	4	6[3]	Berries[4]	Canes	Root[5]
Bakersfield	Foliar Spray	0	**3**	**2**	ND	0	0	0
	Needle Inoculation	0	**2**	0	ND	0	0	0
	Soil Drench	0	**3**	0	ND	0	0	0
	Control	0	0	0	ND	0	0	ND
Napa	Foliar Spray	0	0	0	0	0	0	0
	Needle Inoculation	0	0	0	0	0	0	0
	Soil Drench	0	**1**	**1**	0	0	0	0
	Control	0	0	0	0	0	0	ND
Temecula	Foliar Spray	0	0	0	0	0	0	0
	Needle Inoculation	0	0	0	0	0	0	0
	Soil Drench	0	0	0	0	0	0	0
	Control	0	0	0	0	0	0	ND

[1]Represents 6 samples from 5 grapevines per treatment per field site (n=30 per grapevine).
[2]Grapevines were removes >14 weeks after inoculations at all locations.
[3]ND = not determined.
[4]Berries were collected during final collection date.
[5]Root samples were taken only from *RAxd* treated vines.

2004 Field Project
Data are not complete and will not be reported here.

CONCLUSIONS
Grapevine inoculations were made after 50-80 days following grapevine flowering at all locations. After flowering, both the xylem and the phloem begin to fill the fruit with fluid (6). Between 60 and 70 days after flowering, the xylem stops filling the fruit, and phloem contributes all fluid for the development of the fruit. This flow continues to 120 days after flowering which is the average number of days to fruit harvest. At all field sites, *RAxd* inoculations were made 26 (Riverside) to 43 (Temecula) days prior to harvest. Considering grapevine physiology, inoculations were made after the xylem ceased to contribute fluid directly to the fruit in all cases. Therefore, it was not surprising that *RAxd* was not found in fruit at any location because it is a xylem-associated bacterium (3).

The most probable explanation for the inability of *RAxd* to survive in grapevines after 4 weeks was its lack of competitive fitness associated with the transgenic organism. Xylem contains diverse and sometimes extensive communities of microbes (2). In greenhouse studies, a strain of EGFP protein-expressing *Axd* was introduced into seedlings of several plant species (3). In that study, the genetically marked bacterium moved readily within the xylem vessels of the plants and was recovered 10 months later. However, presence of a well-established microbial community may have restricted the growth and colonization of transformed *Axd*, ultimately leading to its demise. Chromosomally transformed organisms are commonly less fit than native bacterial species due to the cost of the genetic insert (5). A comparison of the genetically modified *Axd* to the native *Axd* showed that the transformed strain was less fit in laboratory cultures (Lauzon, unpublished data). Although the experiments were not designed to test the relative fitness of transgenic *Axd,* the bacterium's inability to persist longer than 4 weeks provides additional support for the theory of reduced fitness.

Given the ubiquitous nature of *Axd* and its ability to colonize several plant hosts, including grapevines, in the greenhouse (3), we expected it to persist longer in field-grown grapevines. Even so, viability of 4 weeks may offer a large enough window for the delivery agent in a symbiont control strategy to dispense the necessary anti-pathogen factors to negatively affect *Xf*. Additionally, re-application of the symbiotic control agent may be necessary. Additionally, reduced fitness offers an internal controlled mechanism that will guard against transformed *Axd* population spread and persistence in the environment or consumer products.

OBJECTIVES

1. Track the movement of *Alcaligenes xylosoxidans denitrificans* (*Axd*) within plants with or without insect involvement and track movement in the environment.
2. Characterize transmission of *Axd* by glassy-winged sharpshooter (GWSS, *Homalodisca coagulata*).
3. Develop an application method for transgenic *Axd* into the xylem of grape plants for delivery of an anti-*Xylella* strategy.

RESULTS

In July 2003, field sites were established at four locations in the state of California; Napa, Bakersfield, Temecula, and Riverside. At the Napa, Bakersfield, and Temecula sites, *RAxd* was applied to grapevines using 3 inoculation techniques; needle inoculation, foliar spray application, and soil drench. These plants were covered with insect-free screening, to exclude arthropods from test plants. Samples were taken throughout the growing season and processed. Grapevines at the Riverside field site were needle inoculated with *RAxd* and three concentrations of GWSS (0, 10, and 50) were placed on the plants to test the affect of GWSS feeding pressure on the translocation of *RAxd* in grapevines. We collected mature grapes and plant parts for analysis from grapevines at all four field sites.

Detection of RAxd in Grapevine Xylem: Napa Field Site

Grapevines were inoculated 41 days prior to harvest. Pre-harvest grapevines xylem samples were collected three times (2, 4, and 6 weeks post-inoculation). Only single samples from 2 of 15 test plants tested positive for the presence of *RAxd* at 2 weeks post-inoculation. These positives were from plants treated by needle inoculation and soil drench. Two weeks later, only a single sample from the soil drench-treated plant tested positive. There were no positive samples collected 6 weeks after inoculation. No control plants tested positive for the presence of *RAxd* on any date.

Bakersfield Field Site

Grapevines were inoculated 33 days prior to harvest. Pre-harvest grapevines xylem samples were collected two times (2 and 4 weeks post-inoculation). Multiple samples from 8 of 15 test plants tested positive for the presence of *RAxd* at 2 weeks post-inoculation. Of these *RAxd* positives plants, 3/5 from foliar spray, 2/5 from needle inoculation and 3/5 from soil drench. Two weeks later, only two plants from the foliar spray-treated grapevines tested positive. No control plants tested positive for the presence of *RAxd* on any date.

Temecula Field Site

Grapevines were inoculated 43 days prior to harvest. Pre-harvest grapevines xylem samples were collected 3 times (2, 4, and 6 weeks post-inoculation). No samples on any collection date tested positive for the presence of *RAxd*.

Riverside Field Site

Grapevines were inoculated 26 days prior to harvest. Pre-harvest grapevines xylem samples were collected two times (2 and 4 weeks post-inoculation). Only 10 samples collected 2 weeks after inoculation were positive for the presence of *RAxd*. Six of the positive samples were from grapevines with no GWSS included, while 4 of the positive samples were from grapevines with GWSS included. No significant differences in *RAxd* presence in grapevines could be attributed to the presence of GWSS (X^2=0.24 df=1, p value=0.624).

Detection of RAxd in Soil. RAxd

Detection of *RAxd* in soil. *RAxd* was not detected in soil samples collected from the base of any grapevines at any locations using the culture methods or RT-PCR.

Detection of RAxd in Grape Berries

In grape cluster samples collected on the date of harvest (Napa Aug. 27, Bakersfield Sept. 3, Temecula Sept. 2, and Riverside Aug. 18), *RAxd* was not detected by RT-PCR in whole grape samples from any location. Furthermore, *RAxd* was not detected in dissected grape berry samples of flesh, veins, seeds, peduncle, or stem from any location.

Detection at the Time of Field Plot Destruction

RAxd was not detected in grapevine, root, or soil samples at the time of removal.

ENVIRONMENTAL FATE OF A GENETICALLY MARKED ENDOPHYTE IN GRAPEVINES

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ABSTRACT
Symbiotic control employs symbiotic bacteria to deliver anti-pathogen compounds to disrupt transmission of the pathogen to new hosts. *Alcaligenes xylosoxidans denitrificans* (*Axd*), an insect and plant symbiotic bacterium, occupies same niche as the plant pathogen *Xylella fastidiosa* (*Xf*) which causes Pierce's disease. We determined the fate of genetically altered *Axd* (*RAxd*) after introduction into grapevines to assess its feasibility as a symbiotic control organism to control *Xf*. *RAxd*, which expresses a fluorescent protein (DsRed), was applied to grapevines by needle inoculation, foliar spray application, or soil drench. The plants were covered with insect-resistant screening, to exclude arthropods from test plants. *RAxd* were detected in stems of several grapevines 2 weeks post-inoculation from each inoculation type. The amount detected at 4 weeks post-inoculation declined, and *RAxd* was absent 6 weeks post-inoculation. *RAxd* was not detected in grape berries or soil samples collected around *RAxd* positive grapevines. This work demonstrated that transgenic *Axd* became established in grapevines in the field but did not thrive there. A limited lifespan of transformed *Axd* in grapevines would keep its population increase in check in that host plant. Re-inoculation of grapevines at 6 wk intervals would be sufficient to keep anti-pathogen products present. *RAxd* thrives in GWSS and citrus. Therefore, there is a good chance that GWSS would pick up the *RAxd* as an antimicrobial symbiont from nearby sources to render GWSS vector-incompetent.

INTRODUCTION
Replacement therapy or symbiotic control employs symbiotic bacteria to deliver anti-disease compounds to target pathogens of plants to make vector insects unable to harbor the pathogen or to prevent a pathogen from being transmitted to healthy plants (1). *Alcaligenes xylosoxidans denitrificans* (*Axd*), was selected for further study and a fluorescent marker gene inserted. We followed the movement of genetically altered *Axd* (*RAxd*) in grapevines and in the vector insect, glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*.

Regulatory and industry acceptance of this approach requires knowing the fate of *Axd* in various locations and in plants at different times of the year. Our current detection methods employ PCR (polymerase chain reaction) and fluorescence microscopy (3, 4). QRT-PCR provides a quantitative measure of bacteria in the samples, which is missing from existing methods. This is important because it allows determining optimum doses and timing for application of the delivery organism and its expression of anti-*Xylella* products.

Fluorescent protein gene markers are now commonly used in genetics and are considered environmentally benign since they are based on natural products. The bacterial transformation cassette was inserted with so-called jumping genes (mobile or transposable elements) originally identified in *Drosophila mauritiana* and called mariners (7). The mariner elements have had their jump mechanism removed (so the inserted gene will not be remobilized) and all antibiotic genes used for selection have been removed (so no antibiotic factors can be moved inadvertently to other bacteria). The resulting transgenic strains are very stable and grow readily in culture. Little or no mutation or reversion has been observed.

Since the marker genes were placed next to an open reading site that is designed to contain the future anti-*Xf* compound, the bacteria we are using now are nearly complete. In other words it is close to the final product. Thus, we can study the biology of the genetically altered vehicle bacterium, *RAxd*, and its behavior in the vineyard ecosystem.

We prefer to do this in commercial vineyards because the laboratory experiments are never fully indicative of behavior in the field. We chose widely separated locations and in California and more than one variety of grapevine to test. A top priority was to determine if the transgenic endophyte lodged in the grape berries or otherwise contaminated the product of the vineyards.

Section 5:
Control Strategies

DETECTION OF *XYLELLA FASTIDIOSA* IN INSECT VECTORS IN CALIFORNIA

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ABSTRACT

Recent spread of *Xylella fastidiosa (Xf)* to several agricultural commodities and ornamental plants in California has prompted great interest in understanding the comparative interactions between *Xf* and native and recently introduced insect vectors. The generally low titer of *Xf* in insect vectors limits the use of serological techniques, such as ELISA, for qualitative and quantitative analyses of *Xf* associated with different insect vectors. *Xf* detection by molecular techniques, such as PCR, can potentially overcome this limitation. The objective of this study was to compare standard PCR for detection of *Xf* in field-collected insects as well as in greenhouse-reared insect vectors using primers RST31/RST33 with newly developed primers HL5/HL6 in standard PCR and in Real Time PCR using the system HL5/HL6 and a probe labeled with FAM. Two native species the green sharpshooter (*Draeculacephala minerva*) and the red-headed sharpshooter (*Xyphon fulgida*), and the recently introduced glassy-winged sharpshooter (*Homalodisca coagulata)* were included in this study. Field-collected insects were obtained from *Xf*-infected grapevines and almonds in the San Joaquin Valley, California. Greenhouse-reared green and red-headed sharpshooters were also obtained from cultures maintained on a non-host of *Xf* in Parlier, California. Five-10 *Xf* cells per µL of insect head DNA sample were detected with the HL5/HL6 primer pair-FAM system. Also, using this system, the number of *Xf*-cells detected in field-collected and greenhouse reared insect was between 10^2-10^3/µL sample/reaction. This concentration of *Xf* cells was detected by visualization of the *Xf*-specific amplicon (221 bp) in gels following standard PCR with the HL5/HL6 primers. This level of pathogen in insect heads was below the limit of detection in standard PCR with primers RST31/RST33. Using Real-Time PCR quantification with the system HL5/HL6-FAM, the total amount of *Xf*-cells per insect head was estimated to be between 10^4-10^5. Implications of these results on the epidemiology of the disease are discussed.

EVALUATION OF A NOVEL, FIELD DEPLOYABLE, ELECTROCHEMICAL DETECTION SYSTEM FOR THE DETECTION OF *XYLELLA FASTIDIOSA* WITHIN GRAPEVINE PETIOLES

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ABSTRACT

We have tested a new electro-chemical detection (ECD) system designed by AnzenBio, Inc. for the quick detection of *Xylella fastidiosa* within grapevine petioles. Like standard ELISA this detection method relies on antibodies against the bacterium, but unlike ELISA it detects movement of electrons through the final product conversion, measuring current rather than color change. Using a hand-held meter and pre-coated chips the test can be done in a fraction of the time (1.5 vs. 5 hrs.). Comparison of 18 Cabernet Sauvignon petioles from a vineyard with Pierce's disease (PD) to 18 petioles guaranteed PD free showed the ECD readings per gram of tissue to be higher for PD petioles (31.3 vs. 6.2 microamps). This difference is statistically different using a t-test ($p<0.0001$). In another trial in South Texas, ECD was used to evaluate the petioles from three different varieties, Blanc du Bois, Black Spanish and Cynthiana, which have been shown to carry differing levels of *Xylella fastidiosa* within this area of high PD pressure. Petioles were also categorized into those from leaves with low, medium and high PD symptoms. Analysis of variance on ECD data from the 9 symptom variety categories with 6 replications showed that ECD could detect distinct significant differences between several of the categories ($p<0.0001$). Analysis of variance on ELISA data run on the same 54 samples found no significance between categories ($p=.43$). ECD appears to give more sensitive readings over a range of bacterial levels, potentially giving fewer false positives.

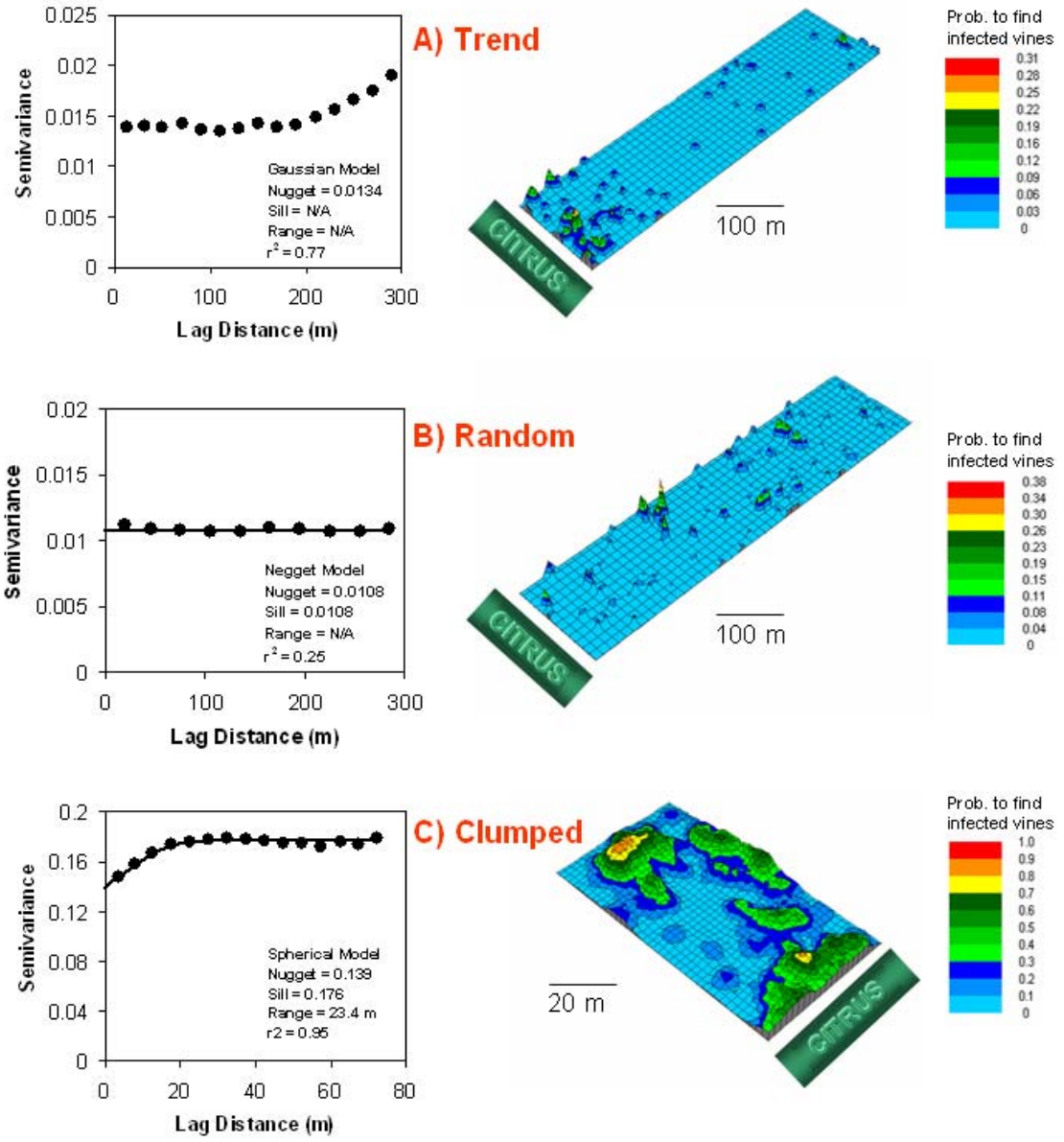

Figure 1. Three main dispersion patterns of PD found in Kern County in 2002. (A) A "trend" spatial pattern from areas of high infection to low infection existed when the infection was between 0.1% and 1.0%. (B) A "random" distribution pattern existed, when the infection was between 1% and 5%. (C) A "clumped" dispersion pattern existed when PD infection was > 5%. When infection was < 0.1% there were no detectable spatial structures.

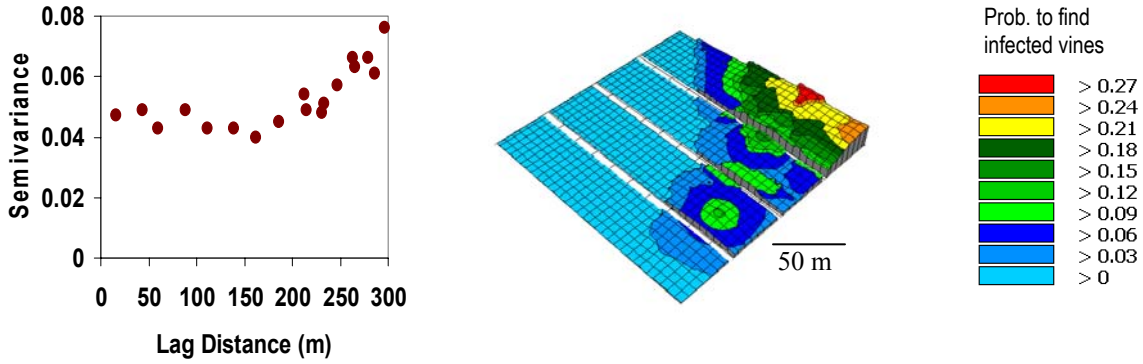

Figure 2. Semivariogram and dispersion map for PD in a Coachella Valley vineyard. The semivariogram indicates a trend dispersion pattern. Within this trend, a random dispersion pattern exists up to a lag distance of 200m. This trend from high to low PD is easily visualized in the dispersion map.

OBJECTIVES

The goal of this project is to develop a sequential grid-sampling program for PD that can characterize the spatial distribution and determine the location of PD based on the spatial structures and patterns of PD distribution in the vineyard. The objectives of this project include:

1. Characterization of the spatial distribution of PD in vineyards.
2. Development of a sequential grid-sampling program.
3. Validation and optimization of the sampling program with cost analysis and sensitivity analysis.

RESULTS AND CONCLUSIONS

We have conducted censuses of Kern County vineyards for the past four growing seasons (2001-2004). This report is focused on the 2002 data. Census data were converted into a GIS database and analyzed with geostatistics. Geostatistics is a set of statistical procedures that can characterize distribution (called *semivariogram modeling*) and generate distribution maps (called *kriging*). The semivariograms show the spatial pattern (e.g., no structure, uniform, trend, random, or clumped) and the structure (e.g., the size of aggregation, spatial correlation, and spatial variability) of PD distributions. Kriging was used to generate distribution maps of the probabilities of PD infections throughout the vineyard.

Census result

We made a census of 215 vineyards in 2002. A total of 135 vineyards were infected with PD. Only seven vineyards had more than 0.1% PD infection, and those vineyards were located adjacent to citrus groves indicating that citrus affects the incidence and severity of PD in nearby grapes. This result is consistent with patterns of PD found in Temecula (Perring et al. 2001). However, as in the Temecula study, proximity to citrus did not affect PD distribution in all Kern County vineyards.

Spatial distribution of PD in vineyards

Determining distribution patterns (e.g., no structure, uniform, trend, random, clumped) is the first step for developing sequential grid-sampling plans for fields in which we do not know the location of infected vines. Geostatistical analyses showed that the distribution pattern of PD could be categorized according to the incidence of PD in each vineyard. When the infection was < 0.1%, there was no spatial structure to the location of infected vines. Vineyards that had between 0.1% and 1% infection showed a distribution pattern of a trend from areas of high infection to low infection (Figure 1A). This type of distribution pattern (i.e. trend) also was found in the Coachella Valley in a field that had a similar proportion of infected vines (Figure 2). When the infection was between 1% and 5%, the pattern of disease was random (Figure 1B), and a clumped distribution existed when infection rate was > 5.0% (Figure 1C).

Our work suggests that knowing the percentage of PD infection and the location of vineyards relative to citrus can predict the distribution pattern of PD in the vineyard. Such inferences from the geostatistical analysis can be used to develop a spatially-oriented sampling program with sampling grids. The development of this sequential grid-sampling program provides three fundamental roles in PD research and management. First, it enables growers to locate vines infected with PD in the vineyard when the proportion of infected vines precludes a vineyard census. Second, using with the geospatial and geostatistical methodologies of the sampling program, growers will be able to identify problem areas in their vineyards. Third, the sampling program provides a method for standardizing PD sampling statewide. Progress in these areas, i.e. locating individual vines, identifying problem areas in a vineyard, and standardizing areawide monitoring, not only will help growers make informed decisions in their own vineyards, but will assist researchers trying to understand the epidemiology of GWSS-vectored PD in California.

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DEVELOPMENT OF A FIELD SAMPLING PLAN FOR GLASSY-WINGED SHARPSHOOTER-VECTORED PIERCE'S DISEASE

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ASBTRACT

Determining the location of grapevines infected with Pierce's disease (PD) in vineyards has been a major question for growers and researchers. Field census has been the only reliable way to identify vines infected with PD in the vineyard. Censuses, however, are difficult when PD incidence is high. In these situations, we need a sampling program that accounts for the spatial structure and pattern of PD in the vineyard. To characterize the spatial distribution patterns of PD, census data from Kern County vineyards were analyzed with geostatistics. These analyses showed that dispersion of PD varied with the amount of PD infection, and with vineyard proximity to citrus. Based on these analyses, our goal is to develop a sequential sampling program for detecting PD in vineyards.

