Pierce's Disease Program

Symposium Proceedings

-- revised Jan. 13, 2004 --

2003
Pierce's Disease
Research Symposium

December 8 – 11, 2003

Coronado Island Marriott Resort

Coronado, California

California Department of Food & Agriculture

Proceedings of the Pierce's Disease Research Symposium

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Proceedings Compiled by:
M. Athar Tariq, Stacie Oswalt, Peggy Blincoe, Roger Spencer, Liz Houser, Amadou Ba, and Tom Esser

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Peggy Blincoe

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To order additional copies of this publication, contact:
California Department of Food and Agriculture
Pierce's Disease Program
2014 Capitol Avenue, Suite 109
Sacramento, CA 95814
Telephone: (916) 322-2804
Fax: (916) 322-3924

http://www.cdfa.ca.gov/phpps/pdcp

E-mail: atariq@cdfa.ca.gov

Abbreviations used in this document:

- ALS = almond leaf scorch
- APHIS = Animal and Plant Health Inspection Service
- ARS = Agricultural Research Service
- AVF = American Vineyard Foundation
- CDFA = California Department of Food and Agriculture
- GWSS = glassy-winged sharpshooter
- PD = Pierce's disease
- PD/GWSS Board = Pierce's Disease and Glassy-winged Sharpshooter Board
- UC = University of California
- USDA = United States Department of Agriculture
- *Xf = Xylella fastidiosa*

Revisions

1. Jan. 13, 2004 - Daane and Johnson, pages 247-249a (missing figures added).
2. Jan. 13, 2004 - Freeman, pages 215-216b (missing figures added).
3. Jan. 13, 2004 - Zalom and Peng, pages 237-240 (dates in Figures 1 and 2 corrected).

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Section 1A: Crop Physiology, Genetics, and Breeding for Resistance

XYLEM CHEMISTRY MEDIATION OF RESISTANCE TO PIERCE'S DISEASE

Project Leader:
Peter C. Andersen
University of Florida, NFREC
Quincy, FL 32351

Cooperators:
Breno Leite
University of Florida, NFREC
Quincy, FL 32351

Maria Lucia Ishida
University of Florida, NFREC
Quincy, FL 32351

Reporting Period: The results reported here are from work conducted from July 1, 2002 to September 1, 2003.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a Gram-negative xylem-limited bacterium that causes Pierce's disease (PD), plum leaf scald, almond leaf scorch, phony peach disease and many other diseases. For many plant species there is no resistant germplasm. Xylem vessels may be filled with exopolysaccharides produced by the bacterium, and pectins, gums and tyloses produced by the plant. There has been considerable interest in the production of exopolysaccharides as a component of disease symptomatology, whereas the formation of gums and tyloses has sometimes been considered an adaptive host plant response to infection (Fry and Milholland 1990). The resultant plant/bacterium interaction between bacterium and plants results in xylem dysfunction, water stress and leaf necrosis, which are characteristic of PD.

Cell multiplication, formation of aggregates and biofilm may be early components of PD. The stimuli for cell multiplication and the formation of aggregates and biofilm may involve specific plant/bacterium interactions and may involve the plant nutrient status of xylem fluid. With the recent development of simple chemically-defined media for *Xf*, it is possible to study the effects of nutrition *in vitro*. We have found that certain chemically-defined media (3G10R and CHARD2) developed in our laboratory promote the development of aggregates and biofilm. The chemistry of xylem fluid typically consists of 95-98% water; amino acids, organic acids, sugars and inorganic ions are the major components of total osmolality. Chemical profiles developed from xylem chemistry of *Vitis* spp. are also being used to test whether xylem chemistry, and specifically xylem nutrient status is related to PD-resistance/tolerance. The chemistry of xylem fluid may be a function of temperature, fertilization and diurnal/temporal alterations (Andersen and Brodbeck 1989 a b, 1991, Andersen et al., 1995). The manipulation of xylem chemistry, whether it is based on the primary compounds or proteins in xylem fluid, is one possible method to affect PD-resistance. An alternative would be the development of transgenic plants with genes encoding for the production of lytic peptides. Transgenic *V. vinifera* cv. Thompson Seedless grapevines have been developed (Scorza et al. 1996) and the technology for the production of these plants has been patented (Scorza and Gray 2001).

A hypothetical model was proposed to explain how *Xf* adheres to xylem vessels (Leite et al., 2002). In this model, a presumed negatively charged surface of *Xf* could be attributed to the presence of sulfur in outer membrane proteins (OMPs). Interaction between bacteria and the formation of aggregates can be facilitated by the formation of disulfide bonds between OMPs. Adhesion may occur between the bacterial cell surface and the negatively charged entities of the xylem vessel wall. Adhesion, in this hypothetical model, may possibly result from the interaction of this negatively charged xylem walls to negatively charged bacterial cell surface via calcium and magnesium bridges (Leite et al., 2002). The presence of *Xf* OMPs with sulfur-containing amino acids (cysteine and methionine) residues with domains localized in the outer membrane region, are being investigated.

OBJECTIVES

1. Determine the relationship between xylem chemistry and resistance of grape *Vitis* spp genotypes to PD after mechanical inoculation with *Xf*.
2. Examine the influence of nutrients on colony number, optical density, aggregation and biofilm formation of *Xf*.
3. In-vitro experiments with cecropin B to evaluate the efficacy against *Xf* and study of stability of this lytic peptide in artificial media and in xylem fluid.
4. Determine the influence of *Xf* cell surface chemistry during the early stages of *Xf* aggregation and biofilm formation.

RESULTS AND CONCLUSIONS

Mechanical inoculation of vines with *Xf* UCLA PD strain during the summer of 2002 produced an average 62 % inoculation efficiency as determined by PCR. Inoculation efficiency varied between 40 and 89% on ten different grape genotypes; however there was no consistent relationship between the known susceptibility of each grape genotype to *Xf* and percentage inoculation success. The percentage of plants positive for *Xf* was much reduced (0 to 43 %) after the winter 2002/2003, perhaps as a result of low winter temperatures (15-20 F). The percentage PCR positive was highest for *V. vinifera* cv. Chenin blanc. We have sampled xylem fluid to test whether or not chemistry is affected by the presence of *Xf*.

Chemically-defined media were developed from the chemistry of xylem fluid (CHARD2 and 3G10-R) (Figure 1). The utility of these xylem chemistry-based media was examined for the PD strains UCLA and STL. All new media were compared to

XF-26 (Chang and Donaldson, 1993) and two genomics-based media (Lemos et al., 2003). The formulation of CHARD2 and 3G10-R were based on the chemistry of the xylem fluid of a PD-susceptible grape genotype *Vitis vinifera* cv. Chardonnay. We have found that growth of *Xf* requires only one amino acid and a much smaller number of compounds than in currently available chemically-defined media. Aggregation, colony size and biofilm formation of *Xf* was mediated by nutrients in the growth media. Our results support the contention that the chemistry of media (or xylem fluid) can greatly affect the behavior of *Xf*. Liquid media of CHARD2 and 3G10-R increased the capacity of *Xf* to form cell aggregates and biofilm, reducing the number of cells in the planktonic state (Figure 1).

Colony growth of two strains of *Xf* (UCLA-PD and STL-PD) after 48 hours of incubation in xylem fluid was determined for *V. rotundifolia* (Michx). cv. Noble (PD-resistant) and *V. vinifera* L. cv. Chardonnay (PD-susceptible). The concentration of total amino acids in xylem fluid was 3.1 to 3.7, and 1.0 to 1.8 mM for *V. rotundifolia* cv. Noble and *V. vinifera* cv. Chardonnay, respectively; glutamine generally accounted for more than 80 % of the profile for both species. Number of *Xf* colony forming units was promoted or inhibited depending on the interaction of *Xf* strain *Vitis* species, source of xylem fluid. Since no treatment completely inhibited colony growth, the bacteriocidal effect of xylem fluid plus lytic peptide was tested. The minimum inhibitory concentration (MIC) for 100% inhibition of *Xf* in agar culture was: cecropin A or B $\leq$ 1 µM, indolicidin 9.5-47.0 µM, magainin II > 80 µM, tetracycline $\leq$ 100 µM and lysozyme > 1000 µM. The activity of cecropin B in xylem fluid of *V. rotundifolia* cv. Noble was progressively reduced with time. There was a substantial amount of bacterial growth at 2 or 10 µM concentrations of cecropin A and cecropin B only after 24 hour duration of cecropin and xylem fluid; shorter time intervals did not degrade the cercopins and kill the bacteria. Cecropin B was less efficient in killing large-sized colonies compared to small colonies (Figure 2). This result suggested that *Xf* cell aggregates may serve as a protective mechanism, whereby external cells are attacked by cecropin B while the internal cells are protected. Tricine SDS-PAGE gel electrophoresis of cecropin B (10 µM) in xylem fluid of *V. vinifera* cv. Chardonnay showed that cecropin B degraded substantially and completely after 96 hours in xylem fluid (Figure 3). Similar result was obtained when cecropin B was exposed to protein phase of xylem fluid. Treatments with cecropin B (10 µM) with the filtrate phase did not show a reduction in the intensity of the cecropin B band after 24 hours, and it was still detected after 96 hours. No degradation of cecropin B was observed (even after 96 hours) when mixed with xylem fluid that had been previously boiled (100 ºC) suggesting that xylem fluid proteins were denatured.

The presence of OMPs of *Xf* with sulfur-containing amino acids was investigated. We verified the presence of OMPs with sulfur-containing amino acids by SDS-PAGE gel using specific dye for thiol groups, 5-iodoacetamidofluorescein (IAF) (Figure 4). Different strains of *Xf* from different hosts showed distinct patterns of OMPs with sulfur-containing amino acids. The results indicate the presence of thiol groups in several OMPs of *Xf*. In this SDS-PAGE gel, serum bovine albumin and the molecular mass marker were not labeled by IAF, indicating the specificity of dye by sulfur residues.

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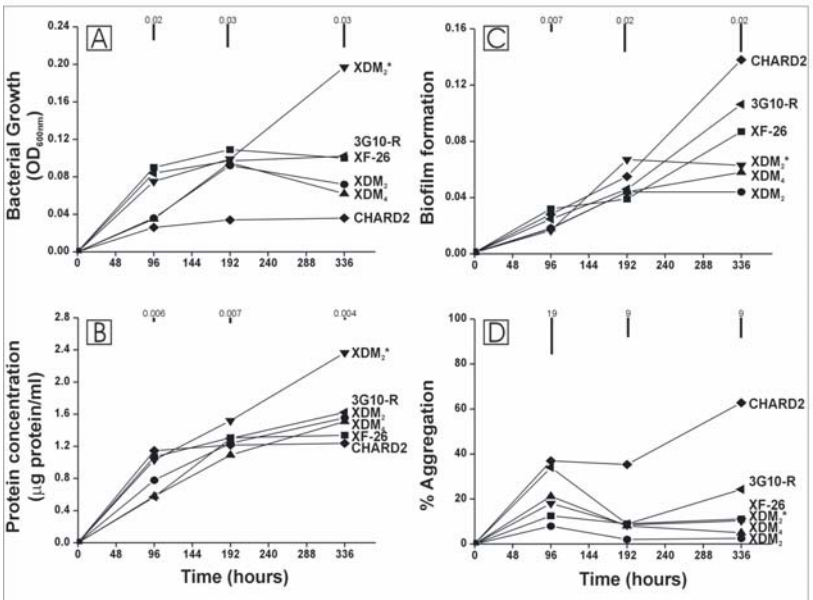

Figure 1 – Growth curves of *Xylella fastidiosa* in chemically-defined media over a period 336 hours at 28ºC. A - OD_{600nm}; B- Protein concentration; C - Biofilm formation and D – Percentage of aggregation. Bars above each data point correspond to lsd, 0.05.

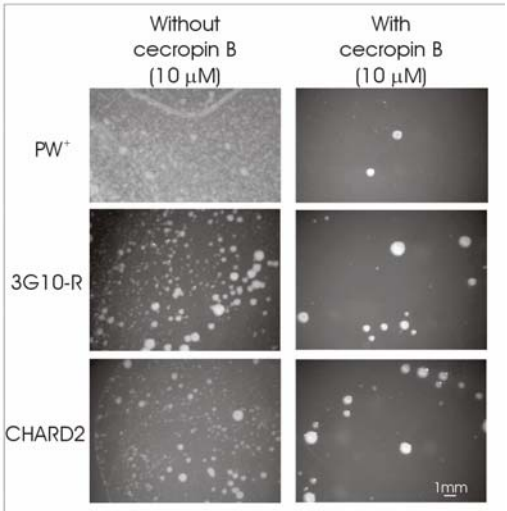

Figure 2 : Colony size of *Xylella fastidiosa* after incubation in cecropin B (10 µM) for 1 hour . Before the treatments *X. fastidiosa* cells were grown in liquid 3G10-R, CHARD2 and PW+ media We assumed that planktonic cells or small-sized aggregates would generate small-sized colonies (0.1mm in diameter); large-aggregates would promote large-sized colonies (>0.1 mm). Bar= 1 mm.

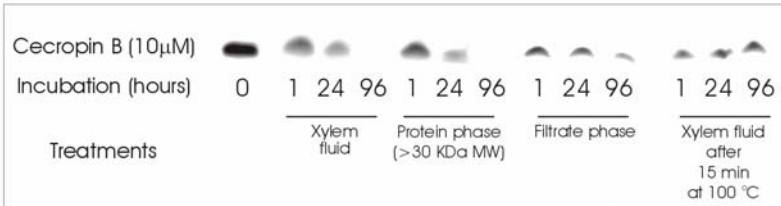

Figure 3: Tricine -SDS-PAGE of lytic peptide after incubation in xylem fluid from *Vitis vinifera* cv. Chardonnay. Cecropin B (10 µM) treated with xylem fluid, protein phase (components of xylem fluid with >30 KDa MW), filtrate phase (components of xylem fluid with <30 KDa MW) and boiled xylem fluid at 0, 1, 24 and 96 h of incubation. Lane MW corresponds to standard molecular weight: myosin (120 KDa), BSA (84 KDa), ovalbumin (60 KDa), carbonic anhydrase (39 KDa), trypsin inhibitor (28 KDa) and lysozyme (18 KDa).