INTRODUCTION

A common sampling technique to detect the presence of PD in vineyards is to visually examine vines, remove symptomatic leaves from possible infected vines, and confirm the presence of PD with enzyme-linked immunosorbent assay (ELISA). Locating vines infected with PD in a vineyard is required for current PD management, and the only reliable method for locating PD-infected vines is to examine every vine in the vineyard. Such a census was used for a county-level PD survey and provided a cost-effective method (< $5 per acre) for identifying infected vines in vineyards when PD infection was very low (Hashim and Hill 2003). As the infection level in a vineyard exceeds 1%, it becomes more difficult to observe and sample every symptomatic vine. It is especially difficult to distinguish PD symptoms when other stress factors, such as drought and salt damage, exist in vineyards. Such difficulties result in high sampling costs because many samples must be taken and confirmed with ELISA. Thus, the development of a cost-effective sampling program appropriate for growers' and researchers' needs and skills is necessary for PD monitoring and management.

By definition, a sampling program employs all available sampling techniques to collect samples that are used to make estimates of population parameters (Pedigo 1994). In our case, we need to estimate the distribution and abundance of PD-infected vines. The sampling techniques consist of the actual equipment and methodologies by which samples are collected (Pedigo 2002). Sampling programs, on the other hand, direct how often and how many samples are to be taken, the spatial pattern to obtain sample units, and the timing of sampling (Pedigo 1994). Sampling programs often include binomial sampling or sequential sampling that makes sampling more cost effective and convenient. However, in PD sampling, such sampling plans cannot be directly adopted because the purpose of PD sampling is not only to estimate the incidence of PD but also to locate individual vines infected with PD. Thus, the sampling program for PD should be spatially oriented to identify the locations of the individual vines infected with PD.

One way to locate infected vines without a census is to use sampling grids that match the spatial structure and patterns of PD distribution. To develop these sampling grids three facts should be known: 1) the spatial structure and patterns of PD distribution, 2) the relationship between PD distribution and the percentage incidence of PD, and 3) the relationship between PD distribution and environmental factors affecting the incidence and spatial distribution of PD. Such knowledge can be obtained with current technology and methods such as the global positioning system (GPS) to locate sampling grids, the geographic information system (GIS) to generate geo-referenced data, and geostatistics analyze spatial data.

Table 1. State variables, process functions and parameters for GWSS-PD Model

Variables	Description	
$A_S(t)$	Susceptible GWSS Adults	
$A_I(t)$	Infectious GWSS Adults	
$S(t)$	Susceptible Vines	
$I(t)$	Infectious Vines	
Process Functions		**Process Sub-Models**
$R(t-T_J)S_J$	Recruitment into the adult stage	$R(t-T_J)S_J = b\left(A_S(t-T_J)+A_I(t-T_J)\right)S_J$
$D(t)$	Death rate for a stage	Linear constant death rate, e.g.: $D_A(t) = d_A A(t)$
$X(t)$	Infection rate for GWSS	$X(t) = \alpha I(t) A_S(t)$
$Y(t)$	Infection rate for vines	$Y(t) = \beta S(t) A_I(t)$
S_J	Survival of stage J with constant death rate	$S_J = \exp(-d_J T_J)$
Parameters		
b	Average birth rate	
T_i	Time in the ith stage or process	
d_i	Constant death rate for ith stage	
a	Transmission rate for GWSS	
β	Infection rate for vines	

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The state variables, process functions and parameters are defined in Table 1. We emphasize that this model is in an early development stage, and undoubtedly will evolve and improve as we develop it further. We used the delay-differential equation (DDE) formalism developed by Murdoch et al. (1987) and Murdoch et al. (2003) for stage structured insects, and to their formulation we will add time dependence (temperature forcing) of the developmental delays (although for simplicity we will not elaborate on this here). The time dependence in the delays can be incorporated according to the mathematical recipes developed by Nisbet and Gurney (1983), Gurney et al. (1983), Gurney and Nisbet (1998) and Nisbet (1998). Methods for setting up the initial history for starting the models are outlined well in Gurney et al. (1983). We will solve our set of equations using a new delay differential equation (DDE) solver, ddesd.m, (with time and system varying delays) developed for The Mathworks (Matlab) by L. F. Shampine (Shampine & Thompson 2001, Shampine 2004). The solver is not yet a part of Matlab itself, but a version is available on the Web at: http://faculty.smu.edu/lshampin/current.html .

Our model system.
The state balance equations are written as a set delay-differential equations (DDEs) with functions for recruitment, infection and death rates as:

$$\begin{aligned}
\text{Susceptible Adults: } & \frac{dA_S(t)}{dt} = R(t-T_J)S_J - X(t) + X(t-T_1)S_1 - D_A(t) \\
\text{Infectious Adults: } & \frac{dA_I(t)}{dt} = X(t) - X(t-T_1)S_1 - D_A(t) \\
\text{Susceptible Vines: } & \frac{dS(t)}{dt} = D_V(t) - Y(t) \\
\text{Infectious Vines: } & \frac{dI(t)}{dt} = Y(t-T_2) - D_V(t)
\end{aligned} \qquad 1.1$$

We adopted and slightly modified the notation of Murdoch et al. (2003) by using $R(t)$, $X(t)$, $Y(t)$ and $D(t)$ to represent recruitment (R), infection of GWSS (X) infection of vines (Y) and death rate (D) functions for each stage, and we then define each of these for our case. These equations indicated that the rate of change of a stage is simply the input to that stage minus output from that stage. The interpretations for each equation are outlined below.

Susceptible adult equation.
The first equation says that susceptible adults have input from reproduction, one juvenile delay period (T_J) in the past times survival going through the juvenile stage, $R(t - T_J)S_J$. Another input to susceptible adults is (possible) recovery from an infectious adult class with a time delay, $X(t - T_1)S_1$ where T_1 is the time that the disease persists in an infected adult, and S_1 is the survival during the infectious period. Outputs from susceptible adults are infection by feeding on an infectious vine, $X(t)$, and death, $D_A(t)$.

Infectious adult equation.
The second equation says that infectious adults have input from the infection process, $X(t)$, (which was output from the susceptible class) and output to (possible) recovery from infection, $X(t - T_1)S_1$, and death, $D_A(t)$.

Susceptible vine equation.
The third equation says that susceptible vines have input equal to death rate of infectious vines, $D_V(t)$, that is, we assume that dead vines are replaced at the death rate. Output from susceptible vines is infection by infectious sharpshooters, $Y(t)$.

Infectious vine equation.
The last equation says that infectious vines have input from the infection process with a latent period time lag, $Y(t - T_2)$, where T_2 is the latent period of the disease in vines after becoming infected. We assume that all vines survive the latent period. Output from the infectious vine equation is by death of infected vines, $D_V(t)$.

Our model system of equations will allow us to simulate the introduction and progress of PD into a vineyard under different conditions and management strategies. What we would like is to see the disease die-out and not invade the vineyard effectively. What we do not understand at this point is how all of the factors influence this scenario and determine its progress and to which factors spread is most sensitive. By studying the dynamic behavior of this model system we can learn how different management options are likely to affect the disease progress in a vineyard, giving us new ideas and methods about how to best control and prevent disease outbreaks.

TREATMENT THRESHOLDS FOR THE GLASSY-WINGED SHARPSHOOTER BASED ON THE LOCAL EPIDEMIOLOGY OF PIERCE'S DISEASE SPREAD (A STAGE-STRUCTURED EPIDEMIC MODEL)

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Reporting period: The results reported here are from work conducted from July 1, 2004 to October 8, 2004.

ABSTRACT
The conditions for the successful invasion of a vineyard by Pierce's disease (PD) are not well understood. To help integrate what knowledge we do have and indicate areas where research is needed we are developing a more biologically detailed model than has been previously available. Fortunately there is a large ensemble of literature from epidemiology regarding this problem, and in addition, much has been done toward solving the kinds of equations that arise in this work in terms of both mathematics and software. Here we outline very briefly our progress to date, and the ways in which these sorts of models can help us to better manage and understand the PD system. Here we describe a system of delay equations for modeling the dynamics of PD vectored by the glassy-winged sharpshooter (GWSS). We will analyze and study this system to derive threshold conditions for the invasion of a vineyard by PD and GWSS. Thresholds for disease outbreaks are common among epidemiological systems and a large literature exists on this subject. In addition new software (not commercially released yet) has been made available to us for solving these kinds of systems. We will attempt to use our model system to bring this methodology to the PD/GWSS problem and find new ways of controlling this disease.

INTRODUCTION
Last year we presented a model to evaluate how the threshold might change in relation to various biological and ecological factors (Perring et al. 2003). It was designed to determine the number of GWSS required to cause a single PD infection in grape. The primary model parameters were the proportion of GWSS carrying PD, GWSS transmission efficiency of PD, proportion of GWSS that will move from citrus to grape, the number of grapevines that a single GWSS will visit, grape varietal susceptibility, and the probability of an infection event resulting in disease. Our recent work, reported in this progress report, is an extension of the previous efforts and is more biologically detailed, allowing us to address more complicated biological processes affecting the epidemiology of PD in grapes. Over eighty years of research in epidemiology has shown that epidemics tend to be triggered when the generation reproductive factor of the pathogen becomes greater than 1.0 (Kermack and McKendrick 1927, Anderson 1978, Diekmann and Heesterbeek 2000, van den Driessche and Watmough 2002, Wonham et al. 2003). This fortunate result is useful in management since it provides us with a target threshold that will trigger a PD epidemic in grapes. More than just a threshold, this approach will provide a function for the basic generation factor of increase of the pathogen, R_0, as a parameter function from the model. The pathogen will grow into an epidemic or decline to zero according to whether R_0 is greater or less than 1.0. It is particularly helpful that this threshold indicator is a function of all of the model parameters, since this indicates what parameters the threshold is most sensitive to and therefore how management can be most effectively focused. Some of the things that we intuitively expect to be important are density of GWSS, pathogen titer of the insects, and their dispersal rate and feeding rate.

OBJECTIVES
1. Develop a model to describe the epidemiology of GWSS transmission of PD to provide a framework for organizing data and examining relationships between data from different research projects.
2. Use the model to develop field-specific treatment thresholds to prevent GWSS transmission of PD.

RESULTS AND CONCLUSIONS
Our results consist of a model system of state equations describing the progress of PD in a vineyard vectored by GWSS. Here we develop our basic model as set of four balance equations, two equations for the GWSS and two equations for grapes.

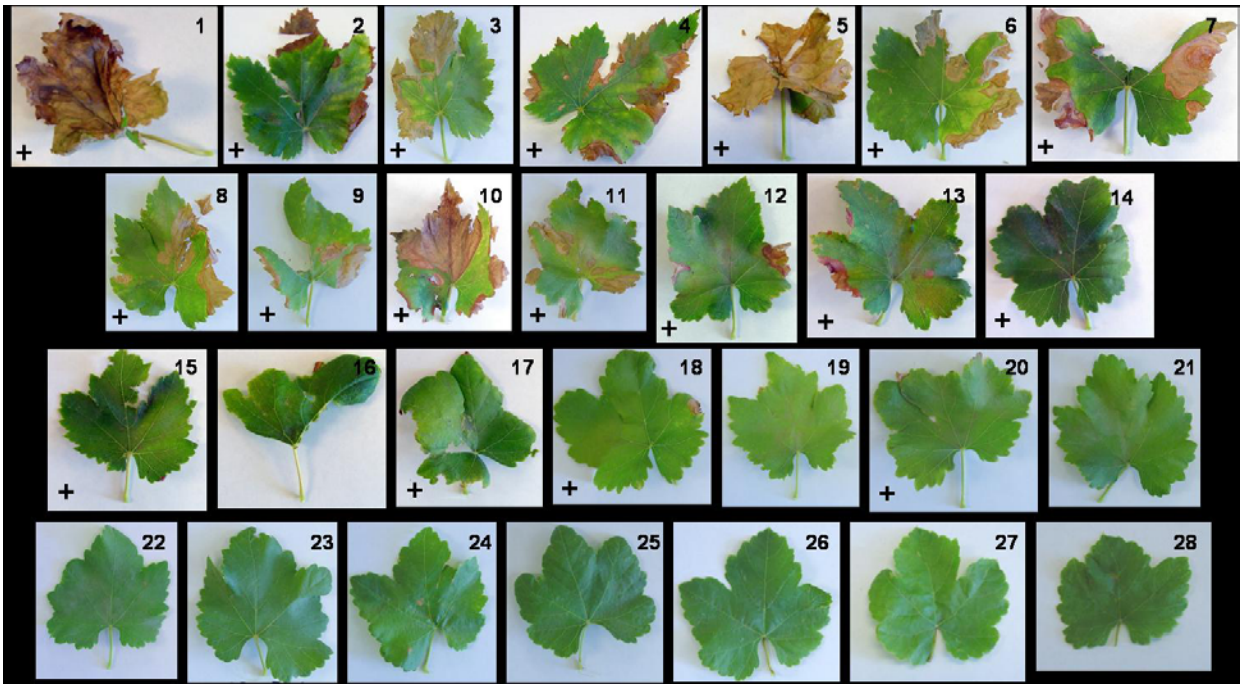

Figure 2. Individual leaves from a single Superior Seedless cane. Number indicates node position with 1 being the most basal node. The plus symbol indicates that the petiole from the leaf tested positive for PD by ELISA.

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two non-infested vines of each variety. On all canes from the Coachella Valley, internodal distance and petiole weight were measured and the number of leaves occurring at cane branches was counted.

Probability of PD Detection Based on Petiole Location

The probability of detecting PD from an individual petiole was greatest in basal portions of the cane (Figure 1). This result follows the suggestion of Hill and Purcell (1995) that the newest growth would not likely contain bacteria because of the incubation time required for spread. Our result is likely most applicable to chronic infections and this has been noted by others (Feil et al. 2003), but not presented by our method of examining infection on a node basis along the length of entire canes.

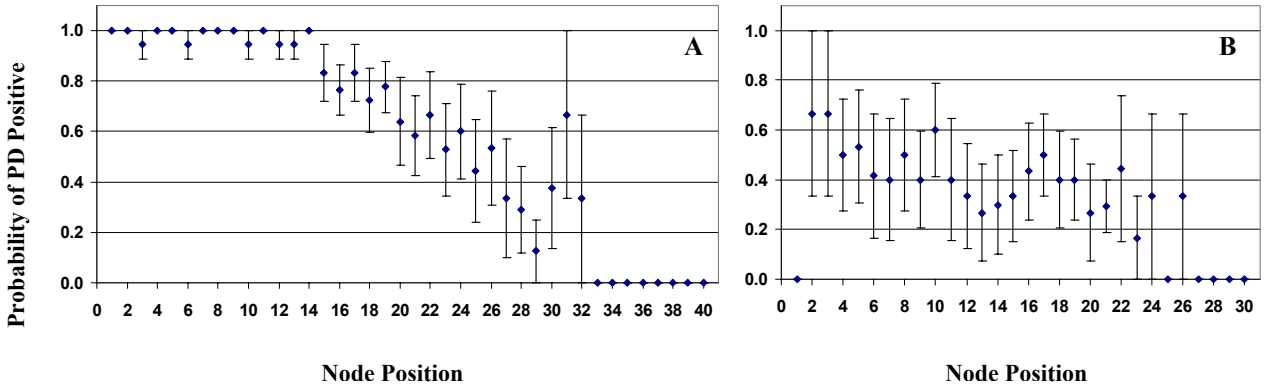

Figure 1. Probability (±SE) of positive PD detection at each node position (1 is most basal) for (A) Superior Seedless (n=6) vines and (B) Chardonnay vines (n=5).

Morphology of Healthy and PD-infected Vines

We did not detect any differences in Perlette (Λ=0.57; df=4, 11; P>0.05; MANOVA) or Superior Seedless (Λ=0.89; df=4, 11; P>0.05; MANOVA) varieties in internodal distance, petiole weight, petiole length, or number of leaves branching off of canes between healthy and infected canes. We measured these factors with the intent to identify a morphological feature that could aid in identifying infected vines, but no differences helpful for this purpose were found.

Effectiveness of PD Symptoms for Sampling

We photographed each leaf from each cane to evaluate the reliability of symptoms for use in identifying PD infected vines. We have begun to examine the visual symptoms in relation to PD infection and will use image analysis to quantify foliar symptoms. In preliminary observations, 9.5% of petioles from PD-infected vines were PD-positive and had asymptomatic leaves, and 16.1% of petioles were PD-negative, but had symptomatic leaves. Healthy vines had 16.7% of petioles with symptomatic leaves that were PD-negative. Generally, symptoms were more severe in basal portions of canes and the likelihood of finding an asymptomatic positive petiole was greater on distal portions of canes (Figure 2).

CONCLUSIONS

- Samples taken from basal portions of grapevine canes were more likely to yield an ELISA positive result. We believe this result applies primarily to chronically infected vines.
- We did not discover cane morphological differences between healthy and PD-infected vines that could be useful in detecting PD infected vines.
- We are in the process of evaluating the relationship between PD foliar symptoms and PD infection and have observed that the likelihood of a PD symptomatic leaf being negative for PD was greater than the likelihood of a PD asymptomatic leaf being positive for PD. Also, distal portions of canes were more likely to be asymptomatic when infected with PD.
- Based on the potential for choosing symptomatic leaves that are PD-negative, we suggest taking petiole samples for PD diagnostic tests from basal portions of grape canes to increase the likelihood of detecting PD positive vines.

IMPROVING DETECTION OF PIERCE'S DISEASE INFECTED GRAPEVINES

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ABSTRACT
Monitoring grapevines for Pierce's disease (PD) is an important component of disease management and epidemiology research. Currently, there are no guidelines for how to choose plant tissue from grapevines for detecting diseased vines. This study was initiated to develop criteria to increase the likelihood of detecting grapevines infected with PD. Grapevines naturally infected with PD were identified from vineyards in the Coachella Valley and Temecula, California. Grapevine canes were removed from three vineyards with three different grape varieties: Perlette, Superior Seedless, and Chardonnay. The probability of detecting a PD-positive cane was greater in petioles tested from basal portions of canes. No differences were found between healthy and PD-infected canes in internodal distance, petiole weight, petiole length, or the number of leaves occurring at branches on canes. In preliminary observations, 9.5% of petioles from PD-infected vines were PD-positive, but had asymptomatic leaves and 16.1% of petioles were PD-negative, but had symptomatic leaves. Healthy vines had 16.7% of petioles with symptomatic leaves that were PD-negative. Symptoms were more apparent on leaves from basal cane portions and asymptomatic PD-infected petioles were more common on distal cane portions. Image analysis to confirm these results is in progress.

INTRODUCTION
A major component of Pierce's disease (PD) research in California has been grapevine sampling to monitor PD incidence in vineyards. Identification of PD-infected vines is important for management and investigating disease epidemiology. University of California guidelines for management suggest removal of chronically infected vines to reduce the possibility of secondary disease spread and increase vineyard productivity by replanting with healthy vines (Varela et al. 2001). Relatively new programs in Kern County (Hashim et al. 2003) and the Coachella Valley (Perring et al. 2003) have been implemented to monitor PD in areas where it had been thought to be uncommon. Most PD monitoring programs have been based on preliminary identification of infected vines based on PD symptoms (Hashim et al. 2003, Perring et al. 2003). Unfortunately, PD symptoms can be similar to other grape diseases and nutrient deficiencies (Varela et al. 2001) and diseased vines may be asymptomatic early in disease progression. To definitively identify infected vines, plant tissue should be tested by a reliable diagnostic method such as culturing, enzyme-linked immunosorbent assay (ELISA), or polymerase chain reaction. Protocols for sampling to detect infected vines in vineyards are needed to reliably detect PD. A first step to preparing such a protocol is determination of the best approach for choosing plant tissue for diagnostic tests.

OBJECTIVES
1. Determine the probability of detecting a PD positive vine based on petiole location on individual grape canes.
2. Compare the morphology of healthy and PD-infected grape canes for potential differences that could aid in identifying infected vines.
3. Evaluate the effectiveness of using PD foliar symptoms for choosing plant tissue for diagnostic tests.

RESULTS
Naturally-infected grapevines with PD were identified from two vineyards in the Coachella Valley and one vineyard in Temecula. Varieties at the three respective locations were Perlette (3 vines), Superior Seedless (6 vines), and Chardonnay (5 vines). Three canes from each vine were removed. Each leaf from the canes was photographed, and intact individual petioles were weighed and tested for PD by ELISA. Additionally, in the Coachella Valley three canes were harvested from

undergraduate to assist. The data storage and web server is currently on order. I plan on presenting the plan for this database with PD investigators at the December conference.

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

SPATIAL DATABASE CREATION AND MAINTENANCE FOR PIERCE'S DISEASE AND GLASSY-WINGED SHARPSHOOTER IN CALIFORNIA

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INTRODUCTION

Whether tracking invasive species, assessing water quality, or monitoring the spread of disease, comprehensive data collection is a key component of scientific inquiry and sustainable natural resource management. Geographic Information Systems (GIS) allow us to unite in one structure spatially referenced data with other information, affording new insights in relationships between variables at multiple scales (original proposal contains full references), as well as assisting in collaborative efforts at natural resource management and multi-disciplinary problem solving. Such is the case with Pierce's Disease, where disparate datasets on PD location and GWSS trap data could, if available in a Geographical Information System (GIS) format with other spatially referenced data "layers" such as crops, hydrography, climate, and roads, aid in management of the disease, as well as in epidemiological research.

Several agencies and individuals have recognized the need for such a geospatial database for PD research and management. Indeed, the University of California Agriculture and Natural Resources "Report of the Pierce's Disease Research and Emergency Response Task Force(http://danr.ucop.edu/news/speeches/executivesummary.html) lists the following recommendations: Support is needed for a coordinated, statewide monitoring, trapping and reporting program involving governmental agencies, the agriculture and nursery industries and UC. The objective is to locate populations of GWSS and BGSS, track the incidence and distribution of Pierce's disease and carry out emergency response programs to slow the spread of PD and its vectors. CDFA or UC should manage a GIS to store, display, manipulate and overlay information collected by statewide monitoring and tracking programs. This data should be available to decision makers, growers and scientists.

We propose to develop a statewide database for PD and GWSS, maintaining the data with the best QA/QC methods, and full metadata (for data ownership tracking), maintained in a GIS format. We also propose to build a mechanism for researcher access to the database via the web, so that data can be downloaded for research purposes, and uploaded to the collection. We are not linking this effort with any analytical proposal, but aim to create the best possible, accessible database for others to use in research. These two components: (1) GIS database storage and maintenance and (2) Internet accessibility, when combined, are called "webGIS", and although not yet widely used in natural resource management, such systems are a promising option for entering and storing heterogeneous datasets, indexed by location, and making them widely available in a visual, dynamic, and interactive format. We use as our model the Sudden Oak Death monitoring project (please see the website at: http://kellylab.berkeley.edu/SODmonitoring) created by the Project Leader M. Kelly and housed at UC Berkeley.

The multi-scale data provide by the database structure described here, and specifically the access to the data, will contribute to finding a solution to PD by allowing researchers to use PD and GWSS data in concert with other spatial data "layers" such as climate, crops, and roads. In this way epidemiological hypotheses about distribution and spread at several scales – from vineyard to county to regional - can be formed. In addition, the data will aid in disease management, as researchers can see the spatial effect of different management options such as vine removal.

We are committed to collaborate with relevant researchers in this pursuit, and understand that there are already existing groups collecting such data. It is not our wish to supercede those efforts, but to lend our expertise to the data collection, storage, and distribution dynamic in support of Pierce's Disease science.

OBJECTIVES

The objectives and priorities for this project are as follows:

1. Create spatially referenced database of PD occurrence from field data;
2. Create spatially referenced database of GWSS trap data;
3. Maintain these data with other relevant spatial data for researchers use; and
4. Develop a web-based tool for researchers to submit data to the database, and for researchers to access existing data. Possibly, we will also develop a tool for the public to report presence of GWSS.

RESULTS

Funding for this project arrived at UC Berkeley on October 11, 2004, so we have no specific data analysis to report. I have a Staff Research Associate – Dave Shaari – who will work half time on this project, and I am in the process of locating an

experiments. We intend for the risk to be very small, and the knowledge gained to be of great benefit in the practical control of PD in the southern San Joaquin and elsewhere in California. We would be happy to work collaboratively with other researchers and cooperators on various aspects of this research.

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multiply to relatively high (easily detectable population sizes) before acquisition becomes efficient (4). Because it multiplies and spreads faster, we hypothesize that bacteria become available for acquisition in an infected grapevine of a susceptible variety earlier in the season than in a vine of a tolerant variety.

Putting these two parts of the hypothesis together can explain why the varietal differences in disease rate were observed. In the most susceptible varieties inoculations occurring later in the growing season can result in infections that survive the winter to become chronic. Because of the faster bacterial multiplication and spread there is still enough time in the growing season to reach a threshold for survival. At the same time, the bacteria multiply in previously infected vines fast enough to become available for acquisition by GWSS earlier in the season. The timing of these two processes results in an overlap, that is a window of opportunity when GWSS can acquire *Xf* from an infected vine, transmit the acquired bacteria to a new vine, and the new infection has enough time to progress to chronic infection and disease. That window of time would close during the seasen, but vine to vine transmissions would still be occurring. However those later season transmissions, after the window of opportunity has ended, would be cured over the winter. So vine to vine transmission occurring within the window would become chronic, and vine to vine transmission occurring after the window would be winter-cured.

Conversely in the tolerant varieties infections must occur earlier in the season in order to have enough time, at the slower rate of multiplication and spread, to progress to chronic disease. At the same time bacteria from previously infected vines also multiply and spread slowly and do not become available for vector acquisition until later in the season. The result is that there is no overlap, no window of opportunity where GWSS can acquire *Xf* from an infected vine, transmit to a new vine, and have the newly infected vine progress to chronic disease. In this case all of the vine to vine transmissions occur too late in the season, and the result is that all the vine to vine infections are cured over the winter.

One question is why do epidemics that are vectored by GWSS result in vine to vine disease spread in susceptible varieties whereas no vine to vine disease spread seems to occur when the traditional native California sharpshooter vector species are transmitting the bacterium? The answer may be related to the feeding and inoculation locations of GWSS vs. other vectors. The GWSS will feed (and therefore inoculate vines) at the base of the canes, but the native vectors all feed almost exclusively at the tip of the cane. Inoculations at the tip of the cane probably require more time to move to an over-wintering refuge, so an early season inoculation is necessary for the infection to survive the winter and become chronic disease. Thus the window for vine to vine transmission leading to chronic disease would not exist. In this case only the early season primary spread from sources outside the vineyard would result in chronic disease ,and because vine to vine transmission cannot begin until mid-season, these infections would be winter-cured.

If this hypothesis is correct, there are a number of possible consequences and conclusions that could improve PD management and control in areas where GWSS is present.

- The risk to growers of tolerant varieties is far less than has been previously assumed.
- There is a critical window of time somewhere in mid-season when susceptible vines need to be protected from vine to vine spread of PD. Chemical vineyard treatments early and late in the season, that is before and after this window, may be less effective than has previously been assumed.
- Economically important rates of secondary spread of PD may only happen in susceptible varieties and when large populations of GWSS are involved. Low but persistent populations of GWSS in Kern County do not appear to have resulted in appreciable losses from of vine to vine spread.
- Better targeted and timed chemical treatments could result in lower costs and be more compatible with other IPM programs.
- Late season vineyard surveys and rouging of infected vines is an important and cost effective management tool.
- The GWSS monitoring programs could be tailored to critical parts of the season, thereby possibly reducing the overall cost of these programs.
- The GWSS population treatment thresholds could be based on better epidemiological information, again possibly reducing overall PD management costs.

Because of the beneficial implications for PD management, it is important to experimentally test this hypothesis. We will be proposing to conduct experiments over the next two years to test the components of this hypothesis. The best experimental protocol would involve experiments conducted in two adjacent working vineyards, one tolerant and one susceptible variety. Ideally the experimental site would be in southern San Joaquin valley with climatological conditions representative of the viticulture areas of Kern or Tulare counties. One experiment would involve inoculations of both varieties vines at intervals throughout the growing season to establish the probability curves for the over-winter survival of *Xf* as a function of time of inoculation. The hypothesis predicts that the probability curves would be significantly different. Another experiment, for year two, would involve acquisition of *Xf* by GWSS at intervals throughout the season from vines of both varieties that were inoculated the previous year. This would establish the probability curves for the acquisition of *Xf* by GWSS as a function of time. The hypothesis predicts that these probability curves would also be significantly different. Other components of the experiments would look for differences between the varieties in the rate of multiplication and spread of *Xf* in the vines. Again the hypothesis would predict differences. It is critically important to everyone involved that these experiments do not create any new local PD problems or outbreaks. We have considered extensive safeguards in the design of these

RESULTS AND CONCLUSIONS

Vineyards were monitored by visually inspecting each vine for PD symptoms, and by collecting and testing (by ELISA) samples from symptomatic vines (2). Thus far in October 2004 all but 2 of the General Beale vineyards have been completed, but much of the other areas of Kern County are still in progress. The results thus far in the General Beale area indicate that the dramatic decrease in the number of infected vines is continuing. From 2002 to 2003 the number of infected vines decreased by 85%, and from 2003 to 2004 the decrease was an additional 68%. Following the survey of these vineyards in 2001 and 2002 the vines found to have confirmed *Xf* infections were removed. The continued decline of *Xf* infection in this area demonstrates that effective PD control can be obtained with a combination of GWSS control, monitoring for infected vines, and removal of infected vines. These projects have demonstrated that vineyard disease monitoring and vine removal is cost effective.

Throughout the county as part of this project vines found to be infected with *Xf* were removed at the end of that season. As a result the surveys in 2003 and 2004 are identifying vines that are newly infected. The rate of infection in all areas of Kern county outside the General Beale and Northern areas is very low, an overall rate throughout the county of less than one new infection per 10,000 vines. By contrast in the General Beale area some of the vineyards developed very high levels of disease within a 2 to 3 year period, peaking in 2002. Several vineyards were entirely lost.

Before the arrival of GWSS, primary spread of *Xf* from sources outside the vineyard accounted for most or all of the PD in California. The rates of new infections in Kern county may be the result of both primary spread and secondary spread, that is vine to vine spread. The low rates of new infections outside the epidemic area is consistent with primary spread, but the rapid rates of infection in many vineyards within the General Beale area is consistent with secondary, vine to vine spread. Perhaps the most startling epidemiological discovery of this project so far was that in 2002, 99% of the PD infected vines in the General Beale area were in Redglobe and Crimson vineyards, the 2 most susceptible of the 6 varieties surveyed. The following year, 2003, these same vineyards accounted for 97% of the diseased vines. These two varieties comprised only 18% of the acreage surveyed in the General Beale area. There were dramatic instances where Redglobe and Flame Seedless were growing in adjacent vineyards, and the susceptible Redglobe vineyards were heavily impacted or totally lost, whereas the more tolerant Flame Seedless vines growing just a few feet away were almost unaffected. The rate of infection in vineyards in General Beale of varieties other than Red Globe and Crimson in any of the three years was less than 14 infected vines out of 337,693 vines surveyed. In the worst epidemic area in Kern County the infection rate in varieties other than Redglobe and Crimson was essentially negligible. The Crimson loss in the General Beale area involved only one vineyard, and these vines were less than three years old. Younger vines are more susceptible to PD than older vines, and it is possible that the losses in the Crimson vineyard were primarily related to their more vulnerable age, rather than a varietal susceptibility. Older Crimson vines may not have been so heavily impacted.

We have developed a new hypothesis that would explain what might be causing this varietal difference. It is based on the timing of when in the season GWSS can acquire *Xf*, when in the season GWSS transmits *Xf* to new vines, and the phenomenon of over-winter curing of *Xf* infections. Over-winter curing of PD has been demonstrated to occur in many areas of California, including the San Joaquin Valley. Populations of *Xf* in grapevines are reduced during the winter dormant season. It has been experimentally demonstrated that if a vine is infected early in the season, the bacterium has enough time left in the growing season to multiply to high enough population levels and spread into areas of the vine where some of the bacterial cells find a refuge and can survive the winter dormancy. The vine then becomes chronically infected and usually eventually dies. Conversely, if a vine becomes infected later in the season, all the bacteria in the vine die over the winter, and the vine is free of disease the following year (1). Also pruning may play some role in over-winter curing. Vines that are inoculated late in the season when there is insufficient time for bacteria to move beyond the inoculated cane would, of course, lose the infection when that cane is pruned. However the bacteria in an un-pruned cane may die over-winter anyway.
Our new hypothesis is predicated on the finding that *Xf* multiplies and spreads faster within a susceptible plant than it does in a more tolerant plant (3). It would reasonably follow that the bacterium would also multiply and spread more rapidly in the more susceptible grapevine varieties of Redglobe or Crimson than it would in the more tolerant varieties such as Flame Seedless or Thompson. The first part of our hypothesis is about when in the season a grapevine must become inoculated in order for the bacterium to survive the first winter dormancy in the plant thereby progressing to chronic Pierce's disease. We hypothesize that the tolerant varieties have to become infected with *Xf* earlier in the season than susceptible varieties in order for the bacterium to have enough time left in the growing season to multiply and spread sufficiently in the vine to be able to survive the winter dormancy period. In general it has been demonstrated that vines must be inoculated before some critical time in the season if the bacterium is to survive the winter (1). However the existence of differences among varieties regarding that critical necessary time of inoculation has not yet been experimentally demonstrated.

The second part of our hypothesis is about when in the growing season the bacterial cells, having over-wintered in a previously infected plant, multiply and spread from their winter refuge into the new growth and achieve population numbers great enough to be efficiently acquired by an insect vector, in this case GWSS. This growth and movement of the bacterium following winter dormancy has to happen before vine to vine spread can begin to occur. It is not possible to detect *Xf* in the new growth of an infected plant until sometime about mid-season, and it ha been demonstrated that the bacterium must

EPIDEMIOLOGICAL ASSESSMENTS OF PIERCE'S DISEASE, AND MONITORING AND CONTROL MEASURES FOR PIERCE'S DISEASE IN KERN COUNTY

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ABSTRACT
Vineyards in the 7 grape production areas of Kern County's area wide management project were surveyed for PD again in 2004. Incidence of PD in the highly affected areas (General Beale and North) peaked in 2002, and declined dramatically in both 2003 and 2004. Treatments to reduce GWSS and to identify and remove PD infected vines each year were associated with these dramatic reductions. Survey and epidemiological data is being processed at CAMFER, a GIS-based research institute at U.C. Berkeley. More than 98% of the vines infected with *Xylella fastidiosa* in the recent epidemic in the General Beale area of Kern County were of the two most susceptible varieties: 6 Red Globe and 2 Crimson vineyards. Thirty-two other nearby or contiguous vineyards of four less susceptible varieties were almost unaffected. A hypothetical mechanism for this varietal difference is proposed.

INTRODUCTION
These two projects have complimentary objectives and methods, and were thus pursued and are being reported here cooperatively. This combination of people and resources has resulted in synergistic efficiency and maximum utilization of resources.

The cooperative area-wide pest management project for the control of GWSS has defined 7 distinct grape growing areas in Kern County. The PD epidemic that peaked in 2002 only affected two of these, the General Beale and the adjacent Northern area. These were also the only areas where the populations of GWSS exploded in 2000 and 2001 to extremely high populations not seen elsewhere in the county. Insect control measures begun in winter 2001-2002 brought the GWSS populations down dramatically. During this time the population dynamics and control methods for controlling GWSS were studied extensively with effective results. However our understanding of how to control the disease (goal of project 1) and the epidemiology of PD when the causal bacterium is transmitted by GWSS (goal of project 2) had been based on limited actual field data. These two projects began in 2002 as 5 year projects to obtain extensive data about the incidence and control of the disease. This disease information would compliment the insect information to enable understanding of the dynamics of the epidemic and methods to control other potential outbreaks. A total of 216 vineyards with 4060 acres and 2,015,698 vines were surveyed, about 4.6% of the vineyard acres in Kern County.
There have been two recent major California epidemics of PD that have been vectored by GWSS: General Beale in Kern County and Temecula in Riverside County. However data about each of these was not obtained until the epidemic was well underway or had already peaked. Because the other five viticulture areas of Kern County did not yet have such high numbers of GWSS, it was thought that disease and insect data from those would provide baseline information in the event that another epidemic such as the General Beale and Northern outbreak might occur, and such an epidemic could be studied from the beginning. Among the other 5 viticulture areas, 4 (Central, South A, South B, and West) have had low numbers of GWSS present since sometime before 2000, and GWSS was discovered in the 5th (Hwy 65-Delano) after 2000. Thus this extensive project to monitor the PD disease incidence in these areas was intended to provide both an understanding of the effect of low populations of GWSS on the incidence of PD, as well as a complete epidemic profile over time if another one should occur in this county.

OBJECTIVES
Project 1: Epidemiological assessments of Pierce's Disease. (BLH)
1. Evaluate the importance of epidemiological factors such as GWSS population size, vine age, cultivar susceptibility, control practices, and GWSS control treatments in vineyards and nearby GWSS hosts or habitat.
2. Make all the epidemiological data obtained available in a commonly acceptable GIS format for analysis by other qualified researchers and epidemiologists.

Project 2: Monitoring and Control Measures For Pierce's Disease In Kern County. (JH)
1. Determine changes in the incidence of PD over time in seven distinct grape-growing areas in Kern County.
2. Develop PD monitoring and management techniques and strategies for use by growers to reduce risk and damage. Update and provide educational materials to assist vineyard managers, pest control advisors, other researchers and government agencies involved in advising growers in the area-wide pest management of the GWSS project.

Table 2. The mean (±SD) ELISA readings and the percentages of *Hippodamia convergens* scoring positive for the presence of chicken egg white or non fat dry milk for up to 35 days after marking. *H. convergens* were scored positive for the presence of each marker if the ELISA value exceeded the mean negative control value by 3 standard deviations.

Application Method	Days After Marking	Egg White Marker			Non Fat Dry Milk Marker[1]		
		Number Assayed	Mean ELISA Reading	Percent Positive	Mean ELISA Reading	Number Positive	Percent Positive
Residual Contact	1	19	0.83 (0.3)	100.0			
	3	19	0.63 (0.2)	100.0			
	5	18	0.29 (0.1)	100.0			
	8	15	0.31 (0.2)	100.0			
	12	12	0.37 (0.3)	75.0			
	13	12	0.49 (0.2)	100.0			
	15	15	0.25 (0.2)	86.7			
	17	5	0.37 (0.2)	100.0			
	19	0	---	---			
	21	3	0.23 (0.2)	66.7			
	34	0	---	---			
	35	18	0.23 (0.3)	94.4			
	Negative Controls	63	0.04 (0.01)	0			
Topical	1	15	1.25 (0.2)	100.0	0.33 (0.1)	17	100.0
	3	26	0.96 (0.3)	100.0	0.34 (0.2)	27	100.0
	5	26	0.62 (0.3)	100.0	0.21 (0.1)	12	100.0
	8	18	0.75 (0.3)	100.0	0.25 (0.3)	2	100.0
	12	33	0.55 (0.3)	100.0	0.17 (0.1)	48	100.0
	13	17	0.23 (0.2)	100.0	0.26 (0.2)	17	100.0
	15	4	0.21 (0.3)	75.0	0.21 (0.2)	8	100.0
	17	20	0.33 (0.2)	100.0	0.25 (0.2)	2	100.0
	19	23	0.24 (0.2)	100.0	0.05 (0.1)	1	33.3
	21	4	0.35 (0.1)	100.0	0.20 (0.2)	20	90.9
	34	23	0.25 (0.1)	100.0	0.11 (0.1)	7	58.3
	35	8	0.27 (0.2)	100.0	---	---	---
	Negative Controls	39	0. 04 (0.01)	0	30	0.04	0.01

[1/]The retention of nonfat milk by contact application was not investigated for *H. convergens*.

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Table 1. The mean (±SD) ELISA readings and the percentages of protein-marked GWSS scoring positive for the presence of chicken egg white or non fat dry milk for up to 35 days after marking. GWSS were scored positive for the presence of each marker if the ELISA value exceeded the mean negative control value by 3 standard deviations.

Application Method	Days After Marking	Egg White Marker			Non Fat Dry Milk Marker		
		Number Assayed	Mean ELISA Reading	Percent Positive	Number Assayed	Mean ELISA Reading	Percent Positive
Residual Contact	1	31	0.49 (0.3)	100.0	8	0.38 (0.2)	100.0
	3	7	0.46 (0.4)	100.0	10	0.38 (0.2)	100.0
	5	19	0.94 (0.4)	100.0	4	0.43 (0.1)	100.0
	8	15	0.71 (0.3)	100.0	5	0.20 (0.1)	100.0
	12	26	0.57 (0.4)	88.5	36	0.36 (0.2)	100.0
	13	7	0.52 (0.3)	100.0	5	0.28 (0.3)	100.0
	15	26	0.31 (0.2)	100.0	6	0.27 (0.3)	83.3
	17	13	0.40 (0.2)	100.0	15	0.11 (0.1)	66.7
	19	13	0.17 (0.2)	76.9	5	0.11 (0.1)	40.0
	21	3	0.10 (0.1)	66.7	6	0.08 (0)	66.7
	34	0	---	---	3	0.06 (0)	33.3
	35	13	0.12 (0.1)	46.2	1	0.15 (NA)	100.0
	Negative Controls	25	0.05 (0.01)	0	20	0.04 (0.01)	0
Topical Contact	1	22	1.62 (0.1)	100.0	16	0.43 (0.1)	100.0
	3	12	1.26 (0.6)	100.0	20	0.40 (0.1)	100.0
	5	8	1.13 (0.5)	100.0	1	0.46 (NA)	100.0
	8	13	1.26 (0.4)	100.0	2	0.64 (0.1)	100.0
	12	16	1.23 (0.5)	100.0	8	0.45 (0.2)	100.0
	13	3	0.66 (0.2)	100.0	3	0.41 (0.2)	100.0
	15	3	0.30 (0.1)	100.0	0	---	---
	17	22	0.46 (0.3)	100.0	6	0.38 (0.3)	66.7
	19	7	0.34 (0.3)	100.0	2	0.40 (0.1)	100.0
	21	1	0.07 (NA)	100.0	1	0.04 (NA)	0.0
	34	7	0.16 (0.1)	57.1	10	0.19 (0.2)	80.0
	35	4	0.16 (0.2)	50.0	1	0.49 (0.3)	100.0
	Negative Controls	20	0.05 (0.01)	0	20	0.04 (0.01)	0

OBJECTIVES

The overall objectives of our research are to:

1. Quantify GWSS and natural enemy dispersal patterns in a complex landscape and
2. Determine which factors influence their dispersal. To accomplish these objectives we must first develop a mark-capture protein marking technique and quantify the protein marking retention intervals for the targeted insects. Field application of better mark-capture techniques will enhance our understanding of the area-wide dispersal patterns of GWSS and its natural enemies.

RESULTS

Direct Contact Marking Method

Dozens of nylon-meshed sleeve cages (66 X 70-cm, 18-cm dia.) were placed on randomly selected citrus branches located at the Agricultural Operations Research Station in Riverside, CA. Adult GWSS and *H. convergens* were then introduced into each cage and sprayed with a 5.0% solution of non-fat dry milk (NFDM) or chicken egg whites (All Whites™). A single cage from each marking treatment was randomly selected on 12 different sampling dates for up to 35 days after marking. All of the surviving GWSS and *H. convergens* in the randomly selected cages were assayed by an anti-NFDM or an anti-egg white ELISA to detect the presence of each respective protein mark.

Residual Contact Marking Method

Randomly selected citrus branches located at the Agricultural Operations Research Station in Riverside, CA were sprayed with a 5.0% solution of NFDM or chicken egg whites. The branches were allowed to dry for several hours, and then nylon-meshed sleeve cages were placed on the branches. Adult GWSS and *H. convergens* were then introduced into each cage. The sampling scheme was the same as the one described above. All of the surviving GWSSs and *H. convergens* in the randomly selected cages were assayed by an anti-NFDM or an anti-egg white ELISA to detect for the presence of each respective protein marker.

The ELISA results for the protein marked GWSS are given in Table 1. Data indicate that both marking procedures, regardless of the type of protein marker used, were retained well on GWSS. As expected, the topical marking procedure yielded higher ELISA values and had longer retention than the residual contact marking method. Generally, the markers were retained on 100% of the GWSS for ≈ 2 and 3 weeks by the residual and topical marking procedures, respectively. The ELISA results for the protein-marked *H. convergens* are given in Table 2. *H. convergens* ELISA reactions were very similar to the reactions yielded by GWSS.

CONCLUSIONS

In the first phase of our research described here, we showed that protein markers can be retained on insects several weeks after marking in the field. This marking technique provides the necessary tool to distinguish GWSS and its natural enemies so that studies of dispersal, migration, longevity, and density can be conducted. Additionally, different protein markers can be used to identify insects released at different times, in different areas, or in different crops. Next, we will use this technique to investigate the landscape-level movement of GWSS (nymphs and adults) and its natural enemies. We propose to use the mark-capture system to simultaneously quantify the intercrop dispersal of GWSS and its natural enemies. Specifically, we will spray large areas (e.g., field plots, whole trees, bushes, etc.) with inexpensive proteins using conventional spray equipment. In turn, insects that are hit by the protein solutions or that eat or walk on plant material containing protein residues will obtain enough protein to be detected by protein-specific ELISAs. Because the two marking ELISAs (chicken egg whites and NFDM) do not cross-react, we can apply the materials to two different host plants in close proximity to one another. Then, insects can be collected using temporal and spatial sampling schemes and analyzed for the presence of each respective protein marker to determine not only the insect's point of origin but the timing and extent to which portions of the population move among different plant species.