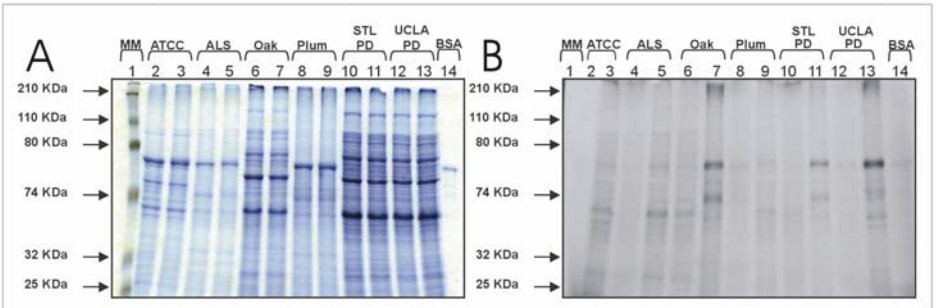

Figure 4: SDS -PAGE gels of surface proteins from different strains of *Xylella fastidiosa* : (A) Gel stained with Coomassie brilliant blue of proteins obtained after cell lysis (lanes 2-13). (B) Proteins isolated from the 5 -iodoacetamidofluorescein (5 -IAF)-labeled cells (lanes 2, 4, 6, 8, 10, and 12) and after treatment with dithiotheitrol (DTT) (lanes 3, 5, 7, 9, 11, and 13). 5-IAF labels sulfur containing amino acids in outer membrane proteins. DTT cleaves disulfide bonds of outer membrane proteins. Notice that proteins bands displayed represent proteins that were localized outside of *X. fastidiosa* cells (facing the environment). Bovine serum albumine (BSA) was used as control (lane14). Prestained molecular mass markers are shown on first lane (MM).

BIOLOGICAL, CULTURAL, GENETIC, AND CHEMICAL CONTROL OF PIERCE'S DISEASE: SIGNIFICANCE OF RIPARIAN PLANTS IN THE EPIDEMIOLOGY OF PIERCE'S DISEASE

Project Leader:
Kendra Baumgartner
USDA-ARS
Davis, CA 95616

Cooperators:
Alexander H. Purcell
Division of Insect Biology
University of California
Berkeley, CA 94720

M. Andrew Walker
Dept. of Viticulture and Enology
University of California
Davis, CA 95616

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ABSTRACT
The goal of this research is to evaluate the significance of riparian hosts in the epidemiology of Pierce's disease (PD) in the North Coast grape-growing region of California. Our first objective is to examine the epidemiological role of seasonal *Xylella fastidiosa* (*Xf*) concentration fluctuations in riparian hosts in the field, where plants are subject to seasonal temperature changes. Among systemic riparian hosts, differences in seasonal *Xf* concentrations and *Graphocephala atropunctata* (blue-green sharpshooter, BGSS) feeding preference affect their importance as *Xf* reservoirs. Temperature affects *Xf* concentrations in plant hosts and, in turn, *Xf* concentrations affect the probability of a BGSS acquiring *Xf* while feeding on an infected plant. We focused on *Xf* concentrations in five systemic hosts: *Rubus discolor* (Himalayan blackberry), *R. ursinus* (California blackberry), *Sambucus mexicana* (blue elderberry), *Vinca major* (periwinkle), and *Vitis californica* (California grapevine). We needle inoculated potted plants of California grape, California blackberry, Himalayan blackberry, blue elderberry, and periwinkle in the greenhouse. After confirming infection with PCR, we transferred infected plants to two sites in the North Coast (Napa County and Mendocino County). Populations of *Xf* reached detectable levels in all five riparian hosts located at our Napa County site in October 2003. Every replicate plant of periwinkle and California grapevine showed typical leaf scorch symptoms of PD, which is not surprising given the high concentrations of *Xf* detected among them. The fact that none of the Himalayan blackberry showed symptoms, despite high *Xf* concentrations, suggests that Himalayan blackberry is more tolerant of *Xf* infection. Assuming our results reflect that of naturally-established riparian hosts in the field, *Xf* concentrations in California grapevine, Himalayan blackberry, and periwinkle are still sufficient for acquisition by BGSS in autumn. Dilution plating results have not yet been obtained from our Mendocino County site and real-time PCR reactions have not been completed for plants from either site. We will continue to estimate *Xf* concentrations in plants at both sites using dilution plating and real-time PCR on a seasonal basis. *Xf* concentration estimates from each plant species at each site will be averaged among replicate plants. The effects of plant species, season, and location on mean *Xf* concentration will be determined using an analysis of variance. Results obtained using the two quantitation techniques will be analyzed separately.

INTRODUCTION
Past research (Purcell 1976, 1981) demonstrated the direct relationship between incidence of Pierce's Disease (PD) in *Vitis vinifera* and proximity to riparian plants bordering vineyards in the North Coastal grape-growing region of California. Vineyard rows closest to riparian plants that occupy the banks of rivers and streams experience the heaviest losses, but rows have fewer diseased vines with increasing distance away from riparian plants. Riparian habitat adjacent to vineyards contains plant species that serve as feeding and breeding hosts for *Graphocephala atropunctata* (blue-green sharpshooter, BGSS), the most efficient vector of PD in the Napa Valley (Hewitt et al 1949, Purcell 1975). Not only do many riparian plant species provide habitat for BGSS, but some also serve as reservoir hosts of the PD strain of *Xylella fastidiosa* (*Xf*) (Freitag 1951). A variety of common riparian plants, including native and non-native trees, shrubs, and herbaceous annuals, are capable of maintaining *Xf* infections without expressing disease symptoms. Purcell and Saunders (1999) found that *Xf* populations are, generally, lower in riparian hosts than in grapevines. The ability of *Xf* to multiply and spread within a plant host varies from species to species. After screening several breeding hosts of BGSS for systemic movement of *Xf*, Hill and Purcell (1995) found that only two, *Rubus discolor* (Himalayan blackberry) and *Vitis vinifera* (grapevine), supported systemic *Xf* populations. These results imply that some riparian hosts are likely more important than others as reservoirs for the spread of *Xf* to grapevines.

Interactions among BGSS, *Xf*, and their host plants are likely to vary from season to season. Aside from the obvious effects of season on BGSS breeding, seasonal changes in BGSS flight activity have been documented (Feil et al 2000). Seasonally variable levels of plant hormones (Hopkins 1985) and changes in temperature (Feil and Purcell 2001) can have major effects on *Xf* concentrations in host plants. *Xf* concentrations change on a seasonal basis in *Vitis labrusca* (Hopkins and Thompson 1984) and they are lower in *V. vinifera* grown at cooler temperatures (Feil and Purcell 2001). The efficiency of *Xf* acquisition and transmission by BGSS is influenced by the concentration of *Xf* in the plant host; the higher the concentration of *Xf*, the higher the probability of BGSS acquiring *Xf* while feeding (Hill & Purcell, 1997). Therefore, we might expect that in

riparian hosts, seasonal fluctuations of *Xf* concentrations may influence the spread of PD to grapevines by affecting the proportion of BGSS that acquire *Xf* when feeding on riparian hosts.

OBJECTIVES

The goal of this research is to evaluate the significance of riparian hosts in the epidemiology of PD in the North Coast. Among systemic riparian hosts, differences in seasonal *Xf* concentrations and vector feeding preference affect their importance as *Xf* reservoirs. Temperature affects *Xf* concentrations in plant hosts. *Xf* concentrations affect the probability of a BGSS acquiring *Xf* while feeding on an infected plant. Probability of *Xf* acquisition is also influenced by how attractive a host is to BGSS; a systemic riparian host that is fed upon more frequently by BGSS will likely serve as a more significant source of *Xf*. The first objective is to examine the epidemiological role of seasonal *Xf* concentration fluctuations in riparian hosts in the field, where plants are subject to seasonal temperature changes. By measuring seasonal concentrations of *Xf* in riparian plants, we will determine if and when concentrations are high enough for acquisition by BGSS. We will focus on five systemic hosts of *Xf*: *Rubus discolor* (Himalayan blackberry), *Rubus ursinus* (California blackberry), *Sambucus mexicana* (blue elderberry), *Vinca major* (periwinkle), and *Vitis californica* (California grapevine). Future research will focus on BGSS feeding preference for these five riparian hosts.

We will test the hypothesis that *Xf* concentrations are the same among five riparian hosts at two sites in the North Coast (Napa Co. & Mendocino Co.). In October 2002, we propagated California grape, California blackberry, Himalayan blackberry, blue elderberry, and periwinkle (100 plants/species) in our greenhouse at UC Davis. In February 2003, we mechanically inoculated all plants with the STL strain of *Xf* (a strain isolated from PD-symptomatic vines near Yountville, CA) in the greenhouse, where there are more uniform environmental conditions for infection development than in the field. In June 2003, we used PCR to confirm infection using PCR primers of Minsavage et al (1994) and Pooler et al (1995). In July 2003, we transferred infected plants from the greenhouse to two sites in the North Coast (50 replicate potted plants x five plant species = 250 plants/site). Plants are in 3-gallon pots and are surrounded by a fine-mesh screen enclosure. On a seasonal basis (except during the dormant season), *Xf* concentrations are estimated from petioles located distal to the stem inoculation site using dilution plating and real-time PCR. *Xf* concentration estimates from each plant species at each site are averaged among replicate plants. The effects of plant species, season, and location on mean *Xf* concentration is determined using an analysis of variance. Results obtained using the two quantitation techniques will be analyzed separately.

RESULTS

We inoculated 100 plants per riparian host in the greenhouse. Using PCR, we confirmed the following numbers of infected plants/species: Himalayan blackberry, 40; California blackberry, 12; blue elderberry, 78; periwinkle, 68; California grapevine, 37. Among infected plants, half were transferred to our Napa County site and the remaining half were transferred to our Mendocino County site.

To date, we collected our first set of petioles for *Xf* quantitation from plants at both sites. Table 1 contains culture attempt results from the Napa County site. (Plates are still incubating from Mendocino County site and real-time PCR reactions from both sites have not yet been completed). Results discussed will focus on autumn *Xf* concentrations in riparian hosts located at our Napa County site as obtained from dilution plating.

Populations of *Xf* reached detectable levels in all five riparian hosts located at our Napa County site in October 2003 (Table 1). Every replicate plant of periwinkle and California grapevine showed typical leaf scorch symptoms of PD, which is not surprising given the high concentrations of *Xf* detected among them. The fact that none of the Himalayan blackberry showed symptoms, despite high *Xf* concentrations, suggests that Himalayan blackberry is more tolerant of *Xf* infection. *Xf* concentrations of at least 10^4-10^5 CFU/g of plant tissue are required for acquisition by BGSS (Hill and Purcell 1997). Assuming our results reflect that of naturally-established riparian hosts in the field, estimated *Xf* concentrations in California grapevine, Himalayan blackberry, and periwinkle are sufficient for acquisition by BGSS in early autumn. Our autumn culture attempt coincided with the increased flight activity of young adult BGSS, which peaks in mid summer and remains high through early autumn (Feil et al 2000). Assuming BGSS feeds on California grapevine, Himalayan blackberry, and periwinkle in early autumn, *Xf* may be transmitted from infected riparian plants to adjacent vineyards before the end of the growing season. Late season infections of grapevines are unlikely to result in chronic disease and infected canes are pruned out during the dormant season (Purcell 1981). However, young adult BGSSs that acquire *Xf* in mid summer to early autumn and survive the winter are still capable of transmitting *Xf* the following spring after budbreak.

Table 1. Culture of *Xylella fastidiosa* from riparian hosts in the field following mechanical inoculation.

Species	Number Infected	Number Contaminated	Mean CFU/g [a]	Range CFU/g	Incubation(days) [b]
Himalayan blackberry	9	0	6×10^5	6.7×10^1 to 2.9×10^6	210
California blackberry	3	0	2×10^2	2×10^2	210
Blue elderberry	1	0	2×10^2	2×10^2	210
Periwinkle	30	0	1.3×10^7	6.6×10^3 to 8.0×10^7	210
California grapevine	5	0	6.6×10^6	6.4×10^3 to 9.7×10^6	210

[a] Colony forming units per gram of petiole tissue.
[b] Number of days between inoculation in spring 2003 and culture attempt in autumn 2003.

CONCLUSIONS

Riparian Revegetation Management is a method of PD control that focuses on removal of host plants of BGSS and *Xf* (host plants other than grapevines), followed by revegetation with native, non-host plants. This method of PD control has been shown to reduce local populations of BGSS (unpublished research, Alexander H. Purcell, Division of Insect Biology, UC Berkeley), but its impact on the riparian area as a reservoir of *Xf* has not been quantified. To obtain approval for a Lake and Streambed Alteration Agreement (1600 permit) from the California Department of Fish and Game, grape-growers interested in pursuing this method of PD control develop a management plan that includes characterizing the plant community in the riparian area, targeting individual plants for removal, and selecting replacement plant species that will provide a similar habitat for wildlife, as a source of shelter, food, and nesting sites. This method of PD control has some positive aspects. With lower BGSS populations, fewer insecticide applications are used in the vineyard. Some of the plants targeted for removal, such as Himalayan blackberry and periwinkle, are invasive weeds. However, removal of large sections of riparian vegetation is very disruptive to wildlife, it increases the probability of streambank erosion, and some of the riparian hosts are extremely difficult to eradicate.

We only have general knowledge of the role of riparian hosts in the epidemiology of PD. Overwintering hosts of *Xf* likely play an important role in the epidemiology of PD in providing a source of bacteria for spring infections, especially near vineyards where infective adult BGSS do not survive the winter (Purcell and Saunders 1999). BGSS transmission of *Xf* from riparian plants to grapevines in spring is more likely than mid- or late-season infections to result in chronic disease (Purcell 1981). Based on the results of this past research, we anticipate that removal of overwintering hosts from riparian habitat adjacent to vineyards will decrease disease incidence. However, not all of these hosts are known to attain adequate *Xf* concentrations for acquisition early in the growing season.

The fewer riparian plants removed before revegetation, the less disruption to wildlife habitat. The success of revegetation management depends on a thorough understanding of how riparian hosts contribute to the spread of PD. We know which plants are *Xf* hosts. We don't know if or when these hosts attain high enough concentrations of *Xf* for acquisition (10^4 colony forming units/g of plant tissue) by BGSS in the field. Some hosts may not attain acquisition concentrations of *Xf* until late summer. Late summer transmission of *Xf* to grapevines is thought to result in local infections of short sections of canes that go dormant (and are then pruned off) before *Xf* has a chance to establish a systemic infection. Systemic infection leads to permanent disease, while local infections, if pruned out, do not. If the results of this research reveal that only a few of the riparian hosts recommended for removal serve as major sources of *Xf* in spring, grape-growers can concentrate on removing fewer riparian plants, thereby reducing the total amount of riparian habitat disruption.

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FUNDING AGENCIES

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FUNCTIONAL GENOMICS OF THE GRAPE-*XYLELLA* INTERACTION: TOWARDS THE IDENTIFICATION OF HOST RESISTANCE DETERMINANTS (CUMULATIVE PROGRESS REPORT)

Project Leaders:
Douglas R. Cook
Francisco Goes-da-Silva
Hyunju Lim
Department of Plant Pathology
University of California
Davis, CA 95616

Cooperators:
M. Andrew Walker
Alberto Iandolino
Department of Viticulture and Enology
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted from April 2001 to October 2003.