FUNDING AGENCIES

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QUANTIFYING LANDSCAPE-SCALE MOVEMENT PATTERNS OF GLASSY-WINGED SHARPSHOOTER AND ITS NATURAL ENEMIES USING A NOVEL MARK-CAPTURE TECHNIQUE

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ABSTRACT
Field cage studies were conducted to compare retention times between two inexpensive proteins, non fat dry milk (NFDM) and chicken egg whites, on glassy-wing sharpshooter (GWSS), *Homalodisca coagulata* and *Hippodamia convergens*. Each marker was applied to the insects by either directly spraying the insects with a conventional spraying device or by exposing the insects to pre-marked leaf tissue. Subsequently, the recaptured insects were analyzed by either an anti-NFDM or an anti-egg white enzyme-linked immunosorbent assay (ELISA) to detect the presence of each respective marker. Data indicate that both protein markers were retained well on both insect species, regardless of the application method. Generally, the topical marking procedure yielded higher ELISA values than the insects marked by contact exposure; however, both methods were sufficient for marking almost 100% of each population for > 2 weeks.

INTRODUCTION
Glassy-wing sharpshooter (GWSS), *Homalodisca coagulata* (Say) feeds on a variety of plants, and in the process transmits the bacterium, *Xylella fastidiosa*, which is the causal agent of Pierce's disease (PD) (Varela 2001). The spread of PD by GWSS now threatens the grape and ornamental industries of California. Due to the polyphagous feeding habit and high dispersal capability of GWSS, control of this pest will require an areawide management approach. Such an approach requires extensive knowledge of the host plant preferences and dispersal characteristics of GWSS and its natural enemies. Unfortunately, very little is known about the dispersal characteristics of GWSS (Blua & Morgan 2003, Blackmer *et al.* 2004) and its associated natural enemy complex. This is due, in part, to the lack of an effective technique for studying insect dispersal at the landscape level.

The first phase of our research plan consists of optimizing a mark-capture procedure for GWSS and its natural enemies that will facilitate future studies of intercrop dispersal. Historically, most studies of insect dispersal have relied on the mark-release-recapture (MRR) technique (Hagler & Jackson, 2001). Typically, mass-reared insects or insects collected *en masse* from the field are marked in the confines of the laboratory and then released at a specific site(s) in the field (i.e., at a central point). The insects are then recaptured using various spatial and temporal sampling schemes to quantify their movement. Unfortunately MRR studies use a relatively small portion of the population and recapture even a smaller proportion of the population (i.e., usually < 1.0%), thus making extrapolations about dispersal to the population level less reliable. The information gained from dispersal experiments could be significantly improved if a large proportion of the insect fauna (e.g., the simultaneous marking of GWSS and its natural enemies) could be marked directly in the field (e.g., mark-capture type experiments) and if several distinctive markers were available for studying intercrop movement of insects.

The development of a protein marking technique (Hagler 1997ab, Hagler & Jackson 1998, Blackmer et al., 2004) solved many of the problems associated with other marking techniques for MRR studies. The procedure is simple, sensitive, safe, rapid, inexpensive (for MRR type studies), invisible, and stable (Hagler & Jackson 1998). Moreover, several distinct proteins are available which facilitate the simultaneous marking of different cohorts of individuals (Hagler 1997a, Hagler & Naranjo 2004). We demonstrated that parasitoids (*Eretmocerus* spp. and *Encarsia formosa*) can be easily marked internally with vertebrate immunoglobulin (IgG) proteins by incorporating the various proteins into a honey diet or marked externally (*Trichogramma* sp.) with a fogging device (Hagler 1997b, Hagler *et al.* 2002). However, the major limitation of this technique is that the IgG proteins are too costly for mark-capture type studies. Recently, we discovered two inexpensive proteins that have potential as markers for mark-capture studies. The proteins are casein (from non-fat dry milk) and chicken egg whites (Egg Beaters™ or All Whites™). In collaboration with Vincent Jones we have developed anti-casein and anti-egg white enzyme-linked immunosorbent assays (ELISA) to each of these proteins. In turn, these ELISAs can be used to detect the presence of each protein on protein-marked insects. In this report, we investigated the feasibility of marking GWSS and *Hippodamia convergens* using two different application procedures. The first method for marking the insects consisted of spraying the markers on the insects in the field using a conventional hand sprayer (e.g., direct contact exposure). The second method for marking the insects consisted of exposing the insects to plant tissue that had previously been sprayed with each protein (e.g., residual contact exposure).

but with most positives falling in the 0.2—0.6 range (Figure 3). However, a few individuals proved to be highly positive for *Xf* with A_{490} readings >1.0, and in one case >2.4 (Figure 3).

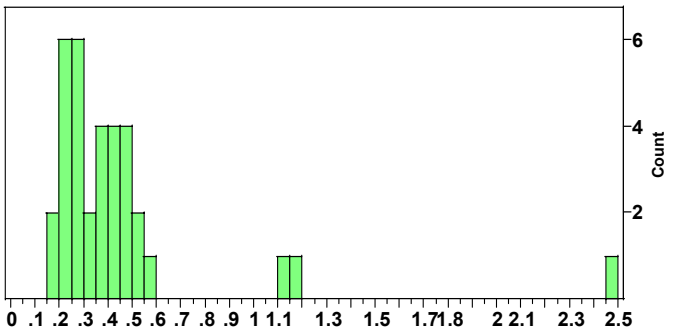

Figure 3. Histogram of Absorbance$_{490}$ readings of GWSS adults collected in Riverside between August and October 2004.

CONCLUSION

The data generated thus far is interesting from the standpoint of the large differences in the number of infected GWSS adults in Riverside compared to Redlands or Piru. As the new summer generation of adults ages, one would expect to find increasing proportions positive for *Xf* as they experience a greater diversity of host plants. This appears to be the case in the Riverside insects, but not for the insects from the other 2 locations. Ongoing collections will help to determine if the location difference is real.

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The almost complete absence of information regarding the degree of *Xf* incidence in GWSS populations has helped fuel much speculation about the future of the GWSS/PD crisis in California. In reality, there is very little that we understand regarding mechanisms of acquisition and inoculation of *Xf* by GWSS adults, either in the controlled conditions of the laboratory and greenhouse, or in the more challenging setting of their natural habitat. While the laboratory approach can provide essential answers to questions regarding the rate of acquisition and efficiency of transmission, it ultimately reflects the conditions imposed by the researcher. For example, the type and age of the acquisition source plant, the isolate of *Xf* used and period of time that the acquisition source plant has been infected, as well as the source of the experimental GWSS individuals and the conditions under which they are provided access to the *Xf* source plant are all variables controlled by the researcher. A dual approach that balances the findings from the laboratory with monitoring information from the field will improve our understanding of how epidemics of *Xf* occur in vineyards and elsewhere. A compilation of data from many sources has contributed to a good understanding of the distribution of GWSS populations within California and the relative intensities of regional infestations. What is now needed is to determine what proportion of individuals within these populations is infected with *Xf* while also identifying the factors that determine a given level of infectivity. I propose that the approaches and methods to be utilized will address a critical deficiency in our understanding of *Xf* epidemiology, i.e. the proportion of the vector population infected and infectious with the pathogen.

OBJECTIVES

1. Monitor GWSS adults from citrus and other sources year-round to determine the proportion positive for *X. fastidiosa* using ELISA, PCR, and media culturing techniques.
2. Perform transmission experiments on a portion of the field-collected adults using grapevine seedlings to determine the seasonal transmission rate.
3. Quantify the titer of *X. fastidiosa* in GWSS adults that transmitted *X. fastidiosa* to grape seedlings using quantitative ELISA and RT-PCR, and determine the relationship between transmission rate and titer in the vector.

RESULTS

As a new project that began July 2004, progress is being made on gathering the materials for carrying out transmission experiments and detection and quantification of *Xf* in field-collected GWSS. A propagation chamber has been assembled that will enable production of experimental grapevines having homogeneous genotypes to be used in the transmission studies. Lateral branch shoots consisting of 4-5 leaves are being cut from certified disease-free parental grapevines (var. Chardonnay) and placed in propagation media until roots are generated. These are transplanted to 4" pots and allowed a minimum of 3-4 weeks to establish before being used in transmission experiments. Ventilated corsage cages will enclose each grapevine plant and provide full access to the entire plants by GWSS adults. A single adult per plant will be confined 3 days for inoculation access followed by recovery and freezing (-80°C) for PCR and ELISA analysis, or for immediate plating to PD 3 media preceded by surface sterilization. An essential component of each of these approaches will be the availability of clean GWSS that are presently being reared. Experimental grapevines will be held a minimum of 2 months to allow for symptom development and then scored. Xylem fluid will be collected from each plant for ELISA/PCR analysis as an independent evaluation to compare with the visual assessments. Experimental and analytical results will be collated to determine which analytical procedure provides the closest agreement with transmission test results.

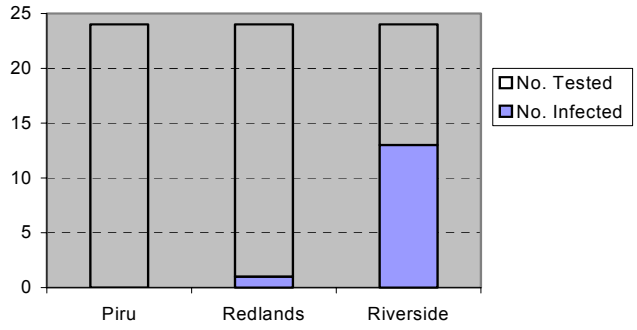

Figure 1. Number of infected GWSS adults from 3 locations collected early October 2004.

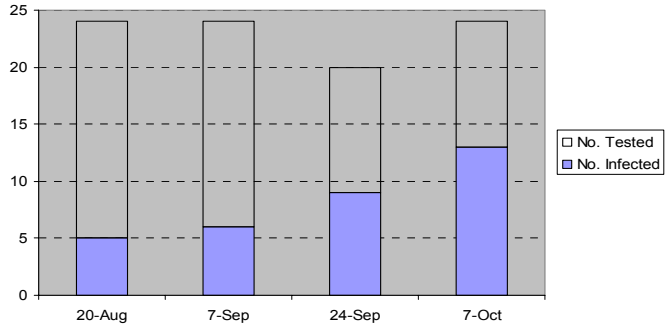

Figure 2. Number of infected GWSS adults out of the number tested for 4 collections from Riverside.

Field collections of GWSS adults that commenced in August 2004 have so far been made in Piru, Redlands, and Riverside. A sub-sample of 24 adults collected from each of these locations in early October 2004 was processed for ELISA detection of *Xf*. More than 50% of the Riverside adults were positive for *Xf* (= absorbance$_{490}$ values > A_{490} mean + 4 standard deviations for the GWSS clean control insects) compared to 4% for Redlands and 0 for Piru insects (Figure 1). A progressive increase in the number of *Xf*-positive insects (Figure 2) occurred between 20 August 2004 (5/24) and 7 October (13/24) in accordance with trends observed from previous years (Naranjo et al. 2003). The distribution of positive A_{490} readings was quite wide,

MONITORING THE SEASONAL INCIDENCE OF *XYLELLA FASTIDIOSA* IN GLASSY-WINGED SHARPSHOOTER POPULATIONS

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ABSTRACT
The seasonal incidence of *Xylella fastidiosa* in GWSS populations will be examined using a combination of analytical and experimental techniques. Collections of live GWSS adults will be made at various locations in southern California throughout the year at regular intervals. Live insects will be confined individually to grapevine plants (var. Chardonnay) to determine what proportion from the field transmit *Xf*. Following a 3 day inoculation access period, each test insect will be processed accordingly for detection of *Xf* by PCR, ELISA, and/or culturing techniques. By examining sufficient numbers of insects from the field and comparing transmission test results to analytical results, the relative efficiencies of each technique at identifying infected or infectious insects will be determined. Moreover, the seasonal occurrence of infectious insects will be determined and may provide guidance for when to be most vigilant for protecting against primary spread of *Xf* into vineyards.

INTRODUCTION
The rate of *Xylella fastidiosa* Wells transmission in the natural environment is a fundamental component of the epidemiology of *Xf*, but one that is thus far poorly defined. As a xylem-limited bacterial pathogen of plants, *Xf* is dependent upon xylophagous leafhoppers for movement from one host to another. The rate that such movement occurs is determined by a large number of factors and interactions among plant hosts, vectors, and bacterial pathogen within the context of variable environmental conditions. Although the inherent complexity of vector-borne diseases defies whole-system approaches to epidemiological studies, specific parameters can be studied towards an overall understanding of vector-borne epidemiology. In the case of *Xf*, the number of leafhoppers feeding upon *Xf*-infected plants, the proportion of those that attain *Xf* through feeding, and the proportion of those that visit and ultimately inoculate uninfected host plants plays a critical role in the spatial and temporal dynamics of Pierce's Disease (PD) and other *Xf*-caused diseases. By investigating the proportion of glassy-winged sharpshooters (GWSS, *Homalodisca coagulata* [Say]) in the natural environment infected with *Xf* (i.e. positive for presence of *Xf*) and determining the proportion of those that are infectious (i.e. positive for transmission of *Xf*) (Anderson 1981), greater understanding of the relationship between GWSS densities and *Xf* incidence in vineyards or other plant stands will be obtained. Measurement of GWSS infectivity and infectiousness may prove invaluable in addressing the issue of whether or not there is an upper threshold of GWSS numbers that can be tolerated in a given region.

Information already available indicates that GWSS is relatively inefficient as a vector of *Xf* in a laboratory setting (Almeida and Purcell 2003). However, large numbers of highly mobile vectors such as GWSS can easily make up the difference lost to poor transmission efficiency, especially if a large proportion in the natural environment is infectious with *Xf*. Regional control efforts made over the past few years in areas such as Temecula and the General Beale Road study area in Kern County have proven very effective at reducing local GWSS populations. However, the question of how many of the remaining GWSS in these regions are infectious is still unanswered. Until some measurement is completed of the proportion of GWSS populations that are infected, and more importantly infectious, our understanding of the relative risks posed by variable densities of GWSS throughout California will be limited. More importantly, policy decisions that process information on relative risks posed by GWSS infestations in particular regions will be compromised without data that describes what proportion of a GWSS population is actually causing new infections in a vineyard or in the urban landscape. Better epidemiological information will contribute to improved basic knowledge and understanding and to more sound policy.

The California grape industry remains at the greatest risk of *Xf* movement and transmission by reason of large acreages spread throughout the state and because of the severity of PD. Primary spread of *Xf* into a vineyard occurs when a cicadellid vector such as GWSS acquires the bacterium from a host outside and subsequently transmits to a grapevine within the vineyard. An infected grapevine can then serve (after an unknown latent period) as a source of secondary spread from infected to susceptible grapevines. Because so little is known about the movement of GWSS in the field and when they become infective with *Xf*, it is unknown whether most grapevine infections occur as a result of primary or secondary spread of *Xf*. What is certain, however, is that secondary spread will not occur until a primary infection has occurred, i.e. at least one grapevine has become infected with *Xf*. This is a critical event that poses a high level of risk to the vineyard because of the establishment of a *Xf* source within rather than outside of the vineyard. It is therefore important that all appropriate measures be undertaken to prevent that first critical infection. Towards this goal, it will be most helpful to know the temporal pattern of *Xf* incidence within GWSS populations so that maximum protection can be applied at the most vulnerable times.

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Table 3. Proportion of GWSS positive for *Xf* after outdoor exposure on a yellow sticky card.

Trial	Mean proportion of GWSS positive for *Xf* [a]		
	Day 0	Day 3	Day 6
1(n=49)	0.388a	0.429a	0.265a
2(n=30)	0.533a	0.333a	0.367a

[a]Means in the same row followed by the same letter were not statistically different (trial 1 χ^2=3.069, df=2, p=0.216, trial 2 χ^2= 2.845, df=2, p= 0.241)

CONCLUSIONS

Our study was conducted to find a means of accelerating a series of steps required to conduct epidemiological studies involving GWSS spread of *Xf,* while maintaining a high degree of detection sensitivity. Epidemiological studies require the examination of a large numbers of samples; therefore, an efficient testing protocol is necessary. Through our investigation, we improved the efficiency of *Xf* detection by streamlining DNA extraction and implementing a QRT PCR-based detection system. The vacuum method was simple, requiring only that heads be removed, pinned into position, and covered with extraction buffer. While time efficiency is the most obvious advantage to using the vacuum extraction method, other advantages also exist which did not impact the studies reported here but may affect detection in field samples. First, no insect tissue is homogenized; it is likely that fewer PCR inhibitors are released to interfere with the PCR reaction and less non-template DNA would be extracted. These factors often hinder detection of pathogen DNA in low concentrations. Second, by flushing the content of the insect's foregut the search for the presence of *Xf* is being concentrated in the area of the insect that will most likely contain the organism of interest. QRT-PCR is a sensitive detection technique that allows low concentrations of bacteria to be detected in environmental samples [13]. Our QRT-PCR detection system improved detect an order of magnitude, from 500 *Xf* cells (with traditional PCR[4]) to 50 *Xf* cells per insect sample. The implementation of such a system is well suited for the detection of pathogen DNA in an insect vector.

A disadvantage of using a molecular technique like PCR for the detection of a pathogen in a host is that detection is based on the presence of pathogen DNA. Unfortunately this does not necessarily mean that the pathogen was alive at the time of collection; the presence of DNA confirms the presence of the pathogen in the host. While other techniques, such as culturing [2], determine the presence of live cells, the sensitivity of such a technique is lower than molecular techniques. The 5-10 d growth period required to see *Xf* colonies on a nutrient agar plate allows time for contaminants to overgrow the plate. Although specialized media are often used for growth, confirmation of bacterial identity is still needed. While morphological and colony growth characteristics are often used, genetically based identification is more reliable and discriminatory.

The mean number of GWSS testing positive varied between trials and between experiments. This was most likely due to natural variation in the ability of GWSS to harbor *Xf* which may be a function of both the insect's age and its exposure to other biotic and abiotic factor that influence the ability of the bacterium to colonize the foregut of GWSS. This does not compromise our objective which was to develop a detection protocol that could be used regardless of field conditions.

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the slow release valve was opened and pressure was slowly returned to ambient. The vacuum application and release was repeated 3 times. In this way, the insect's forgut and mouthparts were flushed out with PBS. The pinned heads were removed and DNA was extracted from the fluid using the DNeasy Tissue kit (Qiagen Inc.). QRT PCR was conducted as described earlier.

To compare our vacuum extraction method to a more conventional maceration technique, heads from GWSS infected with *Xf*, as above, were either macerated in PBS buffer with a pellet pestle in a disposable 1.5mL microcentrifuge tube (Kontes Glass Company, Vineland, NJ) or vacuum extracted in PBS buffer. In further experiments insects were collected and immediately extracted (n=24) as previously described or stored at -4ºC for 10 d either submerged in mineral oil (n=24) or not (n=24). Finally, infectious GWSS were placed by hand on yellow sticky cards (Trécé Inc., Adair, OK). Yellow sticky cards were placed outside in a sunny location. GWSS were removed from the traps for DNA extraction at 0, 3, and 6 d after placement. DNA was extracted individually from GWSS heads using the vacuum technique and QRT-PCR was used for detection of *Xf*.

DNA Extraction

The vacuum extraction technique developed in this study improved the speed and efficiency of extraction. Extraction of DNA using traditional maceration with the Qiagen DNeasy tissue kit averaged 90 minutes for 24 samples. About 30-40 minutes of the extraction was preparing for and executing the maceration step of the procedure. Using the vacuum extraction technique we prepared 24 samples in an average of 15 min. The vacuum extraction technique neither improved nor compromised our ability to detect *Xf* in GWSS heads. No statistical differences were revealed between maceration-extracted and vacuum-extracted samples in any trial for either the number of positive samples or the relative amounts of *Xf* DNA measured (Table 1). However, in 5 of 6 trials mean positives and mean relative fluorescence levels were greater for macerated samples than vacuum-extracted samples (Table 1).

Table 1. Proportion of GWSS positive for *Xf,* and mean relative fluorescence using vacuum (VE) and maceration (MP) sample collection prior to DNA extraction (n=24).

Trial	Mean Positive[a]		Mean relative fluorescence[b]	
	VE	MP	VE	MP
1	0.458a	0.542a	1.137a	6.299a
2	0.464a	0.789a	1.728a	5.879a
3	1.000a	0.917a	0.112a	0.125a
4	0.917a	0.958a	0.001a	0.003a
5	0.750a	0.917a	0.009a	<0.001a
6	0.917a	0.792a	<0.001a	<0.001a

[a]Means in the same row followed by the same letter were not statistically different (χ^2>6.6, df=1 , $p > 0.359$).
[b]Relative fluorescence correlates to cell number. Means in the same row followed by the same letter were not statistically different (χ^2<3, df=1, p<0.01).

Comparison of Sample Storage Methods

On either collection date, there were no significant differences in mean number of GWSS testing positive for the presence of *Xf* that could be attributed to the method of storage following GWSS collection (trial 1 χ^2=1.626, df=2, p=0.443; trial 2 χ^2=2.4, df=2, p=0.3;) (Table 2).

Table 2. Comparison of *Xf* detection in GWSS following storage by three methods (n=24)

	Storage method (n=24)[a]		
Trial	Directly off Plant	-4ºC (10 d)	-4ºC in mineral oil (10 d)
1	0.875a	0.792a	0.917a
2	0.833a	0.750a	0.917a

[a]Means in the same row followed by the same letter were not statistically different (trial 1 χ^2=1.626 , df=2, p=0.443; trial 2 χ^2=2.4, df=2, p=0.3).

Detection Capabilities Following Insect Trapping

Exposure to the elements after capture on sticky cards had little effect on the ability to detect *Xf* in GWSS samples (Table. 3). Chi-square test for goodness of fit revealed no statistical differences among means from trial 1 (data taken 0, 3, and 6 days following capture, χ^2=3.069, df=2, p=0.216), or trial 2 (data taken 0, 3, and 6 days following capture, χ^2= 2.845, df=2, p= 0.241).

DEVELOPING A METHOD TO DETECT *XYLELLA FASTIDIOSA* IN THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT

A rapid and reproducible technique to detect *Xylella fastidiosa* (*Xf*) in the glassy-winged sharpshooter (GWSS) is important for epidemiological studies, and monitoring programs in support of Pierce's disease management. Such a technique must be amenable to large samples sizes, while remaining sensitive enough to detect pathogen DNA in low amounts. In this study we have improved the speed of tissue extraction by developing a simple vacuum step that replaces labor and time-intensive tissue maceration, and is compatible with manufactured DNA extraction kits and a SYBR Green® based real-time (QRT) PCR system. No statistical differences in the ability to detect *Xf* were found among samples that were extracted using traditional maceration vs. our vacuum extraction method. Further experiments using our vacuum extraction methods detected no significant differences among samples immediately extracted, or stored for 10 d at -4ºC, dry or in mineral oil. In another experiment we placed *Xf*-fed GWSS on yellow sticky cards in a sunny location for 0 to 6 d. We found that there was no significant reduction in our detection capabilities for insects left on the cards.

INTRODUCTION

Grapevines infected with *Xylella fastidiosa* (*Xf*), the bacterium that induces Pierce's disease of grapevine [12], usually die within three to five years after infection due to the occlusion of xylem vessels [17]. The glassy-winged sharpshooter (GWSS) has recently become an important vector of *Xf* in California, spreading *Xf* to grapevines that traditionally had little or no Pierce's disease [2, 17]. This vector can disperse widely [5], and has a large host range [18] resulting in alarming spread of *Xf* to new areas [11]. The presence of GWSS in new regions of California, greater incidences of *Xf*-induced diseases in several crops, including grapevine [15], almond [1], oleander [10], and the threat of citrus variegated chlorosis (not currently found in the US) has lead to great concern over the ecology of this pest/pathogen interaction.

Over the past several years control programs have focused on reducing pathogen spread by managing vector populations [18]. Improvements of these strategies can be achieved through studies examining patterns of disease epidemiology [15, 20], and GWSS population densities and dispersion [5, 11, 21]. Most epidemiological studies of this system have involved *Xf*'s interaction with host plants [3, 6, 15, 20] or the population and behavioral ecology of the pest insect [5, 11]. Investigations of the interactions between *Xf* and insect vectors have largely been limited to laboratory and greenhouse studies [2, 4, 10].

Molecular protocols, such as PCR, to detect *Xf* in plants have been developed and are currently being used in epidemiological studies in other disease systems [8, 9, 14, 16, 19, 20]. Unfortunately, methods adapted to detect *Xf* in insects are inefficient. Detection methods designed for epidemiological studies, from collection of insect specimen to analysis of samples for the presence of *Xf*, need to be rapid, reproducible, inexpensive, and amenable to large sample sizes. We recently developed a DNA extraction protocol using the DNeasy tissue extraction kit (Qiagen Inc.) in conjunction with a SYBR Green® based real-time (QRT) PCR system to detect *Xf* in infectious GWSS [4]. Using this protocol, we reliably detected 50-500 *Xf* cells with GWSS background. This method used labor-intensive maceration of tissue to extract *Xf* from insect tissue where the bacterium resides in infectious insects [7]. The speed and efficiency of this method could be improved by simplifying this extraction step.

OBJECTIVES

Our overall goal is to develop a method of detecting *Xf* in infectious GWSS that would allow us to conduct epidemiological studies and optimize plant protection. To this end, the objectives for this study are to develop an efficient method to remove *Xf* cells from the foregut and mouthparts of GWSS for PCR based detection.

RESULTS

In this study we tested a vacuum extraction protocol for removal of *Xf* cells from GWSS foreguts for detection by QRT PCR. GWSS adults, collected from orange trees at the University of California, Riverside, were placed in rearing cages and allowed to feed for a 6 d acquisition access period on cuttings of *Xf*-infected grapevines that showed Pierce's disease symptoms. GWSS heads were removed, and because they float, an insect pin was placed through the back of the insect head and forced through the frons, so that the tip of the pin protruded slightly. The pinned head was then placed in a microcentrifuge tube (one per tube) and 500µl phosphate buffered saline (PBS) was added to the tube so that the head was completely submerged. Tubes were loaded into a tube rack and placed in a glass vacuum desiccator. With the desiccator lid in place, vacuum was applied to 20 bars slowly, to keep buffer from being displaced from its tube, and held for 15 s. Then,

probing activities). In preliminary experiments, longer feeding durations did not influence the number of cells transmitted. Other data are too preliminary to present at this time.

CONCLUSIONS

We have the tools in place to determine transmission rates at the molecular level. Experiments are underway to determine the number of *Xf* cells that are transmitted under certain conditions. Until recently the molecular tools were not available to monitor the movement of single cells in the manner that QRT PCR allows. Almeida et al. [1]encountered difficulty in detecting levels of *Xf* in GWSS that can successfully inoculate a grapevine. That is, they found GWSS that were able to inoculate plants with *Xf* that did not test positive for the pathogen. The most reasonable explanation for these "false negatives" is that these GWSS harbored a titer of *Xf* that can cause infection in grapevines, but were below detection limits. Theoretically, one cell can cause a chronic infection; however, the probability is very low. We suspect the number of cells that are likely introduced into plants is greater than a single cell, but lower than the detection threshold of the method used by Almeida et al. [1], which is 10^2 cells. We need to embrace the molecular tools that are available to accomplish our objective.

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QUANTITATIVE ASPECTS OF THE TRANSMISSION OF *XYLELLA FASTIDIOSA* BY THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT

Transmission of *Xylella fastidiosa* (*Xf*) by the glassy-winged sharpshooters (GWSS) involves a series of events from acquisition of the bacterium to inoculation of *Xf* to a new host. While this process is often over-simplified, certain insect/pathogen interactions may be necessary to achieve a successful transmission event and the number of *Xf* cells acquired or inoculated may govern whether or not transmission will occur. In our preliminary studies, neither higher titers of *Xf* nor longer feeding periods by GWSS result in higher rates of transmission nor a greater number of bacteria transmitted.

INTRODUCTION

Solutions to Pierce's disease (PD) are coming out of an understanding of basic biological aspects of the vector, the pathogen, their hosts, and especially the interactions among these three divergent organisms that culminate in a disease epidemic. The most important of these interactions is the transmission of the pathogen by the vector to a non-infected plant. Transmission is a product of vector acquisition of the pathogen from an infected plant, and inoculation of the pathogen into a non-infected plant. It is a complex process involving sharpshooter host finding and feeding behaviors, and probabilities that a critical titer of bacterium will be acquired from an infected host by a feeding sharpshooter, and once acquired, will be inoculated into an uninfected host. In addition, for an inoculation event to lead to infection, a critical titer of bacterium must be inoculated into plant tissue that supports reproduction and movement.

Recent advancements in technology allow us to examine quantitative aspects of *Xf* transmission with high sensitivity, unlike traditional means. This includes two techniques we have mastered in our laboratories. First, we are currently using a quantitative real-time (QRT PCR) technique in conjunction with commercially available DNA extraction kits to detect and quantify low titers (currently ca 5 X 10^1 cells) of *Xf* in plant and insect tissue [2]. Second we have developed a low-cost method to rapidly extract DNA from GWSS and plant tissue in 96-well micro-titer plates.

Species of sharpshooters differ widely in their transmission efficiency, which ranges from a high of over 90% for the blue-green sharpshooter (*Graphocephala atropunctata*) to 1% for several others including *Oncometopia facialis, Acrogonia virescens,* and *Homalodisca ignorata* [3]. Recently, rates of *Xf* transmission efficiency for the GWSS from grapevine to grapevine were found to be as high as 20% [1]. These observations bring up two questions: First, what aspects of *Xf* transmission by sharpshooter vectors vary in ways that cause a wide range in efficiencies among vectors? Second, can we exploit an understanding of transmission efficiency to reduce PD spread? We seek to understand quantitative aspects of *Xf* transmission by GWSS. We are hopeful that this unique approach to investigating the transmission of an insect-vectored plant pathogen will lead to new tactics to manage disease spread.

OBJECTIVES

Our long-term goal is to understand quantitative aspects of the process of *Xylella fastidiosa* (*Xf*) transmission by *Homalodisca coagulata* (glassy-winged sharpshooter, GWSS) in order to develop a means of reducing the efficiency with which the pathogen is spread from an infected plant to a non-infected one. Our specific objectives for this project are to:

1. Determine relationship between the time a GWSS spends on a PD-infected grapevine and titer of *Xf* they acquire.
2. Determine the relationship between the time a GWSS spends in post-acquisition on a non- *Xf* host and titer of *Xf* they contain.
3. Determine the relationship between the time an infectious GWSS (ie, one that had acquired *Xf*) spends on a non-infected grapevine and the titer of *Xf* it inoculates into the grapevine.
4. Determine the relationship between the titer of *Xf* inoculated into a plant and the probability that it will become diseased.

RESULTS

Our preliminary laboratory experiments show that we can quantify the titer of *Xf* delivered to a stem by a single infectious GWSS immediately after a 24hr inoculation access period (IAP). In this experiment, field-collected GWSS adults were allowed to acquire *Xf* from grapevines showing Pierce's disease symptoms for a 72 hr acquisition access period (AAP). GWSS were then allowed access to cut chrysanthemum stems for 2, 4, 6, or 8 h. During this IAP, time lapse video was used to determine the amount of time GWSS feed on the stem and number of times the insect left the stem (indicating multiple

Section 4: Pathogen and Vector Monitoring and Action Thresholds

GENETIC VARIABILITY OF *XYLELLA FASTIDIOSA* STRAINS ISOLATED FROM TEXAS GRAPES AND OTHER PLANT RESERVOIRS

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ABSTRACT

Pierce's disease is a serious threat to the burgeoning Texas wine industry. Evaluation of the ecology and epidemiology of the disease in Texas may also be of significant scientific value for other areas of the country. We have begun a molecular biological evaluation of the genetic variability of *Xylella fastidiosa* (*Xf*) strains in Texas using small, established primers for creation of diagnostic banding patterns (REP, ERIC, and BOX primers). Cloning and sequencing of amplicons using RST31-33 primers resulted in little genetic difference between strains if one considers the error rate of *Taq* polymerase. However, priming with the small diagnostic primers resulted in differential banding patterns among *Xf* isolates across Texas. Based on these patterns, some vineyards had genetically distinct isolates and others genetically identical isolates. Vineyards may also contain more than one isolate. Analysis of *Xf* from a non-*Vitis* species showed a high distinct banding pattern suggesting broad genetic variability within Texas. Indirect immunofluorescence on *Xf* isolates also supports significant genetic variability within Texas, as there is differential antigen localization among several strains.

Thus, to remain alive each living bacterium in a sample must retain the plasmid to continue producing antidote. We will test the two different types of addiction modules that have been identified in bacteria. The first type of addiction system consists of a toxin that is encoded by a stable mRNA, but expression of the toxin is limited by the antidote, which is a small unstable antisense RNA molecule that blocks mRNA translation. The antisense mRNA antidote is produced as long as the plasmid is retained. Both the *hok/sok* system of plasmid R1 and the *pnd* locus of plasmid R483 utilize this mechanism of establishing addiction. Inclusion of the *hok/sok* system has been shown to successfully stabilize engineered plasmids in divergent species of bacteria including *Escherichia coli*, *Salmonella typhi*, *Pseudomonas putida*, and *Serratia marcescens* (3).

The second type of addition system consists of a stable protein toxin and an unstable antitoxin protein. Similar to the previous example, antitoxin is produced as long as the plasmid is retained. One of the best characterized of this type of addiction system is the *parDE* system from the broad-host range plasmid RK2 (also called RP4). Addition of a region of RK2, which includes the *parDE* system, to a poorly maintained plasmid has been shown to enhance stability of a wide range of bacteria such as *Alcaligenes eutrophus, Alcaligenes latus, Azotobacter chroococcum, Klebsiella pneumoniae, Pseudomonas aeruginosa, P. putida,* and *E. coli* (1, 9). Interestingly, placing more than one type of plasmid addiction module onto the same plasmid provides an additive effect on plasmid stability (6). Thus we will also evaluate whether placing the two different types of plasmid addition system leads to additional plasmid stability in *Xf*.

OBJECTIVES

1. Develop a stable plasmid vector for *Xf*.
   A. Evaluate the potential of various plasmid addiction systems for the ability to convert plasmids known to replicate in *Xf* into stable vectors.
   B. Evaluate how plasmid maintenance by *Xf* is affected by other genetic mechanisms known to affect plasmid stability, such as systems for multimer resolution and active partitioning systems.
2. Evaluate the stability of the newly developed plasmid vectors when propagated in *X. fastidiosa en planta*.

RESULTS

This report summarizes the goals of a new project focused on constructing a stable plasmid vector to aid genetically based studies of *Xylella fastidiosa*.

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PLASMID ADDICTION AS A NOVEL APPROACH TO DEVELOPING A STABLE PLASMID VECTOR FOR *XYLELLA FASTIDIOSA*

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INTRODUCTION

Current approaches to understanding the progression of Pierce's disease are limited by the lack of genetic techniques that can be used to study the biology of *Xylella fastidiosa* (*Xf*). In particular, extrachromosomal elements, such as plasmids, having long-term stability in *Xf* when grown in lab cultures or *en planta,* have not yet been satisfactorily developed. We will develop vectors that exhibit stable maintenance by *Xf* by adapting previously described genetic and microbiological techniques. Our particular research efforts will focus on taking advantage of a well-studied bacteriological phenomenon called plasmid addiction (2, 4, 10). The major mechanistic principle of plasmid addiction is that the plasmid carries a genetic trait that the host bacterium requires for viability. The trait does not affect the metabolic properties of the bacterium nor does it affect reproduction. However, loss of the plasmid-encoded trait is a lethal event, so by definition plasmid addiction ensures vector stability. In addition, we will systematically evaluate other genetic mechanisms for increasing plasmid stability including multimer resolution and active partitioning systems. Finally, we will examine the stability of each of the newly developed vectors for *Xf in vitro* and *en planta*. The results of this analysis will allow us to construct one or more stable plasmid vectors that can be used by all researchers using genetic approaches to develop methods that limit *Xf*-related diseases.

Xylella fastidiosa is a Gram-negative, endophytic bacterium, which is responsible for a number of economically important plant diseases (for recent reviews, see (5, 7, 8)). Diseases that are important to the California agricultural economy include Pierce's disease of grapevine, almond leaf scorch, alfalfa dwarf, and oleander leaf scorch. Some strains of *Xf*, such as the Pierce's disease strains, have very wide host ranges and are capable of colonizing the xylem of widely divergent plant species. In many plant species, infection by *Xf* does not provoke symptoms or noticeable distress. However, the colonization of certain plants, such as grapevines, leads to the development of disease symptoms and of plant decline. Although the specific details of the disease process are not fully understood, it is known that *Xf* forms a biofilm within xylem vessels that has a major impact on the movement of sap within the xylem tissue. Disease symptoms seem to be dependent on the rate and extent of colonization of the xylem tissue by *Xf*. Some of the symptoms observed in infected grapevines include leaf marginal necrosis, severe leaf scorch, and dieback.

Another important aspect of the disease cycle involves the insect vector. *Xf* is transmitted from plant to plant by xylem-feeding insects including the glassy-winged sharpshooter (5, 7, 8). The insect vectors acquire the bacterium by feeding on infected plants. Since the Pierce's disease strain can colonize numerous plant species, the source of inoculum can be infected grapevines or symptomless plants present in the riparian habitats surrounding the vineyard. In vectors showing the highest transmission efficiencies, *Xf* is present as a polar biofilm in the insect foregut and is transmitted to uninfected plants during subsequent feeding events. In susceptible plants, efficient transmission of *Xf* occurs at low bacterial cell numbers (<100 cultivable cells per insect head).

Thus, an important feature of the *Xf* infectious cycle is the ability of this pathogen to colonize and interact with the xylem tissue of plants and the foregut of insect vectors. Successful colonization of these hosts is dependent on the ability of *Xf* to subvert host defense networks and to acquire essential nutrients. To better understand how *Xf* survives in and interacts with its hosts, many research laboratories have been working to identify genes important for virulence and nutrient acquisition. However, rapid progress in this area is affected by the lack of genetic and molecular tools necessary to investigate the contribution of *Xf* genes to the infection process. One extremely important tool that is needed to advance these studies is a plasmid that is maintained by *Xf* throughout the infectious cycle. The goal of our project is to develop this type of plasmid. Plasmid-addiction systems consist of a pair of genes that specify two components: a stable toxin and an unstable antidote (for recent reviews, see (2, 4, 10). When a bacterium looses the plasmid harboring one of these addiction systems, the cured cells loose the ability to produce the unstable antidote and, as a result, the lethal effect of the stable toxin kills the bacterium.

several species; however, additional studies must be completed to further elucidate the role of this pathogen in causing widespread disease in the urban setting as well on crops of agronomic importance in California.

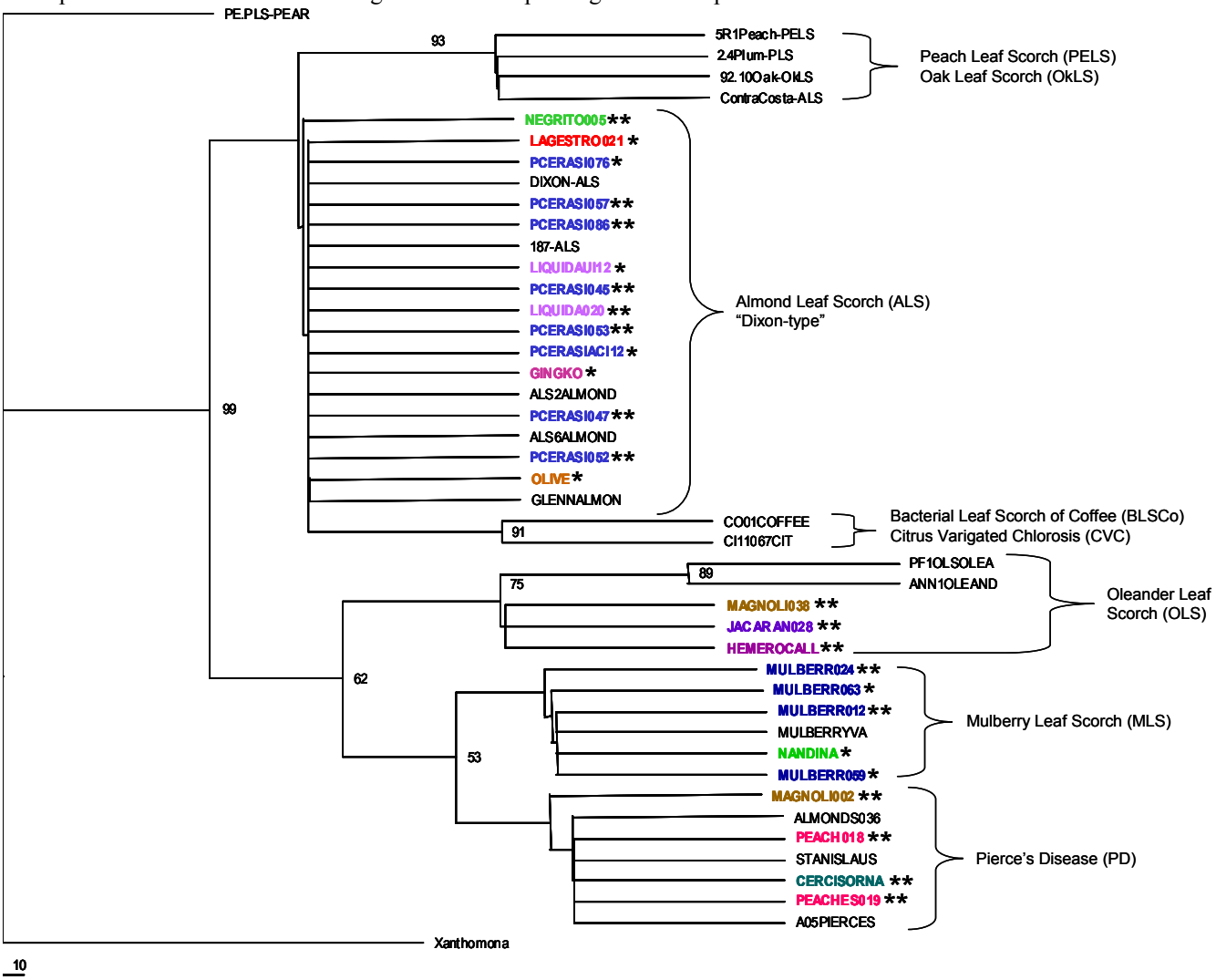

Figure 1. Preliminary phylogenetic tree constructed using the neighbor joining method, based on 16S rDNA sequence data for *Xylella fastidiosa* with the sequence of *Xanthomonas vesicatoria* (AF203392) as the outgroup. The numbers above the branches represent bootstrap values obtained for 100 replications. * Indicates isolates collected in 2003, ** indicates isolates collected in 2004.

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Magnolia002 showed more identity (99.41%) to PD strains. For isolates from Hemerocallis and Jacaranda, they showed 100% identity between them and showed to be more closely related to oleander strains (99.22%)
Gingko, olive, liquidambar and some ornamental plum strains showed to be closely related to the Dixon almond leaf scorch strain (100% identity). Some ornamental plum strains showed divergence amongst them (97.86% identity) and from ginkgo, olive and liquidambar, but all of them grouped together with the Dixon strain. Lastly, the strain isolated from a yet to be identified host (nicknamed "*negrito"*) showed slight differences from the ornamental plum, liquidambar and olive isolates. None of the isolates grouped with plum leaf scald, phony peach, oak leaf scorch group or with citrus variegated chlorosis and coffee leaf scorch strains.

Table 2. *Xf* isolates collected in 2004 surveys.

Host Scientific name	Common Name	Isolate designation
Cercis occidentalis	Western Redbud	Cercis050
Hemerocallis	Day Lily	Hemerocallis034
Jacaranda mimosifolia	Jacaranda	Jacaranda028
Liquidambar styraciflua	Liquidambar	Liquidambar020
Magnolia grandiflora	Magnolia	Magnolia038
Magnolia grandiflora	Magnolia	Magnolia 002
Morus alba	White Mulberry	Morus012
Morus alba	White Mulberry	Morus024
Nerium oleander	Oleander	Oleander031
Nerium oleander	Oleander	Oleander028
Prunus cerasifera	Ornamental Plum	Pcerasifera057
Prunus cerasifera	Ornamental Plum	Pcerasifera086
Prunus cerasifera	Ornamental Plum	Pcerasifera047
Prunus cerasifera	Ornamental Plum	Pcerasifera052
Prunus cerasifera	Ornamental Plum	Pcerasifera053
Prunus dulcis	Almond	Almond036
Prunus persica	Peach	Peach018
Prunus persica	Peach	Peach.019
Unknown species	'negrito'	Negrito005
Vitis labrusca 'Concord'	Grape	Grape153
Vitis labrusca 'Concord'	Grape	Grape154
Vitis vinifera 'Red Flame'	Grape	Grape155
Vitis vinifera 'Red Flame'	Grape	Grape156
Vitis vinifera 'Thompson Seedless'	Grape	Grape149
Vitis vinifera 'Thompson Seedless'	Grape	Grape150
Vitis vinifera 'Thompson Seedless'	Grape	Grape151
Vitis vinifera 'Thompson Seedless'	Grape	Grape152

Objectives 4 and 5

Eight characterized strains of *Xf* collected from the landscape in 2003, plus an oleander and a grape strain, were inoculated into their host plants of origin in glasshouse assays. Strains used were Almond276, Ginkgo, Lagestroemia02 (crape myrtle), LiquidambarUI12 (liquidambar), Morus069 (mulberry), Nandina065, Olive AC12 , Pcerasifera076 (ornamental plum), Riverside3 (oleander), GrapeA05. These same eight strains were also used to inoculate grapevine and oleander. Briefly, isolates were grown on PW media for two weeks from which a suspension of 1 x 10^9 CFU in sterile phosphate buffer was obtained. Plants were needle inoculated on three to four sites per plant using the needle-stab technique described by Hill and Purcell (1995). Approximately 25 plants were used for the inoculation studies. All plants were tested by ELISA prior to inoculation to ensure that they were *Xf* free. Starting approximately three months after inoculation, plants were ELISA tested and attempts were made to isolate the pathogen from positive plants. *Xf* cultures have been obtained from some hosts testing positive by ELISA and have been confirmed as *Xf* by PCR, namely those from mulberry inoculated with the Morus069 isolate. Isolation and characterization studies from these test inoculations are currently underway for the rest of the test plants and *Xf* isolates.