INTRODUCTION

Pierce's disease (PD), caused by the bacterial pathogen *Xylella fastidiosa* (*Xf*), is one of the most destructive diseases of grapevines (Purcell and Hopkins, 1996). All genotypes of *Vitis vinifera* are susceptible to the PD pathogen and only certain non-vinifera species (e.g., *V. arizonica*), typically not suitable for wine production, are able to resist or tolerate this pathogen. Development of resistant varieties through classical breeding is complicated by the desire to retain varietal phenotypes in cultivated species, and by the generally poor agronomic properties (e.g., fruit quality) of these non-vinifera species. An alternative approach for developing disease resistant germplasm is to characterize the molecular basis of resistance and susceptibility in *Vitis* species, and to use this information to design rational strategies for crop protection.

In this project we are pursuing a genomics approach to identify transcriptional pathways that are correlated with susceptible or resistant interactions in *Vitis* species. The comparison of these two distinct interactions should reveal functional elements of the host resistance response, or conversely host functions that confer susceptibility. Such information will considerably increase our knowledge of the grape-*Xylella* interaction and potentially provide the basis for developing resistance to the PD pathogen in *V. vinifera*. A side benefit of these activities will be information that is either of direct relevance to Pierce's Disease (e.g., identification of novel *Xylella*-responsive promoters for gene regulation, improved molecular markers for traditional breeding of PD resistance, and alternative diagnostic methods based on host gene expression), or that is enabling to grape improvement generally (public EST databases, genome-wide molecular markers (e.g., SSR and SNPs), and a public grape oligonucleotide microarray).

OBJECTIVES

1. Construct and archive cDNA libraries from (a) infected and non-infected grape genotypes representing both susceptible *V. vinifera* and resistant/tolerant *Vitis* spp., and (b) a range of tissues and developmental stages to increase gene discovery.
2. Conduct sequencing reactions for 60,000 cDNA clones and submit cleaned, high quality sequence reads to the National Center for Biotechnology Information (NCBI).
3. Develop an analysis pipeline and web-accessible relational database for the grape transcriptome, including an annotated unigene set and the identification of candidate *Xylella*-induced genes.
4. Conduct transcriptional profiling to characterize host gene expression in susceptible and resistant/tolerant grape-*Xylella* interactions.

RESULTS AND CONCLUSIONS

A. EST sequencing

Susceptible *Vitis vinifera*. 120 plants of Cabernet Sauvignon were sampled over a six month period to identify infected and non-infected individuals. Tissue collected from these plants was subject to mRNA extraction, cDNA library construction, and sequencing of Expressed Sequence Tags (ESTs). Because symptom development in *Vitis vinifera* is a function of both bacterial infection and plant development, we sequenced cDNAs from plants collected early in the growing season when the pathogen could be detected but symptoms were not evident, as well as late in the growing season when symptoms were evident on infected plants. Eight cDNA libraries and >30,000 sequence reads were generated by the project.

Resistant *Vitis* germplasm

Working with Dr. Andrew Walker of the Department of Viticulture and Enology at UC Davis, we obtained tissue from resistant progeny of a cross between *Vitis rupestris* and *Vitis arizonica*. Two cDNA libraries were prepared, picked into 384-well plates, and archived at -80°C. 10,000 cDNA sequences were obtained and analyzed to assess variation between the *rupestris/arizonica* cross and the more extensive dataset of *Vitis vinifera* (see below).

Continued sequencing in *Vitis vinifera*

Nine additional cDNA libraries were constructed from *Vitis vinifera*, with the goal of expanding the number of characterized genes available for development of an oligonucleotide microarray. In total, 46,493 sequencing reactions were completed from these cDNA libraries, resulting in the submission of 36,966 sequences to the National Center for Biotechnology Information EST database.

Table 1. Overview of the UC Davis *Vitis* sequencing project: 2001-2003.

Tissue	Total	Submitted	Funding
infected leaves	13206	9590	APHIS
healthy leaves	12764	8114	APHIS
berries*	21170	15237	ARS
cmpd bud	4992	4362	ARS
flower**	9984	8176	APHIS/CDFA
stem	5355	4700	CDFA
petiole	4992	4491	CDFA
Vitis spp	10752	6533	CDFA
Total	**83215**	**61203**	

*Berry libraries: Pre-veraison, Stage I; Pre-veraison, Stage II; Veraison; Post-veraison.
**Flower libraries: pre-anthesis and post anthesis.

B. Bioinformatics

We developed a laboratory information management system (LIMS) database in Oracle 8 to organize sequence information. The associated chromatogram pipeline downloads information from our ABI 3730XL sequencer nightly, processes chromatograms through a standard QC pipeline consisting of the PHRED and X-match algorithms, and generates NCBI submission files. Cleaned and trimmed FASTA format files are submitted to the NCBI GenBank EST database. We subsequently retrieve NCBI accession numbers and store the hyperlink in our Oracle database so that chromatograms can be automatically traced to the public record.

We have also developed an on-line Oracle relational database for analysis of the complete set of public *Vitis vinifera* EST data. The database begins with a contig assembly pipeline consisting of MegaBLAST and CAP3. The following analyses are performed on all EST singletons and contigs: (1) BLASTX against the NCBI non-redundant database to assess protein-coding similarity, (2) BLASTN against all *Vitis* data to identify close homologs (potential orthologs) from other *Vitis* species, and (3) identification of potential polymorphic sites as simple sequence repeats, including automated oligonucleotide primer design for genetic mapping. We have developed a controlled vocabulary that organizes genes according to three ontologies: (I) genotype (cultivar or species), (II) development (e.g., berries pre-veraison, or flower pre-anthesis), and (III) stress (e.g., biotic *Xylella fastidiosa*, or abiotic drought). A related query tool permits users to query the data for genes expressed in grape under specific conditions of genotype, development or stress. For example, one can construct a search to identify genes whose expression is enhanced in *Xylella*-infected tissue, or expressed in Stage II of berry development, or in pre-anthesis flowers. The database can also be queried based on unique identifiers (e.g., GenBank accession numbers), key words (e.g., chalcone synthase), at the nucleotide level based on BLASTn, or for specific simple sequence repeat motifs.

Because it is important to view all *Vitis* data simultaneously, we routinely download public *Vitis* data and incorporate them into our assemblies. As of October 15, 2003, the NCBI dbEST contains 135,601 *V. vinifera* sequences. The current *Vitis* unigene set consists of >25,000 predicted transcripts, including ~12,500 contigs. An important feature of many of these contigs is that they contain paired end reads from single clones, where sequence reads overlap. This feature of paired end reads is a consequence of our strategy to sequence both ends of every cDNA clone. The results of this strategy are (1) accurate 3' data to facilitate design of microarray oligonucleotides, and (2) experimental validation of transcript structure, with rapid identification of full-length or near-full length cDNA clones.

The current version of our *Vitis* EST database (http://cgf.ucdavis.edu) is based on the analysis of ~84K sequences available as of May 1, 2003. Our programmers are in the process of analyzing the October 1, 2003 data freeze that will constitute the basis of the Affymetrix microarrays. This new build of the database will include ESTs from the following species: 135K ESTs from *V. vinifera*, 6,500 ESTs from a genetic cross of *V. arizonica x V. rupestris*, and several thousand ESTs from a range of related *Vitis* species.

C. Genetic variation in Vitis: Analysis of diversity at the sequence level

One question that was raised by reviewers of our original proposal was whether EST data obtained from one variety of grape would be sufficiently representative of *V. vinifera* to develop a common microarray for use across the species. Similarly, reviewers questioned whether sequence from *V. vinifera* would be suitable for developing microarrays that would function in other *Vitis* species, in particular those with "resistance" to Pierce's Disease. To address these questions, we conducted sequence similarity analyses both within *V. vinifera* (between varieties) and between *V. vinifera* and other *Vitis* species.

Variation between *Vitis vinifera* varieties

We analyzed the public data available as of May 13, 2003, composed of 37,902 ESTs from Cabernet Sauvignon (this project), 40,437 ESTs from Chardonnay, ESTs from 8,191 Shiraz, and 1,157 ESTs from other *Vitis vinifera* varieties. The genome of all grapes is highly heterozygous, and therefore allelic variation is common even within a single variety. To quantify allelic variation we conducted multiple sequence alignment of the public ESTs to give us the first detailed glimpse of genetic variation at the species level. In particular, we analyzed the structure of contigs that contained sequences from at least two different varieties, and where each variety was represented by multiple sequences within the contig. This practice allowed us to distinguish random sequencing error from systematic base pair variation (i.e., variation present in multiple sequences within a contig). Such systematic sequence variation is presumed to represent single nucleotide polymorphism (SNP), the most frequent type of natural genetic variation found within species. Two general themes emerged from this analysis. First, the majority of sequences from Cabernet Sauvignon, Chardonnay and Shiraz are 100% identical, and (when present) the level of allelic variation observed within a varietal genome is not substantially higher than variation between genomes (typically >99% identity).

Variation between *Vitis* species

To address the issue of DNA sequence variation between *V. vinifera* and other *Vitis* species, we compared the following data sets:

***V. vinifera* x *V. aestivalis*.** On a contract basis, the CA&ES Genomics Facility sequenced 2,500 cDNAs of *Vitis aestivalis*. These data were deposited at NCBI and were therefore available for comparison of sequence conservation with the much deeper collection of *Vitis vinifera* sequence information. BLASTn analyses and the corresponding pairwise alignments for these two species revealed that the vast majority of sequences possess identity in the range of 98-100%, indicating that the transcripts of these two genomes are remarkably well conserved.

***Vitis vinifera* and *V. arizonica/V. rupestris*.** A similar analysis was conducted on the contig information generated by our *V. arizonica* X *V. rupestris* sequencing effort. The vast majority of comparisons to *Vitis vinifera* have e-values of zero and sequence identities in the range of 98-100%.

The high levels of sequence similarity between *V. vinifera* and other *Vitis* species suggests that it will be possible to produce oligonucleotide arrays with comparable specificity for all of these genomes, using the *Vitis vinifera* data as the reference. Such cross-species use of oligonucleotide arrays are not uncommon in the genomics community, as recently applied in the comparison of human-chimps-monkeys and mice.

Identification of single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs) in the grape genome(s)
Allelic variation is the basis of many types of genetic studies, including association genetics and genetic mapping, and a methodical listing of allelic variation should have utility for current projects that aim to map traits for PD resistance. The *Vitis vinifera* GeneIndex contains a SNP (single nucleotide polymorphism) link for all contigs where SNPs have been identified, and technologies such allele specific oligonucleotide (ASO) primer extension can be used to rapidly convert this information into gene-specific genetic markers. Equally useful, however, is a class of genetic variation termed Simple Sequence Repeat (SSR) markers. Thus, we have implemented an SSR identification pipeline to mine the grape unigene set for SSR motifs. The data is organized in an Oracle database, and is accessible via the "http://cgf.ucdavis.edu" web site. Both of these data types are collateral benefits of our funded EST project and should impact many areas of grape improvement, including the genetic analysis of PD resistance.

D. Analysis of the grape transcriptional response to pathogen challenge

The analyses 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.

Identification of *Xylella*-induced genes in *Vitis vinifera*.

We have identified several (~35 in total) genes that appear to be up-regulated in response to infection by *X. fastidiosa* (see table below). The most abundant contig (1007061) shares homology with a stress-related RNA from Arabidopsis, although the function is unknown in any system. Interestingly, this gene is upregulated in infected plants, prior to symptom development, making it a top candidate for an early and sensitive marker of Pierce's Disease. Some 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 below) we are poised to isolate the promoters from these genes from genomic DNA libraries. The potential application of such promoters to drive *Xyella*-induced and/or tissue specific expression of transgenes is planned as a topic of a future grant proposal.

Development of Real-Time reverse transcriptase PCR for gene expression analyses and improved diagnostic tools for pathogen detection
Ultimately, detailed analysis of transcriptional responses will require methodical analysis by means of microarray studies, to be initiated in 2004. At the same time, the current list of putatively *Xylella*-induced genes may provide leads for further analysis by means of real time reverse transcriptase PCR. We are particularly interested in developing host transcripts that can serve as markers of *Xylella* infection; such markers may be more sensitive (e.g., expressed systemically in locally-

infected plants) than pathogen-based PCR primers for diagnosis of Pierce's Disease. This strategy has been referred as "transcriptional fingerprinting", and is considered to hold great potential for diagnostic analyses.

We have generated primers and probes for real-time reverse transcriptase PCR analysis of 20 candidate *Xylella*-induced transcripts. These primers and probes are being used to monitor gene expression of infected and non-infected plant samples collected from the Napa Valley of California in the months of July and September, 2003 and ultimately from plants grown in growth chamber conditions (a more controlled environment). Results from these analyses will be presented.

Transcriptional profiling

In grapes the development of transcriptional profiling tools that function broadly across all species of interest (i.e., susceptible *Vitis vinifera* and resistant *Vitis* spp.) has the potential to impact grape improvement on multiple fronts. In the case of Pierce's Disease we can anticipate the following outcomes: (1) transcriptional fingerprints for diseased plants may facilitate more reliable diagnosis, e.g., by detecting systemic responses in the host, (2) critical evaluation of the physiology of the host during symptom expression, examining long-standing but untested hypotheses such as (a) the relationship between water stress and disease, (b) the relationship between host development and disease symptom expression, or (c) how source-sink relationships change in infected versus healthy plants during fruit development, and (3) identifying transcriptional response pathways that are correlated with disease resistance, tolerance or susceptibility. Many of the genes induced in resistant interactions may be causal to resistance, for example the induction of insecticidal or anti-bacterial proteins and peptides, or the induction of host secondary metabolic pathways leading to anti-bacterial or insecticidal compounds, would be prime candidates for the development of resistance to *Xylella fastidiosa* in *Vitis vinifera*.

We anticipate having Affymetrix microarrays available in February 2004. Using the Affy arrays, we will initiate experiments to address the following questions: (1) which host genes are induced during resistant, tolerant and susceptible interactions with *Xylella fastidiosa*; (2) what is the relationship between water stress (drought) and infection by *Xylella fastidiosa*?

Genome-wide identification of transcripts in *Vitis vinifera*

As a preliminary effort towards transcriptional profiling we have used statistical tools to analyze gene expression in the public grape EST datasets. Our initial analysis focused on the 84,000 ESTs available in the National Center for Biotechnology Information (NCBI) EST database (DbEST) as of May 2003. These expressed sequence tags (ESTs) were representative of different cultivars, different stages of plant development, and tissue exposed to biotic (e.g., infection by *Xylella fastidiosa*) and abiotic (e.g., drought, salt and cold) stress factors. Correlation analysis was used to select 2821 contigs (preidcted genes) for clustering based on Euclidian Distance and Principal Component Analysis. As expected, both types of analyses revealed that gene expression profiles were highly predictive of plant development. Thus, gene expression profiles resolved leaves, from roots, from berries. Moreover, developmental stage was also highly correlated with gene expression, such that young leaves clustered together and separate from old leaves, while pre-veraison berries from Stage I and Stage II had were more similar to one another than to veraison or post veraison berries, etc.

In terms of Pierce's Disease, there are three potentially important trends in the data. First, the statistical analysis confirmed our previous selection of genes correlated with Pierce's Disease (described above and in the Figure below). Second, transcripts that cluster with putative *Xylella*-induced transcripts become strong candidates for further study, now involving dozens of transcripts. Third, principal component analysis (data not shown) suggests that on infected plants late in the season (when fruit would normally be maturing) there is a significant shift in gene expression, such that they are more similar to young leaves. This change may reflect a shift in source-sink relationships brought as a consequence of aborted berry development in infected individuals.

FUNDING AGENCIES

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ROOTSTOCK VARIETY INFLUENCE ON PIERCE'S DISEASE SYMPTOMS IN GRAFTED CHARDONNAY AND CABERNET SAUVIGNON (*VITIS VINIFERA* L.) GRAPEVINES

Project Leaders:
Peter Cousins
Plant Genetic Resources Unit, USDA-ARS
New York State Agricultural Experiment Station
Geneva, NY 14456

Jiang Lu
Center for Viticulture and Small Fruit Research
Florida A&M University
Tallahassee, FL32317

Cooperators:
Zhongbo Ren
Center for Viticulture and Small Fruit Research
Florida A&M University
Tallahassee, FL

Reporting Period: The results reported here are for work conducted from November 1, 2002 to October 31, 2003.

ABSTRACT
Chardonnay and Cabernet Sauvignon are *Vitis vinifera* scion varieties that are susceptible to Pierce's disease (PD). We evaluated the effect of rootstock variety on PD symptom expression in these varieties grown in a vineyard with high natural PD pressure and abundant vectors. No rootstock completely eliminates PD symptoms in the susceptible scions. However, rootstocks differ in the level of PD symptoms in the susceptible scions grafted to them and the percentage of vines bearing fruit varies with rootstock variety. Rootstocks appear to contribute to PD management in susceptible scion varieties, but are not independently capable of rescuing susceptible scions. Evaluation of PD management practices should emphasize vineyard testing to encourage the efficient identification and development of field suitable techniques and methods.

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

OBJECTIVES
Evaluate the impact of rootstock variety on expression of Pierce's disease symptoms in naturally infected PD susceptible *Vitis vinifera* scion varieties Chardonnay and Cabernet Sauvignon.

RESULTS AND CONCLUSIONS
Chardonnay (*Vitis vinifera*) vines grafted on nine rootstocks and own-rooted Chardonnay vines were planted in Tallahassee, Florida in the vineyard of the Center for Viticulture, Florida A&M University in the spring 2001 planting season (Table 1). The vineyard site has a high incidence of Pierce's disease and glassy-winged sharpshooters inhabit the site. Fruiting and vine vigor observations were made on June 19, Pierce's disease (PD) symptoms were evaluated on July 19, and survival observations were made in early October, 2003. PD symptoms on leaves were assessed and vines given a numerical score from 0 to 5, with 0 representing no symptoms, 1 = minor symptoms up to 15% of leaves with marginal necrosis (MN), 2 = 15-30% of leaves with MN, 3 = 30-50% of leaves with MN, 4 = 50-75% of leaves with MN, 5 = over 75% of leaves with MN or vine dead. There were four replicates for grafted vines with each replicate consisted of two vines of the same treatment (eight vines total per stion). The mean score of the two vines is recorded as the score for that replicate.

2003 is the first year in which the percent of vines fruiting was recorded. Chardonnay vines demonstrated substantial variation in number of vines that fruited (Table 1). Own rooted vines were almost all dead and no fruiting was observed on surviving vines. In contrast, half of the surviving vines grafted on O39-16 fruited, and more than a third of the original vines grafted on 5BB fruited (no vines grafted on 5BB have died to date). Impact on fruit yield is the critical feature of PD-foliar and cane symptoms, while severe in a given season, may not necessarily prevent fruit set and maturation. At the experimental vineyard site, PD pressure is sufficiently high that even some muscadine grapevines (*Vitis rotundifolia*) show PD symptoms. However, these muscadine vines do not succumb to PD, but recover. It may be that vines on selected rootstocks are recovering in a similar manner. Thus reduction in PD symptoms per se may not be as critical as promotion of regular bearing, since the experience with muscadines is that symptoms do not tell the entire story. Loomis (1952, 1965) reported that a rootstock with *V. vinifera* and *V. rotundifolia* parentage extended the life of susceptible scions in Mississippi,

but even Chardonnay on O39-16 showed severe PD symptoms in this trial-although note that the fraction of surviving vines in fruit was fairly high, at 40%, and that the best rootstock for promoting vine survival and fruiting was 5BB (vines grafted on 3309C had higher vigor, but lower fruiting percentage).

At the end of the 2002 season we observed high PD symptom scores in Chardonnay vines across all rootstocks. The recovery and fruiting of vines on selected rootstocks indicates that while reduction in PD symptoms may be occurring, regrowth or survival following infection may be more important for promoting and sustaining yield.

Cabernet Sauvignon grapevines grafted to nine rootstocks were planted in spring, 2002 and PD symptoms were scored on July 18, 2003. Vines grafted on 110R showed the fewest symptoms, while vines grafted on Freedom showed the most severe symptoms (Table 2). No Cabernet Sauvignon vines fruited this year.

Additional rootstocks grafted to Chardonnay and Cabernet Sauvignon were planted in spring 2002 to further investigate the possible influence of rootstock on PD expression. The rootstocks Dog Ridge, 161-49C, and Lenoir are of special interest. Dog Ridge and 161-49C have been reported as increasing vine longevity in areas of high PD pressure (Loomis 1952, 1965). Pierce (1905) suggested Lenoir as a rootstock to manage this disease.

No rootstock has produced 100% fruiting vines at the end of the third season of growth. However, all eight of the vines grafted on 5BB are still alive and three of these fruited this season. In contrast, only one quarter of the own-rooted vines are still alive (2 of 8) and neither of them fruited. Rootstock variety appears to have an impact on the survival and yield potential of susceptible scion varieties, judging from this limited study. Chardonnay and Cabernet Sauvignon grafted to additional rootstocks are in cultivation and will provide more information in coming years.

Rootstocks do not appear capable of completely rescuing or preventing PD infection of and damage to susceptible *Vitis vinifera* scion varieties (here Chardonnay and Cabernet Sauvignon). However, some rootstocks appear to enhance vine survival and fruiting-echoing the results of (Loomis (1965, 1952) and Magoon and Magness (1937). Combinations of particular rootstocks with other management strategies may be one way to enhance individual techniques which used alone are not wholly satisfactory.

This study testifies to the importance of field and vineyard studies and natural infection when investigating Pierce's disease management strategies and techniques. The study described here relies on high natural populations of vectors and abundant *Xf* populations, which are found at the test vineyard in Florida. In contrast, artificial inoculation may introduce unnaturally high levels of bacteria to the plant, obscuring potentially useful results by an overly strenuous test. Greenhouse and laboratory screening is expensive and typically requires constant plant care and maintenance, with daily care often necessary. In contrast, our grapevines in the vineyard in Florida are managed according to standard viticultural practices, including natural rainfall in place of artificial irrigation or individual pot watering, and consequently the cost for care of individual plants is quite low. Vineyard testing of management practices in a naturally infective environment is an efficient and effective approach to evaluating PD control strategies.

Table 1. PD symptom scores and vine survival, fruiting, and vigor ratings of Chardonnay grapevines.

Rootstock	Number of surviving vines	Percent vines fruiting	PD symptom score	Vine vigor	Survival index
O39-16	5	40	4.0	2.8	1.8
101-14 Mgt	7	14.3	3.5	3.7	3.3
110R	4	25	4.4	3.8	1.9
3309C	7	28.6	3.3	4.5	3.9
44-53M	4	25	4.0	3.4	1.7
5BB	8	37.5	3.5	3.5	3.3
5C	6	50	3.6	4.5	3.3
Freedom	3	33.3	4.0	4.2	1.8
Own-rooted	2	0	4.6	1.7	0.4
Ramsey	6	50	3.8	4.0	3.0

Vine vigor ratings:
1 = shoot growth below 1/2 main trunk
2 = shoot growth between 1/2 to 2/3 of the main trunk
3 = shoot growth between 2/3 to the top of trellis wire
4 = 1 cordon with shoots
5 = 2 cordons with shoots
Survival index = % vine survival x vine vigor (calculated from means)

Table 2. Pierce's disease rating of Cabernet Sauvignon grapevines.

Rootstock	Number of vines	PD symptom score
Freedom	7	4.4 a
101-14 Mgt	7	4.0 ab
Ramsey	7	4.0 ab
44-53M	8	3.6 ab
5BB	8	3.1 ab
5C	8	3.1 ab
O39-16	6	3.0 ab
3309C	8	2.9 ab
110R	8	2.7 b

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DEVELOPING TRANSCRIPTIONAL PROFILES AND MICROARRAY EXPRESSION ANALYSIS OF GRAPE PLANT RESPONSE TO *XYLELLA FASTIDIOSA*

Project Leader:
Hong Lin
Exotic & Invasive Disease & Pest Research Unit
USDA/ARS
Parlier, CA 93648

Cooperators:
Andrew Walker
Department of Viticulture and Enology
University of California
Davis, CA, 95616

Reporting Period: The results reported here are from work conducted from April 1, 2003 to October 16, 2003.

ABSTRACT
The goal of the project is to characterize the molecular events in the grape / *Xylella fastidiosa (Xf)* interaction. We are developing a genomic approach to identify transcriptional pathways correlated with susceptibility and resistance. Highly resistant and susceptible genotypes were selected from a *Vitis rupestris* x *V. arizonica* population. Reciprocal suppression subtractive hybridization (SSH) cDNA libraries for both resistant and susceptible genotypes that represent the complete spectrum of gene expression profiles in response to the *Xf* infection are being constructed. This strategy is efficient for cloning and identifying differentially expressed genes associated with signal recognition/transduction and potentially regulated interactions between pathogens and host plants. Based on annotation results, a subset of candidate genes will be selected for cDNA microarray expression analysis. The information derived from this study will help reveal the details of metabolic pathways of host responses and molecular mechanism(s) of grape resistance and susceptibility to *Xf*.

INTRODUCTION
The Pierce's disease (PD) threat to the California viticulture industry has been exacerbated by the recent introduction and establishment of the glassy-winged sharpshooter. Host plant resistance is critical component of integrated crop management and molecular genetic techniques are now available to optimize the development of *Xf* resistant plants. The sequences of four *Xf* strains from citrus, grape, almond and oleander are now available on Genebank (Simpson et al., 2000; Van Sluys et al., 2003) and provide new insights into all aspects of PD research. Information derived from the sequences allows us to understand the molecular basis of the bacterial pathogenicity. Characterization of host responses to *Xf* infection at the molecular level is an important step toward understanding mechanisms of host pathogenicity and resistance. It is clear that PD resistance exists in grape plants (Mortensen; 1967, 1977). Genetic breeding strongly supports inheritable PD resistance. Molecular mapping has linked DNA markers to *Xf* resistance (see Reports from Walker's grape breeding projects). However, details regarding how the genetic information transfers from DNA to RNA and eventually to functional gene products, and details on the molecular basis of pathogen recognition and subsequent activation of defense response in grape plants are very limited.

SSH (suppression subtractive hybridization) is a powerful tool that enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population, but not in the other (e.g. resistant genotypes vs. susceptible genotypes, infected vs. control). By using this molecular technique, we are able to selectively enrich these differentially expressed genes and clone them. There are several advantages of using this technique:

1. This approach is capable of removing most housekeeping genes during library construction and therefore increases the efficiency of cloning pathogen-induced genes.
2. The system works well with paired comparisons between segregated genotypes. In the case of PD, we used highly resistant and susceptible sibling progenies from a *V. rupestris* x *V. arizonica* cross. Thus the differences in gene expression patterns between genotypes and between experimental treatments likely reflect the molecular basis of resistance and susceptibility.
3. The SSH cDNA libraries approach normalizes expressed cDNAs during library construction and therefore significantly increases the chance of cloning expressed genes that are in low abundance. This is particularly important because many pathogen-related genes are expressed at low abundance, and limited to particular tissues or cell types (Caturla et al., 2002). Some of these genes are less likely to be cloned if a standard EST cloning method is used.

We will perform BLAST (Basic Local Alignment Search Tool) search from publicly available grape ESTs resources, and orthologous analysis against *Arabidopsis* and other plant databases, to annotate sequence information derived from SSH cDNA libraries. Differentially expressed genes associated with *Xf* resistance will be selected and analyzed using cDNA microarray technology. This high throughput process allows the parallel assessment of gene expression for thousands of genes. Combining SSH, cDNA microarray, and bioinformatic tools is an innovative way to effectively dissect gene expression profiles of grape plants in response to *Xf*. These gene expression patterns underlying metabolic pathways can help

to elucidate possible mechanisms involved in resistance and pathogenicity (Gu and Martin, 1998). This project is in conjunction with the PD resistance breeding/mapping program. We will also develop DNA molecular markers from resistance genes, which will help accelerate the PD resistance breeding program.

OBJECTIVES

1. Construct tissue-specific reciprocal Suppression Subtractive Hybridization (SSH) cDNA libraries from two sets of plants (resistant genotype vs. susceptible genotype; infected tissue vs. non-infected tissue).
2. Sequence and annotate expressed genes. Identify differentially expressed genes associated with disease development and resistance. Submit annotated sequenced genes to public domain.
3. Expression gene profile analysis using cDNA Microarrary technology. Identify genes associated with pathogenicity and genes linked to *Xf* resistance. Elucidate metabolic pathways involved in the pathogenicity and resistance mechanism(s).

RESULTS AND CONCLUSIONS

The project was funded in May 2003. Research is under way. Currently, we have completed green house experiment and working toward cDNA library construction.

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FUNDING AGENCIES

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FIELD EVALUATION OF GRAPE ROOTSTOCK RESPONSE TO NATURAL INFECTION BY PIERCE'S DISEASE

Project Leaders:
Jiang Lu
Zhongbo Ren
Center for Viticulture and Small Fruit Research
Florida A&M University
Tallahassee, Florida 32317

Peter Cousins
Plant Genetic Resources Unit, USDA-ARS
New York State Agricultural Experiment Station
Geneva, New York

Reporting Period: The results reported here are for work conducted from November 1, 2002to October 31, 2003.

ABSTRACT
To understand the adaptation of grape rootstocks commonly used in major grape production areas worldwide to Florida, where Pierce's dsease (PD) is the primary limiting factor in grape production, ten important grape rootstocks were cultivated at the experimental vineyard, Florida A&M University, Tallahassee, Florida. Disease resistance and symptoms and growing performance were evaluated. PD symptoms were scored in September and October 2002 and 2003, with leaf symptoms the basis of scoring. None of the grape rootstocks was completely resistant to PD and the severity of PD varied with rootstock cultivar. Ramsey and St George showed least PD symptoms, while Freedom and 3309C had the highest PD scores. Vine vigor was evaluated in the fall of 2002 and 2003, and varied among the rootstocks as evidenced by trunk diameter, annual shoot length, annual shoot node number, internode length, and shoot diameter. Shoot death rate in 2002 ranged from 7% to 56%, with Freedom the highest and O39-16 the lowest. The overall growth performance suggested that St George and Ramsey are the most suitable rootstocks in northern Florida environment where natural infection by PD is very high and vectors and inoculum are abundant.