CONCLUSIONS

The results of the study do indicate that there are a number of landscape hosts that are harboring different strains of *Xf* in southern California. Of the new isolates characterized, it appears that new hosts have been identified for a number of strain groups: Pierce's disease (magnolia, peach, western redbud), oleander leaf scorch (magnolia, jacaranda, day lily), mulberry leaf scorch (heavenly bamboo), and almond leaf scorch (ornamental plum, crape myrtle, liquidambar, gingko, olive). Inoculation tests appear to have confirmed the role of *Xf* in causing mulberry leaf scorch in California, while other tests await completion. It does appear that new methodologies will have to be developed to successfully obtain or test for *Xf* in a number of ornamental plant species. The role of *Xf* infections in landscape hosts does appear to have a significant impact on

pathogen from these positive samples yielded only a small number of isolates (see next section). PCR testing (Minsavage 1994) was performed on a subset of the samples collected using a modification of the published methodology. Briefly, petioles and midveins from leaves were chopped in sterile water, tissues were allowed to sit in the water for several minutes to allow for the release of *Xf* from the tissues and then DNA extracted from the water. Results were greatly improved using this method, and *Xf* was detected in 23 species tested (Table 1). PCR testing of additional species testing positive by ELISA is continuing on species from which isolates could not be obtained.

Table 1. ELISA, isolation and PCR results for 23 of 122 species tested for *Xf*.

Plant Name	Common Name	#Tested	#ELISA(+)[a]	Culture(+)[b]	PCR(+)[c]
Albizia julibrissin	Silk Tree	6	5		yes
Cercis occidentalis	Western Redbud	4	3	yes	yes
Ginkgo biloba	Maidenhair Tree	15	6	yes	yes
Hemerocallis	Day Lily	9	5	yes	yes
Jacaranda mimosifolia	Jacaranda	49	24	yes	yes
Juglans	Walnut	2	2	no	yes
Lagerstroemia indica	Crape Myrtle	17	5	yes	yes
Lavandula dentata	Lavender	4	4	no	yes
Ligustrum lucidum	Glossy Privet	7	5	no	yes
Liquidambar styraciflua	Liquidambar	19	7	yes	yes
Magnolia grandiflora	Southern Magnolia	31	18	yes	yes
Morus alba	White Mulberry	3	2	yes	yes
Nandina domestica	Heavenly Bamboo	20	3	yes	yes
Nerium oleander	Oleander	3	3	yes	yes
Olea europaea	Olive	6	5	yes	yes
Phoenix reclinata	Senegal Date Palm	2	2	no	yes
Prunus cerasifera	Ornamental Plum	12	7	yes	yes
Prunus dulcis	Almond	3	3	yes	yes
Prunus persica	Peach	5	2	yes	yes
Rosmarinus officinalis	Rosemary	13	8	no	yes
Vitis labrusca 'Concord'	Concord Grape	2	2	yes	yes
Vitis vinifera 'Red Flame'	Red Flame Grape	2	2	yes	yes
Vitis vinifera 'Thompson Seedless'	Thompson Seedless Grape	5	5	yes	yes

[a] denotes number of samples testing positive using a commercial *Xf*-specific ELISA kit
[b] denotes if an *Xf* isolate was successfully obtained from at least one sample
[c] denotes if PCR-amplification using RST31/33 primers from plant tissue was successful for at least one sample

Objective 2

Twenty-seven isolates (from 13 host species) were obtained from samples testing positive by ELISA (Table 2). Isolation of the pathogen from samples, even those testing strongly positive from ELISA, was not always possible. Briefly, samples were washed in soapy water, soaked for 1 min in 70% ethanol, 1 min in 20% bleach, then triple rinsed in sdH_2O. Samples were then sliced into 1-2 mm pieces and soaked in PBS. Fifty microliters of the PBS buffer was then plated onto PW media with or without the addition of 25 ppm of cycloheximide. The failure to obtain isolates from all samples testing positive by ELISA suggests that specific methodologies need to be determined for specific tissue types from different hosts as a general isolation protocol may be inadvertently killing the pathogen, the pathogen may be highly irregularly distributed in host tissues, or the commercially available ELISA kit may be generating a high number of false positives due to non-specific interactions with host tissue.

Objective 3

Collected isolates were confirmed as being *Xf* by extraction of the DNA from the cultures using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA) and subsequent PCR amplification with the RST31/33 primer pair. Isolates were further characterized by amplification and sequencing of the 16S-23S ribosomal DNA intergenic spacer region as described by Hendson et al. 2001. All the 16S-23S rDNA sequences were aligned using the clustalX program (Thompson *et al*., 1994) and their relationship was analyzed with the PHYLIP program (Felsenstein, 1995) with the sequence of the *Xanthomonas vesicatoria* (AY288080) as an outlying group (Figure 1).

Two strains isolated from mulberry (Morus024 and Morus012) showed 99.41% identity with the previously reported mulberry-VA strain from the eastern U.S. (Huang and Sherald, 2004), while Nandina065, Morus059 and Morus063 showed a 100% of identity with the same strain. For the two peach isolates, Peach018 showed 100% identity with previously reported Pierce's disease strains (AO5) while Peach018 showed a little less identity (99.41%), but both grouped with PD strains. The Cercis050 strain also grouped with PD strains (99.61% identity). Strains isolated from Magnolia showed just 98.44 % identity between them. Since Magnolia038 was more closely related to Oleander leaf scorch (OLS) (99.02% identity) while

DOCUMENTATION AND CHARACTERIZATION OF *XYLELLA FASTIDIOSA* STRAINS IN LANDSCAPE HOSTS

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Reporting Period: The results reported here are from work conducted from October 15, 2003 to October 12, 2004.

ABSTRACT

To better understand the impact of *Xylella fastidiosa* on the urban environment and the potential for ornamental hosts to serve as reservoirs for agronomically important diseases caused by the bacteria, a survey project was initiated to document and characterize strains of the bacteria harbored in landscape plants. Targeted sampling of 122 landscape species either symptomatic for bacterial scorch or testing positive for *X. fastidiosa* by ELISA in 2003 was performed. Of the 830 samples, 321 tested positive by ELISA (representing 77 of the 122 species tested). *X. fastidiosa* was also detected in 23 species by PCR-amplification using *X. fastidiosa* specific primers. Twenty-seven isolates from 13 host species were obtained from samples testing positive by ELISA. Isolates from plants not previously reported as hosts in southern California urban environments included mulberry, heavenly bamboo, magnolia, day lily, western redbud, jacaranda and peach. Genetic characterization of these isolates by 16S-23S rDNA sequencing distributed these isolates amongst previously characterized strain groups: almond leaf scorch (crape myrtle, ornamental plum, liquidambar, gingko, olive), Pierce's disease (magnolia, peach, western redbud), mulberry leaf scorch (mulberry, heavenly bamboo), and oleander leaf scorch (magnolia, jacaranda, day lily). The role of some *X. fastidiosa* strains in their ability to cause disease is presently being tested by fulfilling Koch's postulates in glasshouse experiments. The data collected from this study strongly suggest that *X. fastidiosa* is causing a number of scorch diseases in the urban landscape, and that strains of agronomic importance may be harbored in this environment.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a xylem-limited, insect-vectored, plant pathogen that can cause severe damage to a wide range of host plants. Diseases caused by this pathogen include Pierce's disease of grapevine (PD), oleander leaf scorch (OLS) and almond leaf scorch (ALS). In 2003, a survey of landscape plants in five urban locations in southern California was initiated to document the incidence of the *Xf* infection in landscape ornamental hosts and to characterize strains existing in these hosts that may prove a threat to landscape ornamentals or crops of agronomic importance. Two hundred twenty one samples (29%) representing 48 species tested positive by ELISA. Ten isolates of *Xf* were obtained from eight plant species (*Fatsia japonica, Ginkgo biloba*, *Lagerstroemia indica*, *Liquidambar styraciflua*, *Morus alba*, *Nandina domestica*, *Olea europea*, and *Prunus cerasfiera*) not previously described as hosts of *X. fastidiosa* in southern California.

Based upon the results of the first year, targeted sampling of host species testing positive by ELISA was performed primarily in the Riverside and Redlands areas in order to obtain additional isolates for characterization. To prove the role of *Xf* in causing disease in previously identified hosts, test plants were inoculated in glasshouse experiments to fulfill Koch's postulates for these isolates, and to determine if they were able to cause disease in grapevine and oleander.

OBJECTIVES

1. Use laboratory methods to identify landscape host species that are infected with *X. fastidiosa.*
2. Secure isolates from these hosts to document infection and provide material for genetic characterization of the *X. fastidiosa* strain(s) involved.
3. Genetically characterize the strains of pathogen in landscape plant species.
4. Confirm pathogenic infection through inoculation studies with specific isolates.
5. Test ability of new strains to infect agricultural crops including grape, olive, and almond.

RESULTS

Objective 1

In 2004, 830 samples from 122 landscape plant species were collected. Sampling focused on plant species that were symptomatic or had tested positive by ELISA in 2003 surveys. Three hundred twenty one samples (39%), tested positive by ELISA. At least one sample from 77 of the 122 species tested was positive by ELISA (63%). Attempts to isolate the

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FUNDING AGENCIES
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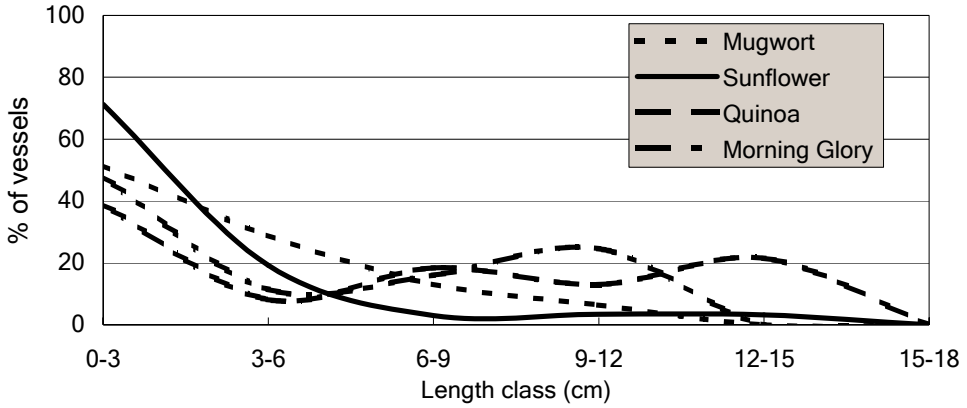

Figure 2: Vessel length distribution in greenhouse-grown annual morning glory, mugwort, quinoa and sunflower.

Blue-green sharpshooters failed to efficiently inoculate *Xf* into both grapes and alternate hosts in three separate attempts from 7/03 to 4/04; only one of 44 grapes became infected. Though the *Xf*-infected source plants had fully developed symptoms and were positive for *Xf* by culture, there may have been nutritional or physiological factors that prevented them from being good sources of bacterial acquisition. We are mechanically inoculating alternate hosts and grapes to generate GFP-*Xf* infected plants for microscopy practice. Because the distribution of *Xf* in an insect-inoculated stem is likely different from a mechanically inoculated stem, we still plan to use insect-inoculated plants when we compare sharpshooter acquisition and bacterial distribution in alternate host stems. Ongoing work focuses on refining microscopic techniques to visualize small numbers of *Xf* in alternate host stems, and generating large numbers of *Xf*-infected grapevines to serve as new sources for sharpshooter bacterial acquisition.

CONCLUSIONS

Three things are required for the development of Pierce's disease in grape: the pathogen *Xylella*, a sharpshooter insect vector, and a susceptible plant host. We are systematically examining the interactions between plants and the pathogen, and the role that host resistance plays in the ability of the vector to acquire *Xf* and spread Pierce's disease. The only significant difference between grape varieties was that tolerant 'Sylvaner' had approximately 20% more rays per stem compared with susceptible 'Cabernet Sauvignon' or 'Pinot Noir'. In grapes, rays are composed of dense parenchyma cells, without tracheids or vessels, and separate the water-conducting xylem into longitudinal zones (3). Perhaps this limits the lateral spread of *Xf* to the zone it is originally inoculated into. While additional work is needed, the vessels of other hosts were approximately 75% shorter than vessels of grapes, limiting the passive spread of *Xf* via xylem sap movement, and are found in bundles separated by parenchyma cells, which may also limit the lateral spread of *Xf*. Additionally, it is likely *Xf* movement between bordered pits is an active process (10); anatomical and biochemical differences in pit structure may explain differences between cultivar susceptibility to *Xf*.

In grapes, electron and confocal microscopy showed *Xf* densely packed in individual vessels, with adjacent vessels empty or containing a few cells (10, 15). Alternate hosts or tolerant grape cultivars with low overall populations may have just a few vessels with bacteria, so acquisition would be highly variable and dependant upon sharpshooters encountering the few colonized vessels while feeding. In symptomatic grape petioles, 13% of vessels were colonized to some extent with GFP-*Xf,* though only 2.1% of all vessels were completely blocked with bacteria (10). Though is it not known how many probes a sharpshooter makes in a given feeding session, glassy-winged sharpshooters can generate multiple salivary sheaths in one insertion, adjacent to vessels and xylem parenchyma cells (6). Sharpshooter acquisition of *Xf* increased along with bacterial populations in infected grapes (9), and a similar positive relationship is expected if the proportion if colonized vessels increases insect acquisition of *Xylella*.

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We will measure *Xf* acquisition by sharpshooters from the alternate hosts and grape cultivars after completing the anatomical comparisons. Insects will be caged on *Xf* inoculated sites for 4 days to acquire the bacteria, and then be placed on another grape seedling for 2 days to determine their acquisition efficiency. Immediately following sharpshooter feeding, the stem site will be examined with confocal microscopy and tested with culture. Three stem cross-sections and three 1-cm long longitudinal sections per site will be sectioned and suspended in 50% glycerol on a depression slide. When illuminated with blue and ultraviolet light, both GFP-*Xf* and the individual vessels are visible, and it is possible to determine the proportion of vessels colonized, the extent of bacterial colonization inside them, and the distribution of colonized bundles. Bacterial populations will be determined by culture from remaining plant material of the same site, and symptom development and severity will be assessed. Since acquisition efficiency has been related to bacterial populations (9), we must separate the effects of bacterial distribution and proportion of colonized vessels from the effect of bacterial population. The number of plants we can evaluate via microscopy is a limiting factor. A maximum of 90 observations per experiment will allow examination of 5 inoculation sites for each of three species or cultivars, which should enable detection of a 20% difference in *Xf* colonization ($\alpha = 0.05$ and $\beta = 0.10$) (14).

OBJECTIVES

1. Describe the bacterial colonization of asymptomatic weed species and grape varieties of varying tolerance to Pierce's disease using an *Xf* strain that continuously expresses green fluorescent protein.
2. Determine the relationship between the pattern of colonization of a plant by *Xylella fastidiosa (Xf)* and the ability of that plant to be a source for bacterial acquisition by sharpshooter vectors.

RESULTS

There were no differences in the total vessel number, the proportion of short vessels, or the longest vessels between resistant and susceptible grape varieties between greenhouse-grown canes of similar length, age, and diameter. The longest vessel measured by paint infusion was 110 cm (Pinot Noir), although most vessels were less than 12 cm long in all cultivars (Figure 1). Cane length had a small but significant influence on longest vessel ($r^2 = 0.20$; $P = 0.02$, $n = 27$), but did not relate to the number of very short vessels. There was no relationship between stem length and vessel length in the other plants.

While more replication is needed, the longest vessel measured in any alternate host was 15 cm long (mugwort). In sunflower, 71% of vessels were less than 3 cm long. Other species had a wider range of vessel lengths, with about half their vessels less than 3 cm long (Figure 2). Mugwort had roughly twice as many vessels (592, $n = 3$) at the stem base than morning glory (217), quinoa (251) or sunflower (286) stems of comparable diameter and age. Sunflower, mugwort and quinoa all had vascular tissues in evenly distributed bundles wide interfasicular regions of parenchyma (4). Annual morning glory had large vessels distributed evenly along the cambium.

Table 1: Comparisons between canes of similar length, age, and diameter belonging to 3 grape cultivars.

Cultivar	Total # vessels at base of cane (SE)	% Vessels < 3 cm (SE)	Longest vessel (SE)	# Rays (SE)
Cabernet Sauvignon	515 (43)	21 (3)	53 cm (5)	34 (1)
Pinot Noir	474 (27)	20 (3)	64 (9)	34 (2)
Sylvaner	514 (38)	18 (5)	69 (9)	40 (2)
one-way ANOVA	(n = 27, $P = 0.67$)	(n = 27 , $P = 0.84$)	(n = 27, $P = 0.35$)	(n = 27, $P = .01$)

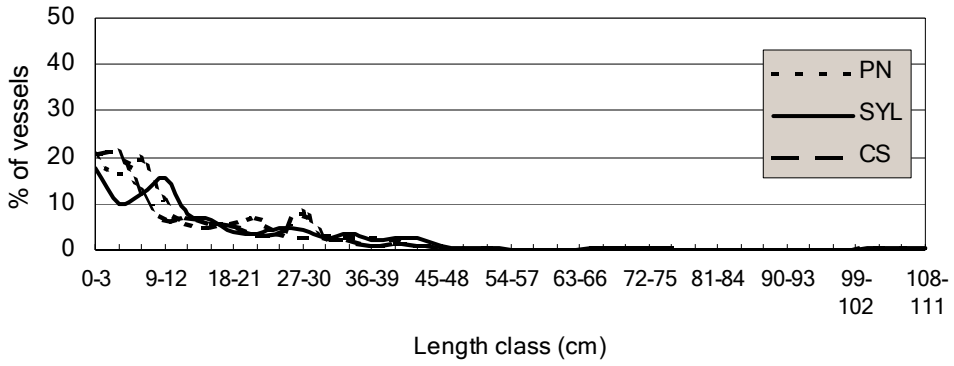

Figure 1: Vessel length distribution in greenhouse-grown Pinot Noir (PN), Sylvaner (SYL) and Cabernet Sauvignon (CS).

PATTERNS OF *XYLELLA FASTIDIOSA* INFECTION IN PLANTS AND EFFECTS ON ACQUISITION BY INSECT VECTORS

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

ABSTRACT
We are studying the effect of host plant tolerance on insect vector acquisition of *Xylella fastidiosa* (*Xf)* from plants tolerant, moderately susceptible, and highly susceptible to *Xf* infection. We are observing *Xf* population and distribution in tolerant and susceptible plants, and its relationship to xylem anatomy, symptom development, and bacterial acquisition by sharpshooters. Since host plant resistance is an important component in the long-term goal of curing PD, it is important to know how resistant plants affect PD spread in areas permanently infested with sharpshooter vectors. We also address the short-term goal of controlling PD spread by comparing grape cultivars in their ability to provide inoculum for vine-to-vine spread of Pierce's disease. Anatomical comparisons of three cultivars, Sylvaner', 'Cabernet Sauvignon' and 'Pinot Noir' showed that all three varieties had similar numbers, lengths and distributions of vessels. The only significant difference was that tolerant 'Sylvaner' had ~ 20 % more rays than the more susceptible 'Cabernet Sauvignon' or 'Pinot Noir' ($n = 25$, $P = 0.01$) in canes of similar age, length and diameter. In all four alternate hosts, morning glory (*Ipomoea purpurea*), mugwort *(Artemisia douglasiana)*, sunflower (*Helianthus annuus)* and annual bur-sage (*Ambrosia acanthicarpa*), the longest vessels measured were less than 13 cm long, while in grapes the longest vessels averaged 62 cm. Though alternate hosts had various vascular morphologies and stem lengths, all had shorter vessels than grapes. Blue-green sharpshooters failed to efficiently inoculate wild-type *Xf* and green fluorescent protein-expressing (GFP) *Xf* into both grapes and alternate hosts; only one of 44 grapes inoculated with BGSS became infected. In order to generate GFP-*Xf* infected plants for microscopy, we are mechanically inoculating alternate hosts and grapes. Ongoing work focuses on refining microscopic techniques to visualize small numbers of *Xf* in plant stems, and generating large numbers of *Xf* infected grapevines to serve as new sources for sharpshooter bacterial acquisition.

INTRODUCTION
Alternate hosts of *Xf* were selected for their different patterns of *Xf* colonization after vector inoculation, lack of stem lignification, varying morphology, and absence of green autofluorescence under blue light. In previous experiments, *Xf*-carrying sharpshooters infected morning glory and sunflower more than 80% of the time. *Xf* spread systemically throughout both plants and reached populations over 10^5 colony-forming units (CFU)/gram. Quinoa and mugwort were less-frequently infected (32% and 16%, respectively) by *Xf* and supported lower bacterial populations (10^3 CFU/g for quinoa, 10^6 CFU/g for mugwort). *Xf* moved systemically to a limited extent in quinoa, but not in mugwort (8, 16). Grape cultivars with varying tolerance to PD selected for evaluation are tolerant 'Sylvaner', moderately susceptible 'Cabernet Sauvignon' and highly susceptible 'Pinot Noir' cultivars of *Vitis vinifera* (12, 13). Both blue-green sharpshooters (BGSS) and glassy-winged sharpshooters (GWSS) will be used to infect plants and assess the efficiency of insect acquisition of *Xf* (1, 7, 11).

We are using wild type and transformed isolates of Temecula *Xf* in our experiment. The transformed isolate continually expresses green fluorescent protein (GFP) when illuminated with blue light. GFP-*Xf* was transmitted by blue-green sharpshooters, retained typical virulence in grape, and allowed examination of plant tissues without the extensive fixation required with electron microscopy. With confocal microscopy, GFP-expressing *Xf* can be observed in small and large colonies in vessels, and passing through bordered pits between vessels in symptomatic 'Cabernet Sauvignon' petioles (10).

Anatomical comparisons between alternate hosts and grape cultivars included measurements of vessel length and number, and vascular bundle number and distribution based on the techniques of Tyson *et al.* (15), and Ewers and Fischer, modified to infuse the pigment via 100kPa pressure applied to the proximal end of the cutting (5). We evaluated primary vegetative growth rather than secondary xylem due to the difficulties in sectioning, culturing from, and feeding BGSS on partially lignified stems. GFP-*Xf* inoculation and colonization of all plants will be measured similarly in all plants: groups of four GFP-*Xf* carrying sharpshooters inoculated a 3-cm stem section, and the plants were evaluated for the presence of GFP-*Xf* approximately 8 weeks after inoculation. Colonized vessels will be counted, and populations estimated by culture on PWG media (2, 8).