INTRODUCTION
Rootstocks are used widely in viticulture to provide resistance against soil pests and pathogens and improve scion performance. Choice of rootstock depends on pest populations, soil, and growing conditions. The grape rootstocks in common use world wide are deployed primarily to provide phylloxera and nematode protection (Bouquet 1980, Einset and Pratt 1975, Winkler et al 1974). In contrast, Pierce's disease (PD), caused by gram-negative bacterium *Xylella fastidiosa* (Wells et al), is the primary limiting factor of growing *Euvitis* grape in the southeast United States (Lu and Ren 2002, Chen et al 2001). Pierce (1905) reported that rootstock variety affected expression of "California vine disease" (now known as Pierce's disease) in grape. Grape rootstock trials in Mississippi showed a large effect of rootstock trial on vine longevity in a region recognized for high Pierce's disease pressure (Loomis 1965, 1952, Magoon and Magness 1937). In humid and hot regions of the United States, such as Florida, bunch grapes often are highly susceptible to pests and diseases (Olien and Hegwood 1990). When the Florida hybrid bunch grape cultivar Blanc du Bois was grafted on to muscadine, which is relatively tolerant or resistant to the bunch grape pests and diseases common in North America, the scion showed a reduction in both PD and anthracnose symptoms and fruiting improved (Ren and Lu 2003). Growing conditions in Florida are harsh-successful rootstock for grape industry in that area must be tolerant to PD and adapted to the environment. Evaluation of rootstock performance and survival in Florida would provide useful information on rootstocks performance for humid tropical and subtropical environments, especially where PD is prevalent. Greenhouse screening has been used to investigate the PD resistance, tolerance, and susceptibility of grape cultivars. However, field screening is more applicable, since conditions closely match those in a commercial vineyard. When relying on natural infection in the vineyard, there is no need to inoculate vines or maintain colonies of *Xf* or insect vectors. Field screening is cheap, requires no specialized equipment and can be accomplished quickly, with symptom expression being used as the main criterion. Northern Florida is an ideal test environment due to heavy PD pressure, with abundant vectors, including glassy-winged sharpshooter, and inoculum, in contrast to many other locations, especially California, which demonstrate substantial cycling of PD incidence.

OBJECTIVES
Evaluate the response of grape rootstocks to natural field infection by Pierce's disease.

RESULTS AND CONCLUSIONS
Ten grape rootstocks (five replicates of two vines each, ten vines total per rootstock cultivar) were planted in the spring of 2001. Vines were bilaterally cordon trained and spur pruned. Pierce's disease (PD) symptoms were scored in 2002 and 2003, with symptoms on leaves assessed in a numerical scale from 0 to 5. For PD, 0 represented no symptoms, 1 = minor symptoms up to 15% of leaves with marginal necrosis (MN), 2 = 15-30% of leaves with MN, 3 = 30-50% of leaves with MN, 4 = 50-75% of leaves with MN, 5 = over 75% of leaves with MN or vine dead. Vine vigor was surveyed later fall in 2002. The annual shoot and node growth was recorded from ten randomly sampled shoots per plant, and shoot diameter was taken in the middle of 4^{th} node. Node length was calculated with total node numbers and the length of each shoot. Twenty (4 x 5) random shoots were investigated for shoot death rate from each vine: 5 shoots in each canopy quadrant area divided by the main trunk and trellis wire. shoot was considered as dead if more than half of the shoot had died. Trunk diameters were measured 50 cm above the ground in fall 2003.

All rootstock vines developed PD symptoms, although the severity varied. The least severe PD scores were seen on Ramsey and St George, with average PD scores of 1.1 to 1.4 in 2002, and 1.0 to 1.7 in 2003. Freedom (3.7 – 5.0), 3309C (3.6 - 4.2) and O39-16 (3.1 - 3.8) had the most severe PD symptoms, and 5C (2.2 – 1.9), 5BB (2.7 – 1.6), 44-53 (2.6 – 2.3), 110R (2.2 – 1.8) and 101-14 (2.2 – 2.4) showed a moderate PD syndrome in the 2 year period (Table 1). The PD score of these rootstocks might be considered as light to severe. These preliminary evaluation data suggested that some of the rootstocks, such as Ramsey and St George, could be used as PD resistant rootstocks in the southeast United States. Interestingly, the muscadine / *Euvitis* hybrid O39-16 showed relatively high PD score. After three growing seasons in Floridas harsh environment, the survival rate was very different among the rootstocks. 101-14, 5BB, Ramsey and St. George showed 100% survival, while Freedom had 10% vine survival (Table 3). It is noted that the vines greatly deteriorated in the third growing season. For example, from 2002 to 2003, the vine losses of Freedom, 44-53 and 3309C were 87%, 70%, and 50%, respectively. Shoot death rate varied significantly among cultivars. Very little shoot death was observed on O39-16, while the shoot death rate of Freedom and 44-53 was as high as 56%. For the remaining rootstocks, shoot death rate ranged from 13% (St George) to around 40% (5BB and 3309C) (Figure 2).

Trunk diameters were different after three growing season. The largest trunks were found in St George, O39-16 and 3309C, with diameters averaging 3.1-3.9 cm. The smallest trunks were found in 44-53, at 1.9cm (Table 2). Annual shoot growth ranged from 66 cm (Ramsey) to 230 cm (5BB) (Table 2). 5BB and 5C grew significantly longer annual shoots than the rest of rootstocks tested, while Ramsey, St George and O39-16 had significantly shorter shoots. Node numbers per shoot differed among the rootstocks, with node numbers of 5BB (27.9 per shoot) and 5C (27.7 per shoot) about 70% more than Ramsey (Table 2). Shoot length is a factor of node numbers and internode length. Here longer shoots generally resulted from more nodes, although internode length did vary (Table 2). Shoot diameters varied among the cultivars. The larger shoot diameters were found in 5BB and 5C, and 3309C, while Ramsey had the smallest shoots (Table 2). In general, higher PD scores coincided with higher shoot death rate and vine death, except 039-16, which showed higher PD scores, but had the lowest shoot death rate (Figure 2). Severe PD symptoms typically related to significant numbers of dead shoots. Similarly, the overall vine survival was correlated to PD severity; rootstocks demonstrating high survival rates had lower PD scores, while the lower survival percentage rootstocks had higher PD ratings (Figure 1).

Rootstock performance in north Florida primarily is a factor of PD response. Cultivars differed in their performance and some were markedly superior—these should be further investigated for their influence on scions. The evaluation of rootstock cultivars in PD limited viticultural regions is important—much PD management research is focusing on augmenting PD resistance and or tolerance in scions, but rootstocks are a critical component of viticulture. As demonstrated here, several rootstocks have substantial levels of PD resistance that should permit their cultivation in PD prone regions, allowing concentration of effort on scion improvement. Field evaluation of PD resistance in Florida is easy due to high PD pressure resulting from high populations of vectors and bacteria in the area and should be continued as a technique to test PD management strategies and screen plant material.

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Table 1. PD symptom scores of the ten grape rootstocks during the 2nd and 3rd growing seasons.

Rootstock	PD score	
	2002	2003
O39-16	3.1bc	3.8b
101-14	2.2d	2.4c
110R	2.2d	1.8cd
3309C	3.6b	4.2ab
44-53	2.6cd	2.3c
5BB	2.7cd	1.6cd
5C	2.2d	1.9cd
Freedom	3.7b	5.0a
Ramsey	1.1e	1.0d
St. George	1.4e	1.7cd

Table 2. Vine vigor, means of 2002 and 2003 growing seasons.

Rootstock	shoot[z]				trunk diameter[y] (cm)
	length (cm)	node #	diameter (cm)	internode (cm)	
O39-16	67.2e	23.4b	56.9abc	2.9f	3.4ab
101-14	99.1cd	20.8bc	54.7bcde	4.9c	2.6c
110R	75.7e	19.4bc	54.7bcde	3.9e	2.6c
3309C	81.0de	19.3bc	47.2cde	4.2de	3.1bc
44-53	120.2c	23.6b	53.2bcde	5.1c	1.9d
5BB	230.8a	27.9a	66.1a	8.1a	2.9bc
5C	176.5b	27.7a	60.3ab	6.2b	2.8bc
Freedom	99.5c	21.4bc	54.6bcde	4.7dc	2.9bc
Ramsey	66.1e	17.4c	45.9de	3.8e	2.6c
St. George	83.2de	20.7bc	55.0bcd	4.1e	3.9a

z. measured in the end of 2002 growing season y. measured in fall of 2003

Table 3. Vine survival of the ten grape rootstocks after three growing seasons.

Rootstock	Number of living vines			Survival %
	2001	2002	2003	2003
O39-16	9	9	6	67
101-14	10	10	10	100
110R	10	10	9	90
3309C	10	10	5	50
44-53	10	10	3	30
5BB	10	10	10	100
5C	10	10	9	90
Freedom	10	8	1	10
Ramsey	8	8	8	100
St. George	10	9	9	90

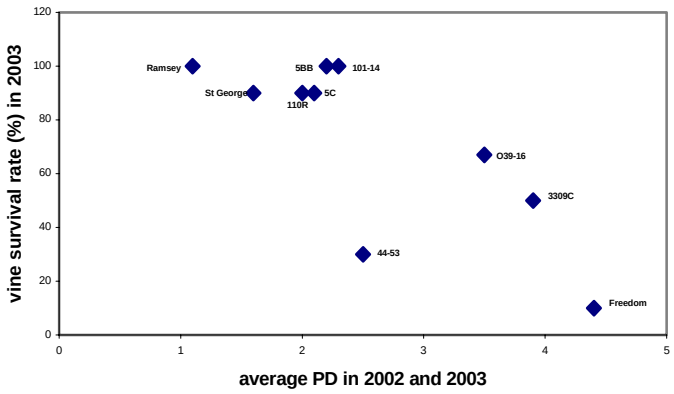

Figure 1. PD score effect on vine survival rate after three growing seasons.

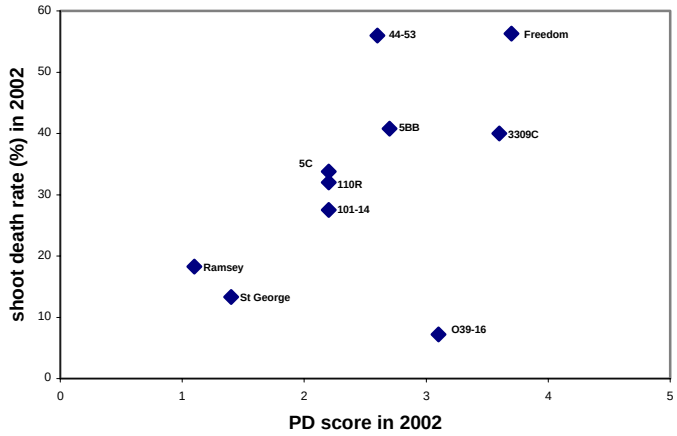

Figure 2. PD scores and shoot death rate (%) in 2002 growing season.

DIRECTING POTENTIAL ANTI-*XYLELLA* GENE PRODUCTS TO THE XYLEM OF TRANSGENIC GRAPEVINES

Project Leaders:

Carole Meredith
Department of Viticulture and Enology
University of California
Davis, 95616

Abhaya Dandekar
Department of Pomology
University of California
Davis, 95616

Cooperators:

Bruce Kirkpatrick
Department of Plant Pathology
University of California
Davis, CA 95616

John Labavitch
Department of Pomology
University of California
Davis, CA 95616

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ABSTRACT

The purpose of this research was to transform *Vitis vinifera* cultivars with the pear polygalacturonase inhibiting protein (PGIP) gene in order to analyze its effect in developing resistance to PD in transgenic plants. A second goal was the transformation of grapevine with several green fluorescence protein (GFP) constructs carrying sequences expected to enhance secretion from the cell to evaluate the effect of signal sequences on the targeting of transgene products to xylem tissue. Some of the transgenic lines expressing *pgip* exhibited reduced PD symptoms, which suggests that *Xyllela* polygalaturonase might be inhibited in transgenic plants. Tests will be conducted in the future to evaluate the development of PD in the field. We also found that the pear PGIP was secreted into the xylem. This is relevant to PD because *X. fastidiosa* is a xylem-limited bacteria. It is also very important that the transgene product was observed to move through the graft union and thus is transmitted to the scion, implying that a few transgenic rootstocks could be used with any scion variety. Fluorescence in plants transformed with GFP fused to the signal peptide sequences of tricosanthin and XSP30 was only detected inside the cells. The absence of fluorescence in the apoplast could be related to GFP expression itself instead of failure of TCS and XSP30 signal peptides.

INTRODUCTION

Genetic engineering offers the possibility of introducing genes that will improve tolerance to Pierce's disease in existing grape varieties without otherwise changing their viticultural or enological characteristics.

One of our targets is a gene coding for a pear PGIP cloned in the Labavitch lab (Stotz et al. 1993). PGIP's are plant cell wall proteins that specifically inhibit fungal polygalacturonases (PG). By inhibiting PGs, PGIP's interfere directly with host cell wall degradation and may prevent degradation of pectic oligomeric elicitors that are inducers of the plant defense response. Their role in plant defense suggests that they may be useful for engineering transgenic plants resistant to pathogen infection. Powell et al. (2000) showed that transgenic tomato plants transformed with the pear PGIP gene exhibited reduced susceptibility to infection with *Botrytis cinerea*. The fact that *Xylella fastidiosa*, the causal agent of PD in grapevines, has genes putatively encoding PG and other cell wall-degrading enzymes (Simpson et al., 2000) led us to hypothesize that PGIP could confer tolerance against *Xylella* in grapes. In order to test this hypothesis, pre-embryogenic calluses originating from anthers of *Vitis vinifera* cvs. 'Thompson Seedless' and 'Chardonnay' were cultivated with *Agrobacterium tumefaciens* harboring binary plasmid pDU94.0928, that contains the pear PGIP gene under the control of the CaMV 35S promoter.

We are also investigating the targeting of transgene products to xylem tissue. Because *X. fastidiosa* is xylem limited, it will be essential that any anti-*Xylella* gene product be present in the xylem in an effective concentration. In order to study protein secretion in grape, pre-embryogenic calli originating from anthers of *Vitis vinifera* cvs. 'Thompson Seedless' and 'Chardonnay' were cultivated with *Agrobacterium tumefaciens* carrying three gene constructs that included the coding sequence for a synthetic GFP (Maximova et al 1998) and GFP fused with amino-terminal of the secreted protein tricosanthin (TCS) (Krishnan 2000) or the xylem specific protein XSP30 (Masuda et al, 1999), all under the control of the Ca MV 35S promoter.

OBJECTIVES

1. Characterize the role of the transgene in the delayed development of PD in transgenic grapevines that express the pear PGIP.
2. Measure the abundance of marker gene product in the xylem sap of transgenic plants and non-transgenic scions grafted into transgenic rootstocks.
3. Evaluate the effect of signal sequences on the targeting of transgene products to xylem tissue.

RESULTS AND CONCLUSIONS

Effect of PGIPs on the development of PD in transgenic grapevines

Active PGIP was found in leaves, roots and stems of transgenic plants obtained from independent transformation events but not in untransformed controls. Secretion of the protein was confirmed with the presence of active PGIP in the xylem sap of transgenic plants and untransformed scions of 'Chardonnay' and 'Thompson Seedless' grafted on transgenic rootstocks. PG inhibition remained high in 10x diluted xylem sap (Figure 1). Five to seven plants of each line were mechanically inoculated with the Temecula strain of *X. fastidiosa.* The development of PD was delayed in some transgenic lines with high PGIP activity, which exhibited reduced leaf scorching, lower *Xylella* titers and better re-growth after pruning than the untransformed controls.

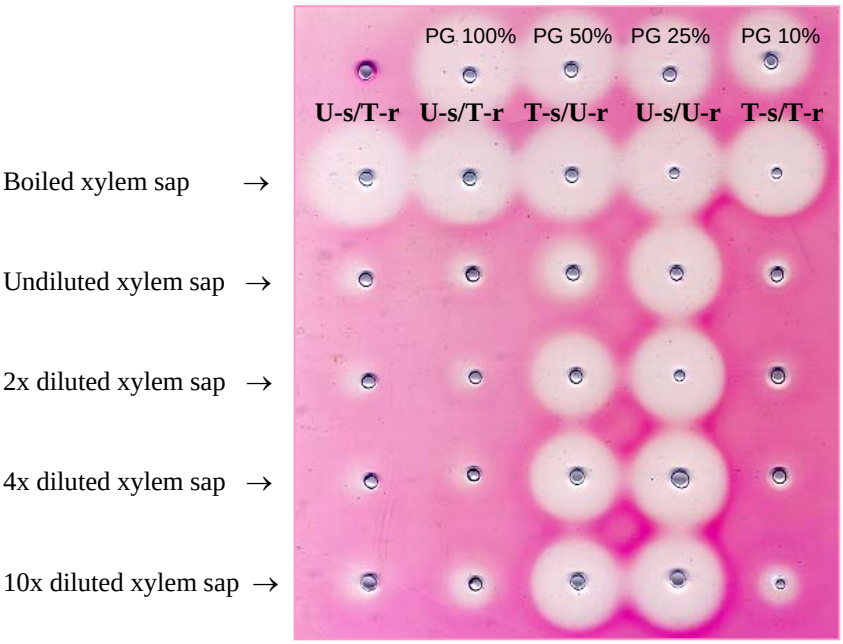

Figure 1. PGIP activity in xylem sap obtained from different graft combinations. A clear zone is indicative of PGs degrading the glycosydic bonds present in the polygalacturonic substrate. First row corresponds to 100, 50, 25 and 10% PG dilutions. Second row are 100% PG with boiled xylem sap samples, rest of the rows correspond to 100% PG with different dilutions of xylem sap. First and second column of treatments are replicate plants. U is untransformed, T is Thompson Seedless transgenic line 77, s is scion and r is rootstock.

Effect of signal sequences on the targeting of transgene products to xylem tissue

Strong fluorescence was found in embryos, roots, stems and leaves of plants transformed with *gfp* and *xsp30-gfp* but the levels of fluorescence observed in *tcs-gfp* transformants were very low. In all cases fluorescence was detected only inside the cells.

CONCLUSIONS

Although all lines tested were susceptible to *X. fastidiosa*, the development of PD was delayed in some transgenic lines with high PGIP activity. Whether these results can be attributed to the inhibition of *Xylella* PG is unclear. The improved growth of PD transgenic grapevines might account for a delay of PD movement along the stem but more information is needed to explain these results. Also it will be important to determine whether the transgenic plants grown in the field exhibit the same characteristics. We are currently growing at least 20 plants per line in the greenhouse to be transplanted to the field this coming spring. Nevertheless, the expression of PGIP has provided useful data regarding the control of the disease. First, our results showed that the pear PGIP is secreted and reaches the xylem. This is relevant to PD because *X. fastidiosa* is xylem-limited and any anti-*Xylella* gene product must be present in the xylem. Second, the signal peptide of the pear PGIP could be used to direct other anti-*Xylella* products to the xylem. And third, the fact that the transgene product moves through the graft union and is transmitted to the scion implies that a few transgenic rootstocks could be used with any scion variety provided that the anti-*Xylella* compound is synthesized in effective concentration in the roots.

Fluorescence in plants with GFP fused to the leader sequences of TCS and XSP30 was detected only inside the cells. The simplest explanation is that these signal peptides were not recognized by the grape secretory machinery but other interpretations are also likely. Alternative possibilities are that the protein was secreted but was not folded properly, or that it is less stable in the apoplast or that it was retained in the ER/Golgi compartments. Consequently more analysis is needed,

e.g., Western blotting of apoplastic fluid, before ruling out the use of the TCS and XSP30 signal peptides for the delivery of anti-*Xyllela* compounds to the xylem.

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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

Project Leader:
Leonard Nunney
Department of Biology
University of California
Riverside, CA 92521

Cooperators:
Richard Stouthamer
Robert Luck
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted from November 1, 2002 to October 15, 2003.

INTRODUCTION

Genomic research provides an extraordinarily powerful new tool for solving applied problems. It can be extremely effective at resolving evolutionary relationships and can be used to extend earlier work defining the interrelationships of the plant-host strains of *Xylella fastidiosa* (see Hendson *et al*. 2001). Similarly, it can provide effective methods for identifying these host strains. Unambiguous identification is of considerable importance for understanding the epidemiology of Pierce's disease and the other plant diseases caused by this bacterium. 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 availability of sequenced genomes allows us to analyze the evolutionary history of not just one or two genes, but of all of the genes that make up this bacterium. In particular, evolutionary genomic techniques developed to detect the action of natural selection (Yang 1998) provide a new approach to identifying genes important in plant-host specificity.

The bacterium *X. fastidiosa* is generally assumed to be clonal. However, horizontal transfer of genes must occur given the presence of unique regions of DNA in the different host strains (Van Sluys *et al*. 2003). Such transfers are assumed to be virally mediated. The possibility of direct inter-strain genetic transfer is more difficult to detect, but needs to be investigated for two important reasons. First, bacteria from different strains are expected to mix at high density within the insect vector, and second, 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.

OBJECTIVES

1. The identification of the rapidly evolving genes in the *Xylella fastidiosa* genome. This is the first step towards achieving our four primary objectives. These are:
2. 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.
3. Identify plant-host specificity candidate genes. We will use our database of rapidly evolving proteins to test for evidence of strong natural selection and for links between the rapid genetic divergence of host strains and specific biochemical functions.
4. Measurement of clonal variation within host strains. Our objective is to assess within-strain genetic variability at rapidly evolving gene loci and to use these results to assess the evidence that all members of a given host strain share common ancestry.
5. 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.

RESULTS

Objectives 1 and 3

We completed our gene-specific database of the four plant-host *Xylella fastidiosa* genomes, PD (Pierce's disease), OLS (Oleander Leaf Scorch), ALS (Almond Leaf Scorch), and CVC (Citrus Variegated Chlorosis). This database identifies all genes that occur in all four genomes, and enables us to compare the evolutionary characteristics of these genes. The genome-wide analysis is ongoing; however, we identified 11 genes to use in the detailed investigation of plant-host strain relationships, based on their significant shifts in Ka/Ks ratio within the phylogeny, and their distribution throughout the

genome (Table 1). Specifically, we measured shifts in this ratio of non-synonymous (Ka) to synonymous (Ks) substitutions between the OLS and ALS branches of the phylogeny. A significant shift indicates a change in the nature of the selection acting on the gene, and may indicate that these genes are important in plant-host adaptation.

Table 1. *Xylella fastidiosa* genes used for strain identification.

Gene ID		Name	χ^2 Value for shift in Ka/Ks
XF0136	holC	DNA polymerase III holoenzyme chi subunit	4.58
XF0257	rfbD	dTDP-4-dehydrorhamnose 3,5 epimerase	6.60
XF0316	nuoL	NADH-ubiquinone oxidoreductase, NQO12 subunit	5.95
XF0318	nuoN	NADH-ubiquinone oxidoreductase, NQO14 subunit	8.82
XF0656	gltT	glutamate symport protein	4.58
XF0832	cysG	siroheme synthase	4.71
XF0910	petC	ubiquinol cytochrome C oxidoreductase, cytochrome C1	3.88
XF1291	eno	enolase	5.29
XF1632	pilU	twitching motility protein	8.88
XF1818	leuA	2-isopropylmalate synthase	6.35
XF2447	lacF	ABC transporter sugar permease	4.28

Objectives 2 and 4

We have so far sequenced 9 of the 11 target genes (a total of 11014 bases) in 13 strains of *Xylella fastidiosa*. Strains have (and will continue to be) selected to represent the geographic distribution of the infections, with a goal of regional replication to enable tests of geographical hypotheses (Table 2).

Table 2. *Xylella fastidiosa* strains so far examined.

	Strain	Host	Location	Strain Identification**
1*	Temecula	Grape	Temecula	PD
2*	Ann-1	Oleander	Palm Springs	OLS
3*	Dixon	Almond	Solarno	ALS
4	STL	Grape	Napa	PD
5	Tulare	Almond	Tulare	PD
6	Conn Creek	Grape	Napa	PD
7	Traver	Grape	Tulare	PD
8	Texas	Oleander	Texas	OLS
9	Riverside	Oleander	Riverside	OLS
10	I03	Grape	Temecula	PD
11	237	Almond	Temecula	PD
12	276	Almond	Temecula	ALS (?)
13	187	Almond	Temecula	ALS

* Strains 1,2 and 3 were the strains used for the PD, OLS and ALS genomes.
** See Table 3

The sequences for 9 of the genes have been compared for the 13 strains using Multi-Locus Sequence Typing (Maiden *et al.* 1998). Any difference in a sequence results in the assignment of a new type (letter) for that gene (Table 3). All the PD strains except Traver in gene XF1632 were identical to the genome sequence for PD strain #1 (and scored the maximum of 100 in the row labeled "Score PD type"). The strain #11 (237) was isolated from almond but was identical to the "type" Temecula PD strain #1. Similarly #5 Tulare, also isolated from almond, showed only a single base difference with the #1 PD strain. The oleander strains showed more variation. The Riverside strain (#9) differed from the Ann-1 genome sequence (#2) at two genes, XF0832 and XF1632. Almond strains had the most variation. Dixon (#3) and 187 (#13) were identical except for one gene, XF0257. Strain #12 (276) had differences from Dixon in nearly all the genes, but still had more in common with Dixon and 187 than with any other strain.

We can also look at the MLST pattern from the perspective of the genes (i.e. rows in Table 3). For the design of efficient and effective strain identification it is important to note that the genes in the first 4 rows show identical patterns (and hence the same information). Similarly, the genes in the next 2 rows have an identical pattern.

Table 3. Multi-Locus Sequence Typing (MLST) for *Xylella fastidiosa*

	Strain												
Gene	1	4	6	10	11	5	7	2	8	9	3	13	12
XF0136	a	a	a	a	a	a	a	b	b	b	c	c	d
XF0656	a	a	a	a	a	a	a	b	b	b	c	c	d
XF1818	a	a	a	a	a	a	a	b	b	b	c	c	d
XF2447	a	a	a	a	a	a	a	b	b	b	c	c	d
XF0318	a	a	a	a	a	a	a	b	b	b	c	c	c
XF0910	a	a	a	a	a	a	a	b	b	b	c	c	c
XF0257	a	a	a	a	a	a	a	b	b	b	c	d	d
XF0832	a	a	a	a	a	a	a	b	b	d	c	c	e
XF1632	a	a	a	a	a	d	e	b	b	d	c	c	e
% Score PD type	**100**	**100**	**100**	**100**	**100**	**89**	**89**	0	0	0	0	0	0
% Score OLS type	0	0	0	0	0	0	0	**100**	**100**	**78**	0	0	0
% Score ALS type	0	0	0	0	0	0	0	0	0	0	**100**	**89**	**22**

Using the sequence data from the 13 strains provides a large dataset to construct a phylogenetic tree showing the interrelationships of the strains. There are several techniques that are used for the construction of phylogenetic trees, principally maximum likelihood, parsimony, and distance methods. All of these methods gave the same overall relationships among PD, OLS, and ALS strains (using CVC to root the tree). The maximum likelihood tree shown (Figure 1) was derived using the software package PAUP. The tree shows 3 well-defined clades, PD, OLS and ALS. These three clades are statistically very robust, with 100% bootstrap support for PD and OLS, and 98% for ALS. The PD strain's closest relative is always the OLS strain.

The PD clade includes strains isolated from almond, but the ALS clade does not include strains isolated from grapes. Furthermore, both the OLS and PD strains show surprisingly little variability in our sample so far, and these sequences of over 10K bases show no geographical structure. For example, the cluster of PD strains (1,4,6,10 and 11) derive from Napa and Temecula. Similarly the geographically separated OLS strains 2 and 8, from Palm Springs and Texas (site unknown), are identical across the sequenced genes.

The finding of little variation within our present strain sample simplifies strain identification, if the pattern holds when more strains are added. Using some of the 11 sequenced genes we have developed and tested 9 gene/restriction enzyme reactions. Each reaction identifies one strain (3 PD, 3 ALS, 3 OLS), thus appropriate combinations of three reactions allow unambiguous positive identification of the three strains. One promising combination involves three PCR reactions (genes XF0136, XF0257, and XF0832) using the same restriction enzyme. We are currently testing mixtures of DNA from different strains as a first step to establishing how effective the method is for detecting a mixture, when one strain is rare.

Objective 5

We did not anticipate finding any indication of recombination at this early stage; however examination of the ALS strain #12 revealed strong (though preliminary) evidence of genetic exchange between strains. When the strains are compared on a gene-by-gene basis, the three ALS strains typically cluster very closely. However, in two genes the #12 sequence clusters closely with OLS and in one gene it clusters closely with PD.

CONCLUSIONS

1. In broad agreement with previous work, we have found that PD, OLS, and ALS strains of *X. fastidiosa* form robust clades, with PD being most closely related to the OLS strain.
2. It appears that the PD strain can infect almond, but there is no evidence that the ALS strains can infect grape.
3. We find no evidence of geographical differences within the PD and OLS strains. In particular, the PD strains from Temecula and Napa are extremely similar.
4. The simple technique of multi-locus sequence typing (MLST) is effective at identifying the three strains.
5. We have developed a simple method to uniquely identify the PD, OLS, and ALS strains by looking for characteristic restriction fragments following PCR of targeted genes. We are currently testing the sensitivity of the method in detecting a rare strain in mixtures of DNA.
6. One strain (#12) provides preliminary support for recombination among strains. The importance of this result is that it raises the possibility that recombination can rapidly generate novel pathotypes.