To determine what genes were affected that resulted in restored transmission, we will clone and sequence the DNA flanking the transposon using standard protocols for determining genomic DNA sequence flanking insertion DNA. The identity of these genes may enable us to grasp key features of the bacterial mechanism driving transmission. For example, we may find that certain adhesins are required for attachment to the foregut if activating transposons near adhesin genes restore transmissibility.

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FUNDING AGENCIES

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

In parallel, an "activating transposon" will be designed to activate transcription of genes normally up-regulated by DSF (Figure 2).

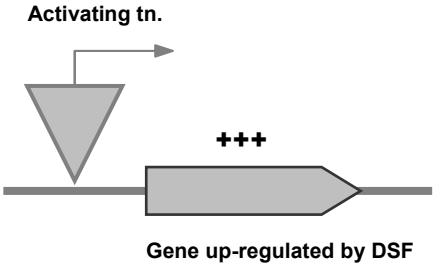

Figure 2: Activating transposon mutagenesis to enhance gene function.

The activating transposon will contain a constitutive promoter that will activate transposition of genes downstream of its insertion site (Newman, 2003). This dual approach will increase the likelihood that we can obtain mutants with restored transmission, and will give us information about those processes that are required for transmission, as well as those processes that must be "turned off" for colonization and transmission to occur. The library will be screened for disrupted gene mutants and then for activated gene mutants.

The insect vectors used for the screen in this study will be GWSS and BGSS. To screen for those mutations that restore transmissibility to the *rpfF* mutant, the gene libraries will be injected into 10 healthy plants of *Vitis vinifera* cultivar Cabernet Sauvignon. The mutant library will be mechanically inoculated into the grape plants. The plants will be kept in the greenhouse and will be monitored periodically for the presence of PD symptoms. Five plants will contain the disrupting transposon mutagenesis libraries and the other five will contain the activating transposon mutagenesis libraries. The source plants will be kept in the greenhouse to allow the strain to reproduce and grow. Group of 100 BGSS, non infective for *Xf* will be placed on the source plants to permit acquisition. The insect vectors BGSS and *Homalodisca coagulata* (GWSS) will feed on the plants containing the mutant collections.

Half of the vectors will be analyzed by bacterial culturing for the presence of *Xf* mutants 14 days after removal from infested plants. The bacteria recovered from these insects will represent mutants that have regained the ability to colonize insect foreguts. Strain KLN61 was only rarely recovered from insects at 7 days, and at 14 days it is expected that that number will be reduced to zero. This will be tested prior to the screen.

The other half of the vectors will be transferred to new healthy plants, and after 6 to 8 weeks, the plants will be cultured for the presence of bacteria. The bacteria recovered from those plants represent those mutants that have regained transmissibility.

OBJECTIVES

1. Create a library of *Xf* mutants in the *rpfF* mutant background using a disrupting transposon mutagenesis to block gene function.
2. Create a library of *Xf* mutants in the *rpfF* mutant background using an activating transposon mutagenesis to enhance gene function.
3. Design and carry out a screen for mutations in *Xf* that restore transmissibility in the non-transmissible *rpfF* mutant.
4. Identify the genes affected in the screen. These will be genes that are important for transmission of Pierce's disease (PD) by insect vectors.

RESULTS AND CONCLUSIONS

Generating the mutant libraries is the main focus of the research during this first year. We have constructed an *rpfF* knockout by allelic exchange mutagenesis using a $Strep^R$ marker carried on pKLN121 plasmid. A total of 200 cfu were yield after the transformation and transferred on new media plates containing a concentration of 100ug/ml spectinomycin and 50ug/ml streptomycin as selective markers. This new $Strep^R$ strain allows compatibility with the transposome system, which confers Kan^R allowing us to proceed with the transposome-mediate mutagenesis technique soon. The transposome approach would permit us to rapidly construct a library of mutants in the *rpfF* background. It has been shown that transposome-mediated mutagenesis was successful in Kirkpatrick's laboratory when applied on *Xf* (Guilhabert et al, 2001).

To construct a mutant library in the *rpfF* mutant background gives an important advantage to this project. A secondary mutation on *rpfF* could short-circuit the need for *rpfF* in transmission, using other important genes involved in the process and restore transmissibility of the mutant strain.

A SCREEN FOR *XYLELLA FASTIDIOSA* GENES INVOLVED IN TRANSMISSION BY INSECT VECTORS

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

ABSTRACT
The sharpshooter vector transmission of *Xylella fastidiosa* (*Xf*) to grape causes Pierce's disease (PD). Identification of genes in *Xf* which are responsible for transmission is an essential step in understanding bacteria-vector interactions and may shed light on biofilm formation by *Xf*.

The aim of this work is to understand the role of the genetic regulon of the rpf (regulation of pathogenicity factors) system in *Xf* and its role in disease transmission. In *Xf*, the rpf system likely regulates genes important for colonization of and transmission by insect vectors. The *rpfF* gene is one of the essential genes of the rpf cell-cell signaling system. Transcriptional control regulates genes by cell-cell signaling. The rpfF gene codes for the enzyme that synthesises the signaling molecule, DSF (diffusible signal factor). This system regulates the expression of a host of genes that are as yet unidentified in *Xf*. The rpf gene cluster of *Xanthomonas campestris* pathovar *campestris* is required for pathogenesis of this bacterium to plants (Dow et al. 2000).

In a transmission experiment with the sharpshooter leafhopper *Graphocephala atropunctata* (BGSS), the *Xf* strain KLN61 (an rpfF knockout mutant) could not perform cell-cell signaling. It was not retained by the insect vector and consequently not transmitted to the plants (Newman, 2004). When the *Xf* rpfF mutant strain was compared with *Xf* wild type, it showed to be hypervirulent, non-transmissible, and lacked biofilm formation. Because the spread of Pierce's disease requires the transmission by insects, this indicates that blocking bacterial transmission by insect vectors may be a strategy for controlling PD. However, this requires a better understanding the role of cell-cell signaling by *Xf* and its importance for transmission.

INTRODUCTION
This research study, during its first year, will focus on constructing mutant libraries. By screening for mutations that suppress the non-transmissible phenotype on the rpfF mutant, we will identify the genes involved in transmission using two approaches. The first approach is to restore transmissibility through mutagenesis by disrupting genes normally down-regulated by DSF with a "disrupting transposon" (Figure 1).

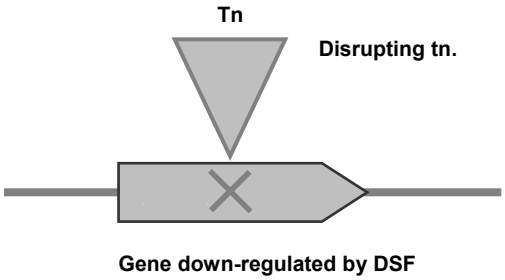

Figure 1: Disrupting transposon mutagenesis to block gene function.

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

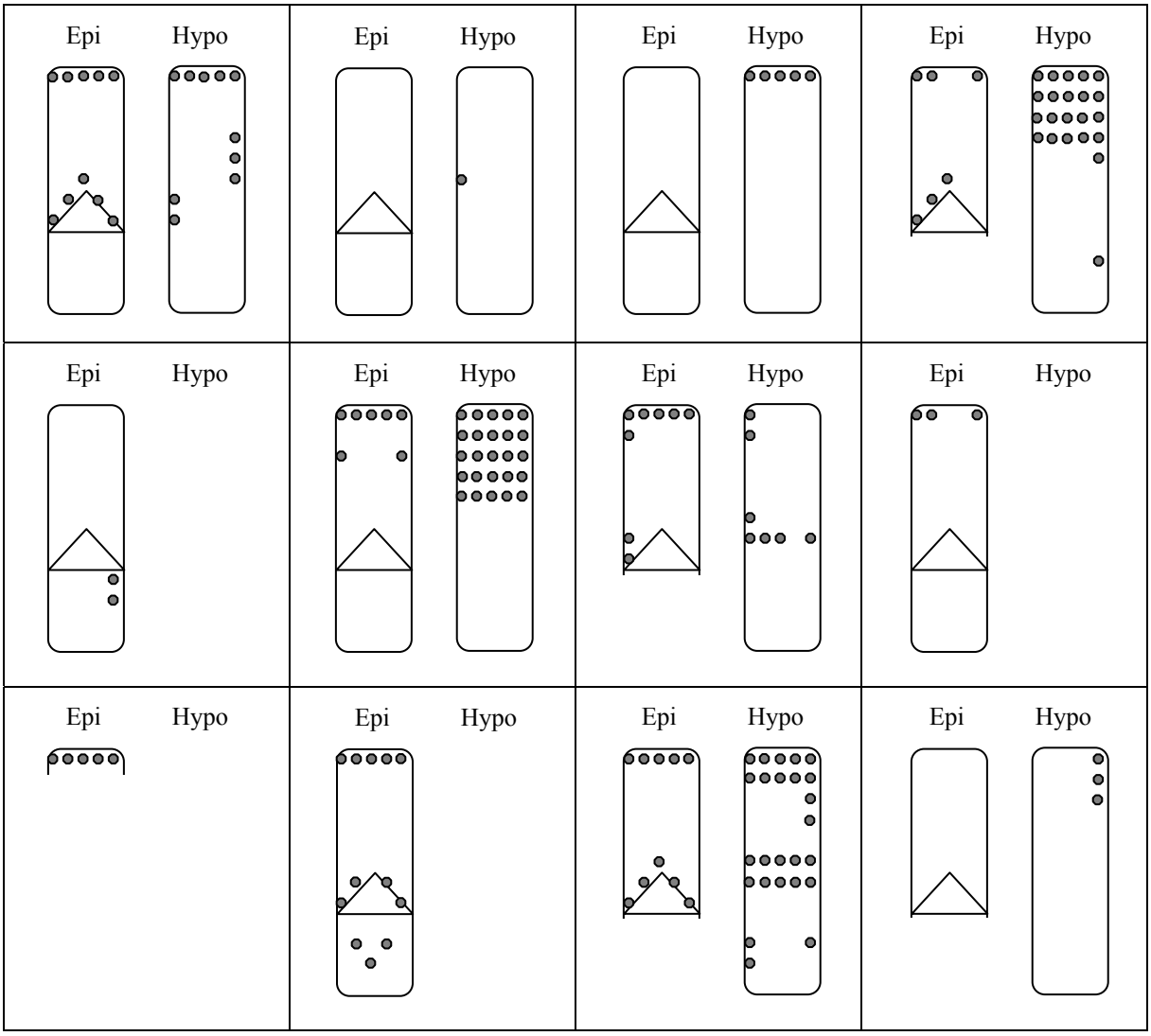

Figure 2. Diagrammatic illustration exemplifying areas with *X. fastidiosa* attached after 1 day AAP and 1 day IAP in the precibarium of 12 *Graphocephala atropunctata*. Epipharynx (Epi) and hypopharynx (Hypo) are represented, the stylets would be below and the cibarium above each figure. Precibarial valve shown as a triangle; filled circles indicate regions colonized by the bacterium. Figures not drawn to scale, sections of cuticle not available for visualization were not included in diagrams.

CONCLUSIONS

Our findings are consistent with the hypothesis that *Xf* must be present in the precibarium, the narrow channel leading from the junction of the mouthparts (needle-like stylets) with the head to the entrance of the cibarium (sucking pump), for successful inoculation to occur. It is also consistent with reports that small numbers of *Xf* cells are adequate for efficient transmission. This suggests that the back-flow of ingested sap from sharpshooters does not have to be a large volume to enable vector transmission.

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RESULTS

Objective 1. We conducted transmission experiments, labeled 'A' through 'C', as shown in Table 1. In 'A' we used long acquisition access periods (AAP) and inoculation access periods (IAP) to increase *Xf* transmission efficiency. We also used a long incubation period to allow bacterial colonization of the precibarium of vectors. 'B' was similar to 'A' when the incubation period is considered, but we reduced the AAP to 8 hours to determine if that had an effect on *Xf* distribution patterns. We also used 1 day AAP followed by a 1 day IAP without an incubation period (experiment 'C'). The objective was to determine regions of initial bacterial attachment in the precibarium before thorough colonization of the canal occurred. Table 1 summarizes these experiments, including results for insects with adequate head dissections but excluding other individuals from the experiment. After plant access periods, heads were prepared for microscopy and the test grape plants kept for later diagnosis. We tested grapes for *Xf* presence by visual symptoms and the culture method (Hill and Purcell 1995). Standard SEM protocols were used for preparation of samples. All individuals not adequately dissected for SEM analysis were eliminated from the experiment.

We obtained very good correlation between presence of *Xf* cells in the precibarium of *G. atropunctata* and its transmission to grape. Only one insect identified as negative, in experiment 'B', transmitted to plants. All other infected plants were associated with insects in which *Xf* was observed. When short incubation and acquisition access periods were used some positive insects did not transmit *Xf* to plants, most likely due to the short IAP used. This is consistent with the many observations that not every infective sharpshooter will transmit at every opportunity. The distribution of *Xf* in the precibarium of vectors in experiments 'A' and 'B' was the same as described in a previous report (2003 PD/GWSS Research Symposium). The length of the AAP did not affect colonization, and 2 weeks seems to be enough time for cells to colonize available surfaces of the precibarium.

Experiment 'C', with short AAP and IAP, provided information on the sites of initial bacterial attachment after acquisition. In all cases *Xf* had not fully colonized the precibarium. Most of the heads were colonized by few clusters of cells. These colonies were assumed to be located at sites of initial attachment on the precibarium by *Xf*. Figure 1 depicts representative photomicrographs of small colonies of *Xf* attached to the precibarium; Figure 2 diagrams examples of *Xf* site observed on the precibaria of 12 insects. All insects that transmitted to plants had micro-colonies on the precibarium. In those cases, cells were found both nearby the valve as well as proximally to it, immediately before the cibarium. In one case cells were only observed below (distally to) the valve entering the valve's pit.

Objective 2. Objective two was completed last year.

Table 1. Summary of transmission experiments and their respective acquisition, incubation and inoculation periods.

	Insect transfer sequence					
Exp	AAP	Incubation	IAP	No. insects [1]	Positive heads	PD plants
A	4 days	7 days	4 days	10	7	7
B	8 hours	13 days	1 days	9	3	4
C	1 days	0 days	1 days	22	12	7

[1] Includes only the number of insect heads that were adequately dissected for SEM analysis.

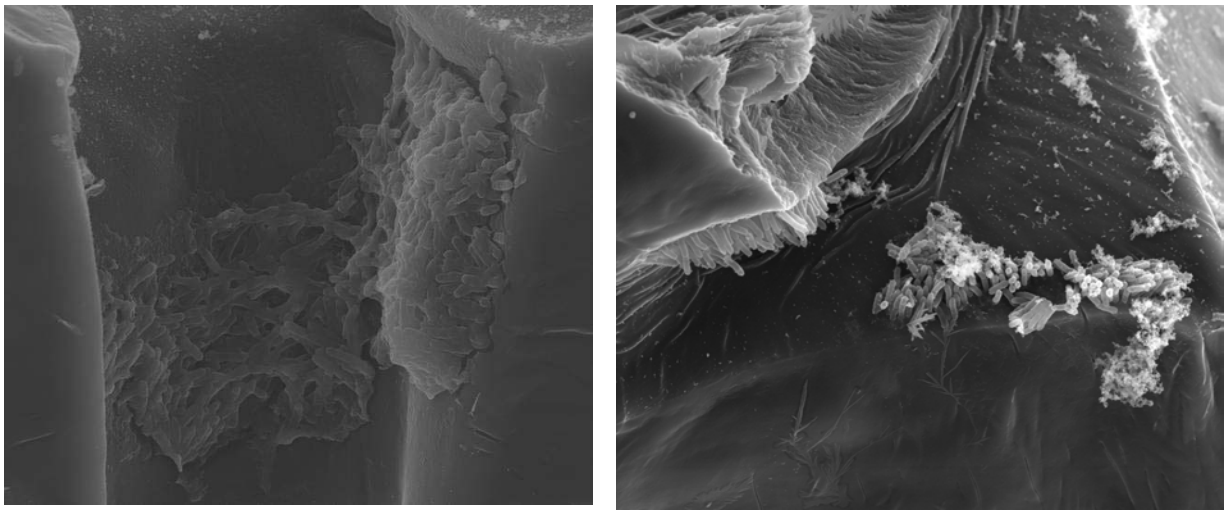

Figure 1. Clusters of *Xf* cells on the hypo- (left) and epi-(right) pharynx of two blue-green sharpshooters after 1 day acquisition feeding and 1 day inoculation feeding (different individuals). On both pharynges the colonies are limited to the proximal section of the precibarium. The clusters formed one micro-colony in the hypopharyngial precibarium (right); there are two clusters of cells on the epipharynx. Note matrix covering some of the cells on the left picture.

ROLE OF BACTERIAL ATTACHMENT IN TRANSMISSION OF *XYLELLA FASTIDIOSA* BY THE GLASSY-WINGED SHARPSHOOTER, AND OTHER FACTORS AFFECTING TRANSMISSION EFFICIENCY

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ABSTRACT
Blue-green sharpshooters (BGSS) that had long acquisition access periods (4 days) feeding on grapes with Pierce's disease symptoms, followed by a week on test plants consistently had monolayers of cells of *Xylella fastidiosa* (*Xf*) in the precibarium, the narrow channel leading from the junction of the stylet mouthparts with the head to the entrance of the cibarium (sucking pump). BGSS given short acquisition and inoculation periods that transmitted *Xf* to test plants also had small colonies or isolated attached cells of the bacterium in the precibarium. Our findings are consistent with the hypothesis that *Xf* must be present in this small area of the sharpshooter foregut and also consistent with reports that small numbers of *Xf* cells in this area are adequate for efficient transmission. These results also suggest that the back-flow of ingested sap from sharpshooters does not have to be a large volume to enable vector transmission.

INTRODUCTION
Xylella fastidiosa (*Xf*) occurs on the foregut ("inner mouth") surfaces of vectors; but the importance of precisely what part or parts of the cibarium are critical for vector transmission of *Xf* is not clear (Purcell et al. 1979). The foregut is formed as an in-folding of the outer body wall. As such, the foregut is lined with cuticle that is shed when the insect molts. Because molting interrupts vector transmission and there is no delay between acquisition and inoculation of *Xf* by vectors (Purcell and Finlay 1979), the foregut is considered to be the site from which *Xf* is transmitted by vectors. The needle-like mouthparts (formed by modified mandibles and maxillae) of sharpshooters transport plant sap to the pharynx, which is formed by the "upper" (epi-) and "lower" (hypo-) parts of the anterior head. The epipharynx and hypopharynx contain narrow grooves that come together to form the precibarium, a circular canal leading to a pump chamber (cibarium or cibarial pump) within the head. A muscle-powered, flexible diaphragm pumps ingested fluid to the gut via a tubular, flexible esophagus. A muscle-powered valve in the precibarium (the precibarial valve) can prevent the backflow of fluid from the pump to the mouthparts while the pump chamber is contracting to move fluid to the gut. Considering the function and position of the precibarial valve, *Xf* cells in the pump chamber would have to detach and move through the precibarium and the food canal of the stylets to be inoculated into plants. The correlation between the occurrences of *Xf* at the entrance of the cibarial sucking pump with its transmission to plants was not consistent, as some insects that transmitted did not have visible bacteria in this location (Purcell et al. 1979). The numbers of viable *Xf* cells was not well correlated to transmission efficiency, as many transmitting sharpshooters had few or no detectable (cultivable on artificial medium) *Xf* within their heads (Hill and Purcell 1995). Later, it was demonstrated that *Xf* also occurs on the precibarium of other sharpshooters (Brlansky et al. 1983), where *Xf* occurs distally and proximally to the valve in the precibarium but did not correlate the abundance or presence of *Xf* or its location in the insect foregut with transmission to plants. We investigated the correlation between the presence of *Xf* attached to the precibarium and transmission of the bacterium to grape by an efficient sharpshooter vector.

The blue-green sharpshooter (BGSS, *Graphocephala atropunctata* [Signoret]) is the most important vector of *X. fastidiosa* in Coastal California (Redak et al. 2004) and is an efficient vector when compared to other sharpshooters (Almeida and Purcell 2003, Purcell and Finlay 1979, Severin 1949). It is so far the most studied vector of *X. fastidiosa* in relation to transmission biology. For these reasons, we used *G. atropunctata* to study the spatial distribution of *X. fastidiosa* on the precibarium of infective sharpshooter vectors and its transmission to plants after short and long incubation periods using scanning electron microscopy (SEM). We previously reported that *Xf* had colonized the precibaria of all BGSS after by 10 or more days after acquiring *Xf* from plants. Because BGSS can efficiently transmit *Xf* even after a short period following acquisition (Hill and Purcell 1995), we used SEM to inspect the precibaria with of transmitting BGSS for *Xf* after short (1 day) acquisition and inoculation feeding periods.

OBJECTIVES
1. Determine the association of *X. fastidiosa* transmission and its location in the vector's precibarium and cibarium.
2. Determine the effects of within-plant location on vector transmission efficiency.

CONCLUSIONS

Our overall objective is to understand the role of "colonization" phenomena in acquisition, retention and delivery of *Xf* by vectors. By manipulating the *in vitro* environment in which wild type *Xf* is cultured, and subsequently presented for acquisition by leafhopper vectors, we hope to understand what factors promote colonization of insect foreguts, and delivery to plants. The use of *Xf* mutants with impaired or enhanced ability to perform some part of the colonizing behavior will be important to understanding the interaction between environment and bacterial behavior affecting vector retention and delivery. Interfering with vector acquisition and inoculation (reducing or avoiding vector populations) are currently the major control methods for Pierce's disease in California. Our findings may reveal currently unanticipated ways of interfering with vector transmission and elucidate features of *Xf* biofilms applicable to this bacterium in plants.

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colonization and biofilm formation of other bacteria living in fluid environments (e.g., Arnold 1999, Korber et al. 1997), and attachment of *Xf* cells to inert surfaces was, in fact, dependent on surface chemistry (Hoch and Burr 2003).

Both the genetic and environmental factors that affect attachment or detachment of *Xf* are amenable to experimentation. Availability of the mutants discussed above has been and will continue to be important in allowing researchers, to expand our understanding of the role of particular colonization behaviors in transmission and virulence by using new mutants. Relevant environmental factors can be experimentally manipulated by the use of artificial diets for *Xf* acquisition by vectors; excised native and artificial substrates for *Xf* colonization; and fluidic chambers to regulate flow of medium over those substrates.

OBJECTIVES

1. Determine whether vector retention (and subsequent delivery) of *Xylella fastidiosa* is related to the chemical and physical environment from which the bacteria are grown or acquired.
2. Investigate how *X. fastidiosa* cells attach (and detach) to specific foregut regions of sharpshooter vectors. *NB: this objective is similar to one proposed from the Hoch/Burr labs with which we propose to collaborate.*

RESULTS

We have begun to address our first objective by measuring *in vitro* survival and growth of wild type *Xf* (Temecula strain) in a variety of media, at different pHs, and in different volumes of media. The media we have used to date are: xylem sap; *Xf*D2, a defined minimal medium developed in this lab (Almeida et al. 2004); and two standard media used for growing *Xf*, PW (Davis et al. 1981a) and PD3 (Davis et al. 1981b). Media pH ranged from 5.2 to 8.0, and volumes varied from 100uL to 30 mL. In all cases, media were inoculated with a 10% by volume of *Xf* suspension of approximately 10^6-10^7 cfu/mL, and samples from each were plated 6-8, 24, 48 and up to 172 h after inoculation. In one assay, media were incubated under lowered oxygen tension. We have also begun to look at a second *Xf* strain, the *rpfF* mutant KLN 61 (Newman et al. 2004).

To date, clear effects of most variables have been undetectable due to inconsistent results even in our controls. The volume of media in which *Xf* are incubated during the assays appears to override the importance of other variables, including any strain differences. For example, control *Xf* in only four out of 12 assays using media volumes of 100 to 200uL survived to 24 h; in 2 mL volumes, three of six control populations survived to 24 h; and in 30mL volumes, all (6/6) control populations survived to 24 h and beyond.

Even in assays in which *Xf* survived, most populations did not grow over 48 hours or more. In all assays so far we have used *Xf* grown from stock on solid media for 1- to 2-weeks, to inoculate the various test media. We have begun to inoculate liquid broth as well, which we will use to subsequently inoculate test media after 5 days of incubation to utilize log-phase cells already growing in liquid (Campanharo et al. 2003).

Preliminary results comparing attachment of two *Xf* strains grown in three media are shown in Table 1. Using a crystal violet assay adapted from Espinosa-Urgel (2000), we compared the relative amounts of the wild-type strain Temecula and the *rpfF* mutant KLN 61 adhering to vessels in which they had been incubated (live *Xf* were not recovered from these media after 24 h, except for strain Temecula in PW, which survived to 172 hours). These results are not yet conclusive and have not been replicated, but show an interesting trend for reduced attachment of the mutant strain, and maximum attachment of the wild-type strain in xylem compared to artificial media.

Table 1. OD_{600} of crystal violet solution eluted from rinsed wells containing *Xf* of wild type Temecula or *rpfF* mutant KLN 61grown in indicated media. n=4 for each strain in each medium. (Calculated by subtracting mean absorbance in each medium from OD of control medium without *Xf*).

	Mean OD_{600}	
Media	Temecula	KLN 61
xylem	0.031	0.010
XfD2	0.021	0.018
PW	0.015	0.008

For our second objective, our plan is to collaborate with the Hoch/Burr labs at Cornell to develop a method for assessing bacterial attachment to vector mouthparts. Together we will examine temporal aspects of cell attachment and colonization under these more realistic conditions of moving fluids through/over sharpshooter mouthparts, using dissected foregut regions placed in microfluidic (flow chamber) devices. In addition, artificial channels that mimic the relevant internal portions of vector mouthparts in flow devices (to be designed at Cornell) will be used to evaluate the effects of high velocity flow conditions on *Xf* cell attachment. We can provide bacteria-free insects and dissected mouthparts to the Cornell labs and test at Berkeley flow devices developed at Cornell. We have previously found that *Xf* colonizes specific regions of the precibarium of insect vectors after bacterial acquisition from infected grapes. This objective addresses our interest in developing an *in vitro* assay to better understand the mechanisms for such site-specific attachment and colonization.

EFFECTS OF CHEMICAL MILIEU ON ATTACHMENT, AGGREGATION, BIOFILM FORMATION, AND VECTOR TRANSMISSION OF *XYLELLA FASTIDIOSA* STRAINS

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ABSTRACT
We have begun work on the effects of chemical and physical factors, including type of media, pH, media volume, and vessel on the *in vitro* survival, growth and substrate-attachment of a wild-type and mutant strain of *Xylella fastidiosa* (*Xf*). The volume of media in which *Xf* is incubated appears to override the importance of other variables, including any strain differences. *Xf* populations incubated in small (200uL) volumes died within 24 h in 50% of assays, but fared better as volumes increased. Preliminary results suggest that attachment to the incubation vessel is greater for wild-type compared to an *rpfF* mutant that does not produce a cell-cell signaling factor.

INTRODUCTION
Under natural conditions, *Xf* attaches to and colonizes the foregut of its leafhopper vectors and the xylem vessels of its plant hosts, creating aggregations of cells attached to their host substrates and surrounded by a polysaccharide matrix, forming a biofilm. Some progress has been made in identifying *Xf* genes responsible for particular colonizing behaviors, and the use of mutants that disable particular functions (e.g. Newman et al. 2004, Feil et al. 2003) is an invaluable aid to studies of transmission and disease. However, much remains to be learned about what environmental factors (of plant or insect origin) affect colonization; and about how such environmental factors interact with bacterial genetic factors to promote or prevent acquisition, retention and delivery of *Xf* by the vector.

The uptake of *Xf* cells by the insect and subsequent detachment of *Xf* as insects probe xylem tissue are essential for vector transmission. These simple requirements, however, belie the more complicated picture that emerges from experimental data. For example, *Xf* added to xylem sap in artificial diets were taken up but not subsequently transmitted to plants by the vector (Davis et al. 1978, Almeida and Purcell, unpublished). In addition, *Xf rpf*F mutants, which were unable to produce a cell-cell signaling factor (DSF, diffusible signal factor), were acquired by vectors; but they were not retained and were not transmitted to plants (Newman et al. 2004). Although other studies have shown that *Xf* could be transmitted within an hour of vector acquisition from plants (Severin 1949, Purcell and Finlay 1979), before anything like a biofilm could form in the foregut, the foregoing data suggest that some rudimentary level of attachment may be necessary for short-term transmission; and that retention, and by implication, colonization and biofilm formation, may be necessary for longer-term ability to transmit. However, the actual role of aggregation/attachment/colonization in the transmission of *Xf* is still largely unknown.

It is clear that both genetic and environmental factors affect colonization of *Xf in vitro*, as well as in insects and plants. Experiments with site-specific mutants of *Xf* have yielded insights into the control of aggregation/attachment/colonization phenomena, though not always in completely unambiguous ways. For example, the *Xf* DSF-deficient mutant formed biofilms and caused severe disease in mechanically inoculated plants, in spite of its inability to colonize the insect foregut (Newman et al. 2004). Cell-cell signaling, therefore, apparently plays different roles in *Xf* colonization behaviors in insects and plants. In the plant pathogen *Xanthamonas campestris,* DSF triggered dispersion of cell aggregates *in vitro*, and was suggested to promote virulence to plants (Dow et al. 2003). Mutants in two other *Xf* genes involved in formation of bacterial fimbriae that aid in attachment, *fimA* and *fimF,* showed reduced aggregation *in vitro*, but were insect transmissible, and caused disease in grapevines (Feil et al. 2003, Feil and Purcell, unpublished).

In both the plant and the vector, environmental factors that putatively affect attachment or detachment would include chemical makeup of sap from which *Xf* cells are acquired; the substrate colonized (insect foregut, xylem vessels); and movement of sap through the xylem or foregut. Media composition has a reportedly major effect on aggregation and biofilm formation of *Xf* (Leite et al. 2004). It is likely that substrate surface characteristics are also important, by analogy with

trees more than 200 miles apart (Temecula and San Joaquin), but they exhibit the same 3 recombinant events. These isolates may represent the evolution of a new pathotype through recombination.

The source of the recombinant DNA could be determined by its sequence identity with the gene from a different strain. This identity suggests that these genetic transfers occurred relatively recently. Thus PD14 incorporated DNA from a multiplex ALS-type bacterium in its cysG gene.

CONCLUSIONS.

1. There are 3 clades of *X. fastidiosa* within N. America, corresponding to subsp. piercei and multiplex, and the newly named taxon sandyi that causes oleander leaf scorch.
2. The 3 clades originated at least 15,000 years ago. This guarantees that the clades could not have developed in response to host plants introduced by Europeans, e.g. oleander.
3. Isolates from the same clade showed very few genetic differences, and we found no evidence of geographical genetic structure within the piercei or sandyi clades. This limited variability within very old taxa suggests strong selection, possibly driven by host-plant adaptation.
4. Multi-locus sequence typing (MLST) is effective at identifying the three clades, and the plant-host strains within the multiplex group.
5. We can detect mixtures of the 3 main types of *X. fastidiosa* using 3 genes subject to restriction digests.
6. We observed 4 examples of recombination in a sample of 257 genes. Three of these recombinations were found replicated in two isolates. This highly non-random distribution is consistent with the possibility that new recombinant forms can rapidly generate novel pathotypes.

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Americas and since most of the plant hosts exhibiting disease symptoms are introduced species, we need to know if these three N. American clades pre-date European colonization. We estimated divergence dates based on the rate of synonymous substitution. Assuming that such substitutions are generally neutral and driven by genetic drift, then we have that the time of origin *T* (in years) of a given clade is $T = K/(nu)$, where *K* is the number of synonymous substitutions per site in a given branch, *u* is the mutation rate per generation, and *n* is the number of generations per year. We used $u=5.4x10^{-10}$ (the E. coli rate, see Drake *et al* 1998) and n=1000, corresponding to a long-term division rate of once every 9hrs. The generation time of *X. fastidiosa* has been estimated at between 9 and 60 hours (Wells *et al* 1987), so our assumption is conservative (reducing T). The resulting estimates are shown in Figure 2. These estimates suggest that the three clades, piercei, multiplex, and sandyi, have been distinct for at least 15,000 years, and possibly much longer.

It is notable that the estimated age of the multiplex clade is 3x less than the estimated age of the parallel piercei/sandyi group. Since they are exactly the same age, the most likely explanation is that the generation time (in nature) of members of the multiplex clade is about 3x longer (i.e. *n* is smaller in eqn 1). Note that this effect is apparent both before and after the split of piercei and sandyi, (20,000 yrs plus 24,000 yrs compared to the multiplex total of 14,700 yrs), and that the rate within the piercei and sandyi clades is extremely similar (24,600 vs. 23,300).

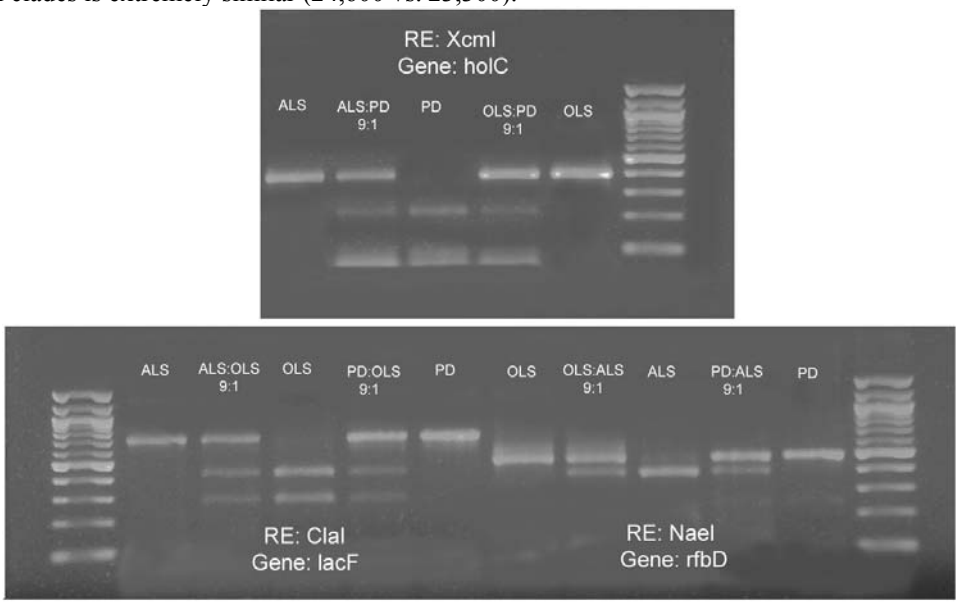

Figure 3. Restriction digests following amplification of single genes from pure-strain DNA, or from a 9:1 mix of the DNA of two strains.

We have shown that the MLST approach of Maiden *et al* (1998) can be used to document both the differences among the three major groups, and the differences among the plant-host isolates of subsp. multiplex (data not shown). The strength of this approach is that MLST data are unambiguous, can be held on a central database, and can be queried through the Web.

Using three of the target genes, we developed a PCR/restriction enzyme essay that separates the major groups of *X. fastidiosa*. We have shown that this method can be used to identify strains from mixtures of DNA (figure 3).

Objective 2: Measurement of Clonal Variation Within Host Strains

It is clear from Figure 1 that there is very little variability within the three clades. Furthermore, we found no evidence of geographical substructure. Using Kst (which measures genetic differentiation between populations relative to within populations) we found no differentiation between 2 northern California isolates of piercei (PD4,6; see fig. 1) vs. 6 southern California isolates (PD1,7,10,14, ALS5,11) (Kst = 0.00 ns), or between three northern California almond (non-piercei) isolates (ALS3,15,22) and 2 southern California isolates (ALS 12,13) (Kst = -0.26 ns). Over a longer distance, the piercei isolate from Florida (PD16) and the sandyi isolate from Texas (OLS8) showed no marked difference from the remaining isolates in their respective clades (all from California). The lack of intra-clade variability results in a phylogeny with long basal branches leading to very short terminal branches. This pattern suggests that the strains experience strong selective pressures from their host plants, eliminating all but the best-adapted clones.

Objective 3: Estimate the Frequency of Recombination

Given the low level of clade variability, the isolates exhibiting inter-strain recombination at one or more of the 10 sequenced loci can be seen quite clearly from fig. 1. They are PD14 (1 recombination), and ALS 12, 22 (recombination in 3 genes). The sites of the recombination can be seen clearly by aligning the sequences. Thus from 257 gene sequences we found 4 independent recombination events, i.e. 1.6%. It is notable that ALS 12 and ALS 22 were isolated in California from almond

OBJECTIVES

During the last year we have focussed on the following objectives:

1. Develop a systematic multigenic method for identifying host strains of *X. fastidiosa*. Our objective is to develop a method that unambiguously identifies the known host strains, and that allows an efficient recognition of the invasion of new strains.
2. Measurement of clonal variation within host strains. Our objective is to assess within-strain genetic variability and geographical substructure at our target gene loci. From this we can infer the probable importance of plant-host adaptation.
3. Estimate the frequency of recombination. Our objective is to look for evidence of both within- and between-strain genetic transfer. Genetic transfer can dramatically increase the rate of evolution, and potentially can increase the rate at which new –more virulent- host strains arise.

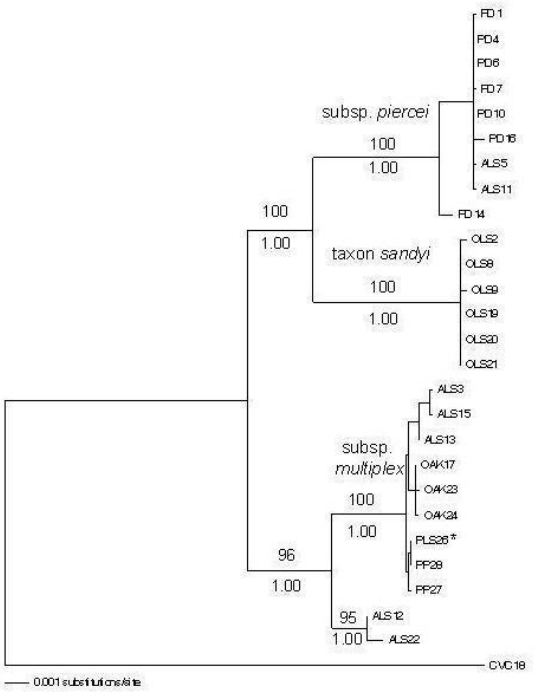

Figure 1. Phylogenetic relationships among 26 N. American isolates of X. fastidiosa from 6 species of host plant, using CVC (from S. America) as the outgroup. The maximum likelihood tree is based on 10 genes except PLS26, which was positioned in the tree based on the sequence of 7 genes. Isolates were from grapevine (PD), almond (ALS), oleander (OLS), oak (OAK), peach (PP), and plum (PLS).

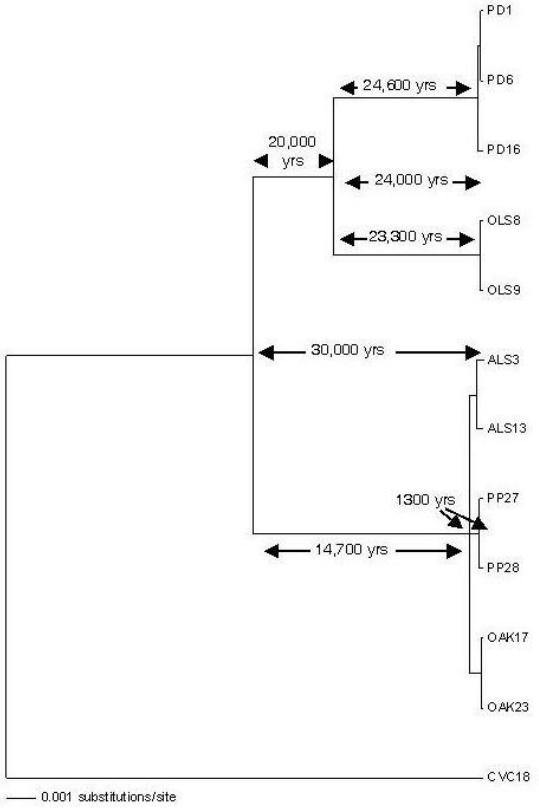

Figure 2. Phylogenetic estimates of the divergence times of the groups of X. fastidiosa based on the rate of synonymous substitution within each branch of the maximum likelihood tree.

RESULTS

Objective 1: Develop a Systematic Multigenic Method for Identifying Host Strains of X. fastidiosa.

To create a statistically robust phylogeny of the host-plant strains of *X. fastidiosa*, we sequenced 10 genes (9288 bp) from each of 25 isolates, and 7 genes from 1 additional isolate. The results are shown in Figure 1 using the S. American CVC strain as the outgroup. The tree shows three well-defined clades that are supported 100% by bootstrap procedures. Two of these clades correspond to the recently named subspecies piercei and multiplex (Schaad et al 2004). Subsp. piercei includes all Pierce's disease isolates. Subsp. multiplex includes a set of isolates from almond plus isolates from a range of host plants from the eastern US (oak, peach, and plum). The third clade contains only isolates from oleander. It is most closely related to subsp. piercei, but shows a high degree of differentiation from that subspecies (2.6% at synonymous sites). In addition, bacteria from these two groups cannot infect each other's major host plant (oleander vs. grapevine) and based on the lack of intermediates, we conclude that the oleander clade constitutes a third N. American subspecies that we have tentatively named sandyi (Scheunzel *et al* 2004).

To begin to understand the evolution of the pathogenicity of the plant-host strains of *X. fastidiosa*, it is important that we have a good estimate of the age of these clades. In particular, since this species of bacteria appears to be restricted to the

GENOME-WIDE IDENTIFICATION OF RAPIDLY EVOLVING GENES IN *XYLELLA FASTIDIOSA*: KEY ELEMENTS IN THE SYSTEMATIC IDENTIFICATION OF HOST STRAINS, AND IN THE SEARCH FOR PLANT-HOST PATHOGENICITY CANDIDATE GENES

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ABSTRACT
We have developed a robust phylogeny of the North American isolates of *Xylella fastidiosa* based on 10 genes (9288 base pairs). This supports the recent division of *X. fastidiosa* into subspecies (piercei and multiplex in N. America), however, we found 1 additional distinct taxon. The oleander isolates form a distinct group (provisionally named sandyi) that separated from the Pierce's disease group (piercei) long before European settlement of N. America, probably substantially more than 20,000 years ago. We used the phylogenetic tree to confirm the effectiveness of multilocus sequence typing (MLST) in identifying the subspecies and (within subspecies multiplex) plant-host isolates. MLST involves sequencing at least 7 genes from pure cultures. We have also developed a simpler method that distinguishes the major groups using restriction enzymes. This method has the advantage of working on mixed cultures and requiring only 3 PCR reactions. Our sequencing has confirmed that *X. fastidiosa* is largely clonal, and that within the piercei and sandyi groups there is very little genetic variability or geographical substructure. This pattern is particularly notable given the age of these groups and suggests the action of strong natural selection favoring specific clones. Finally, we found 4 (1.6%) examples of interstrain recombination, and the clustering of 3 in each of 2 isolates suggests that recombination may drive the rapid evolution of new pathotypes.

INTRODUCTION
We are utilizing the extraordinary power of genomic research to investigate aspects of *Xylella fastidiosa*'s evolutionary history. This history provides information essential for controlling and solving the problem of Pierce's disease. At a minimum, it provides an understanding of the origin of the Pierce's disease (PD) strain of *X. fastidiosa*, and the relationship of the PD strain to other isolates of *X. fastidiosa*. Knowing the level of variability within the PD strain provides important information regarding the nature of these bacteria. Low variability would suggest that the PD strain is subject to significant constraints that may make controlling the pathogen simpler. On the other hand, evidence of high variability and high levels of recombination would suggest that the rapid evolution of resistance to control measures could be a severe problem.

A high priority is to place the PD strain within a robust phylogeny, extending earlier work defining the interrelationships of the plant-host strains of *Xylella fastidiosa* (e.g. see Hendson *et al.* 2001). Schaad *et al.* (2004) have recently named the PD strain as subspecies piercei, based on DNA hybridization. They identified two N. American subspecies (piercei and multiplex). It is important to determine if that taxonomy is sufficient to describe all N. American isolates.

Given a robust phylogeny, genomic data can be used to develop effective methods for identifying host strains, using either simple assays (e.g. restriction enzymes) or more sophisticated methods. MLST (multiple locus sequence typing) (Maiden *et al* 1998) is a valuable technique for identifying bacterial strains. Unambiguous identification of strains is of considerable importance for understanding the epidemiology of Pierce's disease and the other plant diseases caused by this bacterium. Previously, this has been approached using a variety of DNA based methods (Banks et al. 1999; Hendson *et al*. 2001; Rodrigues et al. 2003; Meinhardt et al. 2003;); however, an effective methodology for identifying the plant-host strains, including when they are mixed together, has yet to be developed.

The bacterium *X. fastidiosa* is generally assumed to be clonal. However, virally-mediated horizontal transfer of genes must occur given the presence of unique regions of DNA in the different host strains (Van Sluys *et al*. 2003). The possibility of direct inter-strain genetic transfer is more difficult to detect, but needs to be investigated. If such transfer does occur, it could lead to the very rapid evolution of novel pathogenic forms. Studying the details of sequence evolution across many genes provides information on the past occurrence of such events and hence their future likelihood.

the likely evolution of new, perhaps more virulent strains. It also is important in cataloging the strains in California so that the invasion of new stains can be detected.

OBJECTIVES

1. Collect *Xylella fastidiosa* samples from a diversity of native and naturalized alien plants in and around the riparian zones in southern and central California.
2. Collect *Xylella fastidiosa* samples from a diversity of adult sharpshooters: *Homalodisca coagulata* (Say) and *Homalodisca liturata* Fowler,
3. Characterize the *Xylella* strains that are recovered using multilocus sequence typing (MLST) and,
4. Determine the associations between specific *X. fastidiosa* strains, their plant hosts, and their geographic distributions.

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