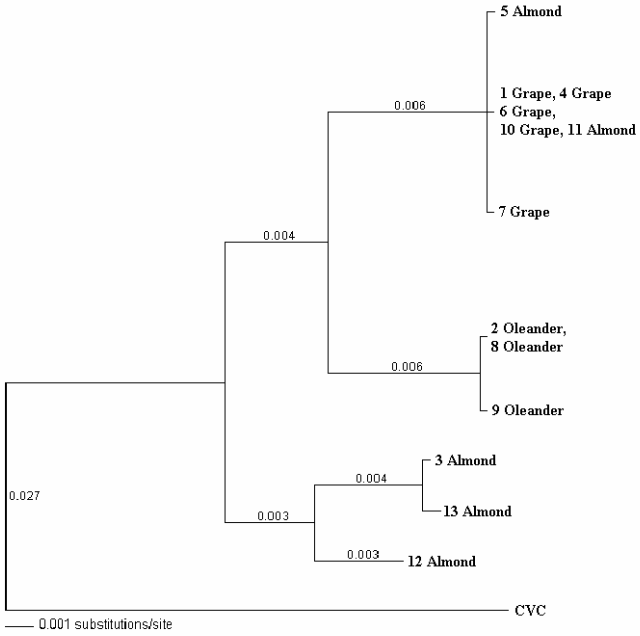

Figure 1: Phylogenetic relationships among 13 strains of *X. fastidiosa*

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TESTING TRANSGENIC GRAPEVINES FOR RESISTANCE TO PIERCE'S DISEASE

Project Leaders:
Bruce I. Reisch
Department of Horticultural Sciences
NYS Agricultural Experiment Station
Cornell University
Geneva, NY 14456

M. Andrew Walker
Department of Viticulture and Enology
University of California
Davis, CA 95616

Julie R. Kikkert
Department of Horticultural Sciences
NYS Agricultural Experiment Station
Cornell University
Geneva, NY 14456

Cooperator:
José Vidal
NYS Agricultural Experiment Station
Cornell University
Geneva, NY 14456

Reporting Period: The results reported here are from work conducted from July 1, 2003 to October 15, 2003. (Some results of prior work are included as well.)

ABSTRACT
Numerous genes involved in plant disease defense have been isolated and when introduced and expressed in transgenic plants, fungal and bacterial diseases have been greatly reduced. This strategy is especially appropriate for grapevines, where the industry is rooted in traditional European grapes with strong name recognition and very high disease susceptibility. Our laboratory has developed a set of transformed grapevines harboring genes that produce anti-microbial peptides (AMPs). Seventy-six 'Chardonnay' lines transformed with the magainin-type genes, mag-2 and MSI99, and with a PGL class gene, were produced. Some are now growing in tissue culture, in the greenhouse, and in the field (Vidal et al. 2003). AMPs are particularly effective against bacteria, and act by disrupting the cell membranes. The primary goal of this project is to study the potential resistance to Pierce's disease (PD) of magainin and PGL-producing vines. In doing so, it becomes important to characterize the expression of these genes in each line. We are also studying whether AMPs can move from a transgenic rootstock to a non-transgenic scion. If so, it might be possible for engineered rootstocks to be used as a means to affect disease development in a range of scion varieties. Our data to date show that between 1 and 4 copies of the foreign gene can be found in each line studied, and that the inserted genes were correctly transcribed in 46 of 76 lines tested (via RT-PCR). An antibody was produced that can detect the presence of both mag-2 and MSI99 peptides. Using this antibody, AMP production was verified in leaf extracts of in vitro vines harboring mag-2 and MSI99 genes. Studies of PD resistance and movement of transgenic proteins from rootstocks to scions are at a very early stage in this newly funded project.

INTRODUCTION
Numerous genes involved in plant disease defense have been isolated (Punja 2001; Mourges et al. 1998). When disease resistance genes are introduced and expressed in transgenic plants, fungal and bacterial diseases have been greatly reduced (Mourges et al. 1998; Punja 2001; Van der Biezen 2001). This disease control strategy is especially appropriate for clonally-propagated crops, such as grapevines, where the industry is rooted in traditional European grapes with strong name recognition and very high disease susceptibility. Moreover, cross-breeding cannot produce disease resistant forms of elite varieties because other characteristics would be altered and varietal identity would be lost.

Our laboratory has developed a set of transformed grapevines in which we have determined that anti-microbial peptides (AMPs) are transcribed. Seventy-six 'Chardonnay' lines transformed with the magainin-type genes, mag-2 and MSI99, and with a PGL class gene, were produced (Vidal et al. 2003). These are now growing in tissue culture in the greenhouse, and in the field. Magainins are small peptides with strong inhibitory activity against numerous bacteria and fungi (DeGray et al. 2001; Zasloff et al. 1988; Smith et al. 1998; Smith et al. 2001). The MSI99 peptide expressed in tobacco and banana was recently shown to be highly effective against several pathogens (Chakrabarti et al. 2003). In studies conducted in 2002 and 2003, we determined that some AMP-transgenic lines of 'Chardonnay' are significantly more resistant to tumorigenic strains of crown gall (*Agrobacterium vitis*). It is logical to think that these plants might have improved resistance to other bacterial diseases as well.

Some AMP producing genes such as *Shiva-1* are effective against Pierce's disease (PD), according to a recently issued patent (Scorza and Gray 2001) but the subject warrants further study. Scorza and Gray described a trial of two lines of 'Thompson Seedless' expressing the Shiva-1 peptide; both eventually succumbed to PD, but one showed milder symptoms, which did not include the typical signs of marginal leaf burn when compared to the non-transformed control plant. Based on this data, the

above-mentioned patent was issued covering the usage of all AMP genes in the development of *Xylella fastidiosa (Xf*) resistant transgenic vines. However, data are not available in the literature to determine if mag-2, PGL, and MSI99 peptides are effective *in planta* against *Xf*. It is the purpose of this proposal to study the potential resistance of our magainin and PGL-producing 'Chardonnay' vines to PD.

OBJECTIVES

1. Quantify the expression of AMPs (anti-microbial peptides) in transgenic 'Chardonnay' vines.
2. Evaluate resistance to Pierce's disease among these transgenic vines.
3. Determine the extent to which an AMP transgenic rootstock can confer PD resistance to the scion.

RESULTS

Objective 1: Because AMPs are very small (~2.7 kD) and easily degraded by host proteases, expression was studied by RT-PCR (for detection of mRNA products from the inserted gene sequences) and ELISA (for direct detection of the presence of peptides). Out of 76 transgenic lines tested by RT-PCR, 46 lines (11 mag-2, 11 MSI99, 8 PGL and 16 mag-2 + PGL) were positive for transcription of the expected mRNA. For mag-2 (23 amino acids) and MSI99 (22 amino acids) peptides, an antibody was developed (by Sigma-Genosys) that recognizes an antigenic sequence common to both peptides. In a series of preliminary ELISA tests using leaf extract from in vitro plants, the peptide could be detected in a number of lines, in agreement with the RT-PCR results. However we were unable to detect the peptide consistently, suggesting the methodology requires some improvement. To improve the ELISA procedure, young leaves from actively growing greenhouse plants will be used immediately after collection.

Objective 2: In a preliminary study, 31 transgenic lines (4 plants/line) were inoculated with two different *Agrobacterium vitis* strains to test for resistance to crown gall disease. Among these lines, 6 harboring *mag-2*, 5 with *MSI99*, 5 with *PGL* and 5 with the combination of *mag-2 + PGL* showed statistically significant gall size reductions ($P<0.05$) compared to non-transformed controls. From among these crown gall resistant lines, the most promising 16 (4 lines per gene construct) were selected and sent in early October, 2003, to Dr. Walker for PD resistance testing. These are currently being grown in an isolated greenhouse at U.C. Davis awaiting sufficient growth to begin inoculations with *Xf*. PD symptoms are expected to appear 8 weeks after inoculation, and stem and leaf symptoms will be recorded 12 and 16 weeks following inoculation.

Objective 3: Plans are to test the hypothesis that rootstocks can transmit peptides to the scion in late winter or early spring, 2004. In the case of the *mag-2* and *MSI99* transformants, the AMP gene was fused to a signal peptide to allow product secretion into intercellular spaces. These products are likely to be xylem mobile and may have the ability to move upwards in the plant. Their small molecular size may further facilitate movement within the plant. These transgenic 'Chardonnay' are not being considered for use as commercial rootstocks. However, if they are capable of suppressing PD, efforts to engineer appropriate grape rootstocks would then be justified.

To test this hypothesis, transgenic 'Chardonnay' vines will be used as rootstocks with non-transgenic 'Chardonnay' as scions. Since we will be using young potted plants, approach grafting will be used to connect the plants. Transgenic and normal 'Chardonnay' plants will be planted in the same 15 cm pot, and, after about 4 weeks, there will be enough shoot growth to produce a tongued approach graft and connect the two plants. The unions will heal in about 4 weeks and the non-transgenic 'Chardonnay' stems will be cut so that they become scions on the transgenic 'Chardonnay' rootstock. Shoot growth on the scion 'Chardonnay' should be ready for *Xf* inoculation in about 4 weeks and the scions will be needle inoculated with the Stag's Leap *Xf* strain. Symptom expression will be evaluated as above. We will also culture *Xf* from stem pieces to further quantify the levels of *Xf* in the 'Chardonnay' scions. ELISA techniques will be used to detect the presence of the rootstock-produced AMP in the leaves and stems of the scions. If an effect is detected, it will be very important to carry out rootstock/scion tests using hardwood cuttings and under field conditions.

CONCLUSIONS

This project is at a very early stage, yet it is progressing very well. We are developing an ELISA technique to detect magainin-like peptides in young tissues, with promising results to date. PD resistance studies are about to get underway, and studies regarding the concept that a rootstock can be used to transmit disease-fighting substances to the scion are planned to take place in the coming months. If successful, this project could result in the development of transgenic versions of important cultivars that resist Pierce's disease while maintaining all their important varietal characteristics. In addition, we hope to learn whether rootstocks could be used to improve the resistance of the scion.

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MAGNETIC RESONANCE IMAGING: A NON-DESTRUCTIVE APPROACH FOR DETECTION OFXYLEM BLOCKAGES IN *X. FASTIDIOSA*-INFECTED GRAPEVINES

Project Leaders:
Ken Shackel
John Labavitch
Department of Pomology
University of California
Davis, CA 95616

Cooperators:
Mark Matthews
Department of Viticulture and Enology
University of California
Davis, CA 95616

L. Carl Greve
Department of Pomology
University of California
Davis, CA 95616

Jeffrey Walton
NMR Facility
University of California
Davis, CA 95616

Alonso Perez
Department of Pomology
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted from October 1, 2002 to October 1, 2003. (**Note**: Funding for the project was not received until September 11, 2003, but work had begun with other funding)

INTRODUCTION

Results from PD research programs led by Matthews, Rost and Labavitch (reported in 2001 and 2002 in San Diego) have provided substantial support for the idea that obstructions in the vine's water-transporting xylem tissue develop rapidly post-inoculation, before an appreciable bacterial population has been established. The results also strongly suggest that these obstructions, and likely other aspects of the Pierce's disease (PD) "syndrome", result from the grapevine's active responses to the presence of *X. fastidiosa* (*Xf*), rather than to direct "action" by the bacterium. Thus, careful analysis of the timing of changes in xylem element anatomy and function relative to *Xf* introduction, as well as to external symptoms of disease development, is important for establishing reliable indicators of the "stage" of PD development. The analyses done thus far have been based on destructive tissue sampling. While the Matthews and Labavitch project continues to test a model of PD development, sampling of infected vines is essentially blind and must, therefore, be destructive. This is particularly so since it has become clear that important internal responses to the bacterium are correlated with the presence of rather few bacteria in the tissues, at a time, post-inoculation, that is well before external PD symptoms are in evidence.

Perez and Walton have has carried out a number of pilot studies aimed at "observing" the development of xylem vessel obstructions in infected vines without damaging the plants. The value of the work is that periodic examinations can be done on each of several vines over a number of weeks (reported in 2002). The NMR images provided clear evidence of the progressive, localized deterioration of water movement capacity in treated vines. The data provide spatial information about where, around the circumference of a vine as well as along its length, obstructions are present. This information should provide specific guidance for eventual destructive sampling to assess the presence and nature of xylem obstructions. Optimization of the analytical approach (our Objective 1) in order to maximize the signal to noise ratio by reducing the noise component will add considerably to the value of the technique. This will lead to studies aimed at addressing whether the vessels cavitate (i.e., air "embolisms" develop) when the glassy-winged sharpshooter feeds on vines and non-destructive testing of the impacts of *Xf* cell wall-degrading enzymes, pectin-derived oligosaccharides, and ethylene on vine water transport, as discussed in the Matthews and Labavitch PD development model (Figure 2, below).

OBJECTIVES

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

RESULTS

Optimization of the use of MRI for visualizing water transport deficiencies in PD-infected grapevines

Progress on this Objective has been delayed because a supplier for a key electronic element of the new MRI probe that has been designed for use with grapevines no longer provides the key part. We are proceeding with the testing of aspects of the PD model using the NMR instrument in its more conventional configuration.

MRI will show non-functional sections in the xylem of a PD-infected grapevine stem

Usually the techniques to evaluate xylem function are destructive. Magnetic Resonance Imaging (MRI) allows us to visualize vessels that are functional and full of movable water. Functional vessels appear as bright spots in an MRI view of the stem cross-section, non-functional vessels lack water and appear as dark spots in the area of the stem where water-conducting cells are found. Figures 1a and 1b show the difference in the distributions of functional vessels in an infected vine at a point where leaf symptoms of PD are apparent (Figure 1a) and nearer to the stem apex at a point where the leaves show no sign of PD symptoms (Figure 1b). Compare these images with that for a healthy vine (Figure 3a). Because the MRI analysis is non-destructive, this technique allows periodic examinations on each of several vines over a number of weeks. These features make MRI an excellent tool to study the time course progression of xylem disruption in PD-infected vines.

We can use MRI to visualize air embolisms in grapevines

The PD model (Figure 2) predicts that air embolisms may occur when the glassy-winged sharpshooter feeds and/or as pit membranes between xylem vessels are digested. Cavitations can be easily observed with MRI (Figure 2). When there are dark spots in the center region of the stem where vessels are located, the dark spots indicate that vessels are non-functional. The loss of vessel function can result from cavitation or obstruction with bacteria or polysaccharide gels that have been observed in infected vines. If dark spots in an image are the result of air embolism, then we should be able to refill the vessels by forcing pressurized water through the stem. That this approach is valid was demonstrated in an experiment in which the image of a shoot in a healthy, well hydrated vine was made (Figure 3a) and compared to another image that was taken after a section of stem through ca. 80% of the stem's cross section was removed, allowing air to embolize the vessels above the cut (where the Figure 3b images were taken). Later the stem segment that contained the embolisms was excised and refilled with pressurized water. A new image that confirmed the refilling of the vessels was taken (Figure 3c). This experiment also demonstrated the more general principle that the dark spots seen in the MRIs of xylem correspond to non-functional vessels.

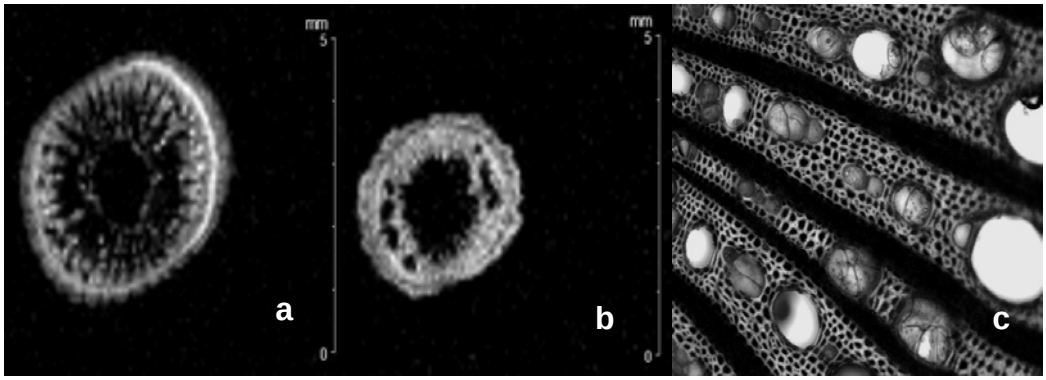

Figure 1. MRI of a PD-infected stem in a basal internode (a), and closer to the apex (b). Bright spots between the central pith (dark) and the ring of vascular cambium show functional vessels (compare to a healthy stem in Fig. 3a). Tyloses (cellular-physical blockages of the vessels) are often associated with dark spots in MRIs of infected xylem, as shown by optic microscopy in (c).

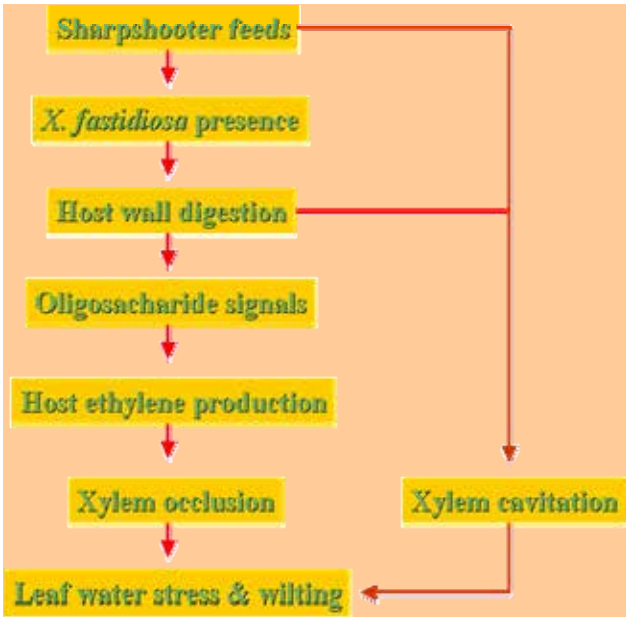

Figure 2. Hypothetical model for PD development. PD starts with a local infection caused by the glassy-winged sharpshooter's introduction of *Xf* locally (i.e., into one or a few vessels). Once *Xf* is in the xylem the bacteria become systemic, which implies that *Xf* must be able to cross (digest away?) the cell wall in the pit membranes that separate two neighboring vessels. The digestion of the cell wall by bacterial enzymes would generate transient oligosaccharides with biological activity. The presence of these oligosaccharides is detected by the plant triggering a series of defensive responses, including a raise in ethylene production. Ethylene has been shown to induce tylose formation. Cavitation of vessels may be also important for the disruption of water transport in the plant. Cavitations may happen during insect feeding or during PD progression. The "bottom line" of our thinking is that PD is primarily caused by the grapevine's responses (local and systemic) to *Xf* presence.

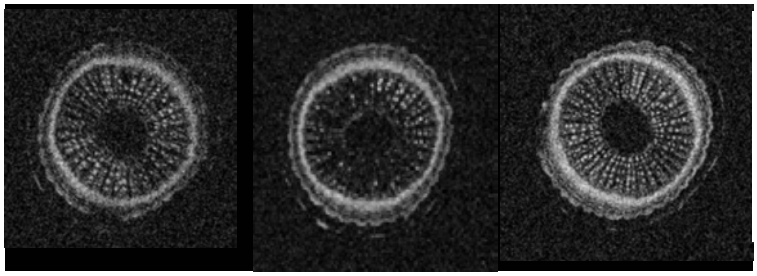

Figure 3. (a) MRI of an intact stem segment in a healthy shoot. (b) Image of the same stem portion after an important part of the cross section has been removed below it, thus causing cavitation of many vessels. (c) The same stem segment after it has been refilled with water.

CONCLUSIONS

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

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FUNDING AGENCIES

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

MARKER-ASSISTED SELECTION FOR RESISTANCE TO *XYLELLA FASTIDIOSA*: ACCELERATED BREEDING OF PIERCE'S DISEASE RESISTANT GRAPES

Project Leaders:
Andrew Walker, Alan Krivanek, and Summaira Riaz
Department of Viticulture and Enology
University of California
Davis, CA 95616

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

ABSTRACT
Efforts at identifying genetic markers tightly linked to *Xylella fastidiosa* (*Xf*) resistance are continuing. These efforts are primarily focused on the 9621 mapping population (D8909-15 x F8909-17) and are in cooperation with fine-scale mapping efforts discussed in our report "Map based identification and positional cloning of *Xylella fastidiosa* resistance genes from known sources of Pierce's disease resistance in grape." This project is adding 200 SSR markers to the 9621 map and has positioned *Xf* resistance from the male parent F8909-17 on the lower arm of linkage group 14, where *Xf* resistance is flanked by multiple markers. Resistance from the female parent D8909-15 maps as a quantitative trait locus. The addition of additional SSR marker is expected to reduce the distance between SSR markers and *Xf* resistance to about 2 cM and lead to the development and utilization of very specific sequence characterized amplified region (SCAR) primers. These markers will be applied to populations in the breeding program derived from D8909-15 and F8909-17 and tested for effectiveness in *Xf* resistance backgrounds derived from other species.

INTRODUCTION
Marker Assisted Selection (MAS) is the process whereby indirect selection on a trait of interest (such as disease resistance) is made by screening for the presence of a DNA marker allele tightly linked to the trait. MAS for disease resistance can be used to eliminate susceptible genotypes in a breeding population early in the selection process, which allows for evaluation of much larger effective populations. Larger effective population sizes increase the opportunity to identify genotypes with high disease resistance and high horticultural qualities (such as good fruit size, color, texture etc.). Other key aspects of the MAS process is that confounding environmental effects on the trait phenotype can be avoided and progress in breeding programs can be accelerated while saving space and time, allowing for more efficient use of resources (Paterson et al. 1991, Kelly 1995). Rapid screening time is particularly valuable when applied to perennial crops such as grape with relatively long generation times (Alleweldt 1988, Striem et al. 1994).

Markers linked to grape resistance genes of other diseases have been published. AFLP and RAPD markers tightly linked to powdery mildew resistance (Dalbo et al. 2001, Pauquet et al. 2001) and downy mildew resistance (Luo et al. 2001) are some examples. To effectively use such linked markers in MAS only requires that the markers be highly reproducible, linked in coupling phase i.e. on the same homologous chromosome, and within 5 cM (cM = centimorgan, a mapping unit representative of the distance between two loci or genes) of the resistance locus (Kelly 1995). Conversion of AFLP and RAPD markers to SCAR primers allows for a more reproducible marker system and identifying tightly linked markers is a direct function of numbers of markers screened. In the case of powdery mildew resistance MAS has already been successfully utilized for screening a grape breeding population (Dalbo et al. 2001) and it is expected that this project will have a high chance of success for developing a functional MAS system for screening PD resistant genotypes. Markers tightly linked to PD resistance should have immediate benefits toward accelerating the breeding of PD resistant wine, table and raisin cultivars.

OBJECTIVES
1. Refine localization of primary QTL's associated with PD resistance derived from *Vitis arizonica.*
2. Saturate regions of primary QTL's with AFLP markers via Bulk Segregant Analysis (BSA).
3. Identify tightly linked flanking markers around PD resistance QTL's and convert to SCAR primers.
4. Confirm candidate marker linkage to resistance within a (8909 x *V. vinifera* table grape) family.
5. Utilize resistance markers to eliminate susceptible progeny within a (8909 x *V. vinifera*) x *V. vinifera* table grape backcross generation and future generations of the continuing UCD/USDA collaborative PD resistance breeding program.

RESULTS AND CONCLUSIONS
Objective 1. This proposal expands upon a portion of a project funded by the AVF and last year by the CDFA entitled "The Genetics of Resistance to PD". That project developed a genetic map in a *Vitis rupestris* x *V. arizonica* population (9621 = D8909-15 x F8909-17; see Walker, Tenscher, Ramming Progress Report in this proceedings for more detail on this population) segregating for *Xylella fastidiosa* (*Xf*) resistance and was based on about 500 DNA markers. The parents of this cross were half siblings sharing a common *V. rupestris* parent which is susceptible to PD. The progeny D8909-15 is a female

vine and derives its PD resistance from a *V. arizonica* collected in Baja California. The progeny F8909-17 is a male vine and derives its PD resistance from an apparent *V arizonica / V. champinii* hybrid collected in northern Mexico west of Monterrey. PD resistance from the F8909-17 male vine has been localized to the lower arm of a single linkage group when resistance is mapped as a single dominant trait. PD resistance for the D8909-15 female parent cannot be localized on the map when scoring PD resistance as a single dominant trait. When scoring PD resistance in a quantitative manner, preliminary results indicate that this resistance is localized at multiple positions (i.e. QTL's within the D8909-15 genome).

Progress has been made on expanding this map with additional individuals and developing a framework map based on highly reproducible SSR markers. The map now includes 188 individuals with SSR marker data and 140 individuals that have PD resistance data. To date, approximately 310 SSR markers have been tested and 165 of them proved to be useful within the 9621 mapping population. Our objective is place a total of 200 SSR markers on the framework map such that each linkage group will have 8-10 highly reproducible markers. Markers will be selected for even spacing across the genome at distances of 5-7cM, which will allow good coverage for refined QTL mapping.

Objective 2. Thus far we have identified one SSR marker and 2 AFLP markers linked in coupling phase within ~ 10cM of the primary *Xf* resistance locus of F8909-17. Following accurate placement of the F8909-17 locus and D8909-15 *Xf* resistance QTL's on the framework map in Objective 1, Bulk Segregant Analysis (BSA) (Michelmore et al. 1991) will be used to saturate regions of resistance loci with AFLP markers. We have arrangements to use a PE 3100 sequencer for large scale marker screening. A goal of 200 primer combinations should lead to identification of 2-4 markers flanking the *Xf* resistance gene within a 2-cM window. Utilization of SSR markers to link the 9621 map other genetic maps should also lead to selective placement of a variety of markers around primary *Xf* resistance QTL's.

Objective 3. Candidate AFLP markers linked to *Xf* resistance identified in Objective 2 will be confirmed by separately evaluating marker patterns on each individual within the DNA bulks. The framework 9621 population will be used to precisely localize all confirmed resistance markers, after which Sequence Characterized Amplified Region (SCAR) primers will be developed from the tightly flanking markers.

Objective 4. A family derived from an D8909-15 x *vinifera* table grape (0023) has been evaluated for PD symptoms based on cane maturation, and *Xf* bacteria numbers in stem tissue based on ELISA data at 16 weeks post inoculation. DNA has been extracted from these screened genotypes and each will be tested for the presence or absence of resistance markers identified in Objective 3, so as to confirm the correlation with the resistance phenotype. Correlation of the markers with resistance will be recalculated if different from the original mapping population.

Objective 5. A backcross population (D8909-15 x *vinifera* table grape) x an advanced seedless *vinifera* table grape was made in the spring of 2002 and 2003. Highly resistant genotypes: 0023-19, 0023-54, 0023-63, 0023-98 (stem bacteria numbers of less than 60,000 cells/ml) were backcrossed to several advanced table grape selections in order to establish a large advanced breeding population. From the 2002 crosses 1,693seeds were collected and 654 seedlings were planted in the field the summer of 2003. Markers shown to be linked to resistance in Objectives 1-4 will be used on this population to identify candidate resistant and susceptible genotypes to confirm the effectiveness and economics of the MAS relative to our greenhouse screening procedure.

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FUNDING AGENCIES
Funding for this research was received in mid October 2003. This proposal was not submitted to other funding agencies. However it is linked to the Walker/Tenscher/Ramming Pierce's disease resistance breeding project funded by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, California Table Grape Commission, California Raisin Advisory Board, and the Walker/Riaz Mapping project. This project was initiated through funding by the American Vineyard Foundation and California Department of Food and Agriculture on the Genetics of Resistance to Pierce's Disease, a project that developed a framework map for the 9621 population. Funding from the Louis P. Martini Endowed Chair in Viticulture has also supported Pierce's disease mapping and marker development projects.

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

Project Leaders:
Andrew Walker and Summaira Riaz
Department of Viticulture and Enology
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted from July 2002 to November 2003.

ABSTRACT
Genetic mapping efforts continue in the 9621 population (D8909-15 x F8909-17). *Xylella fastidiosa* (*Xf*) resistance is segregating in this population both the *Vitis arizonica* and a *V. arizonica/V. champinii* are resistant. We have increased the mapping population size from 116 to 188 individuals, thus increasing recombination frequencies and taking advantage of 96-well plate based techniques. We are adding the original 375 AFLP markers to the additional 72 individuals. Three hundred and ten SSR markers have been tested of which 192 amplified and 165 were polymorphic in the population – these have been added to the map. Fourteen of 20 EST markers from Doug Adams' lab were also added. We have also added 16 EST derived SSR markers from Doug Cook's database of ESTs with the goal of adding 100 to 150 more by June. *Xf* resistance maps to linkage group 14. There are two flanking SSR markers on one side and an SSR and AFLP marker on the other side of the *Xf* resistance locus derived from F8909-17.

INTRODUCTION
This project expands upon and continues a genetic mapping effort that has also received funding from the California Grape Rootstock Improvement Commission, the Fruit tree, Nut tree and Grapevine Improvement Advisory Board, the California Table Grape Commission and the American Vineyard Foundation. Last year's CDFA GWSS/PD Research Board proposal was entitled "An Expanded Genetic Map of *Vitis rupestris* x *Muscadinia rotundifolia* for Fine Scale Mapping and Characterization of Pierce's Disease Resistance". The name was changed to reflect current knowledge of the mapping population's true parentage. The project has been mapping resistance to *Xiphinema index*, the dagger nematode, and *Xylella fastidiosa* (*Xf*) in an "F2" population made from "siblings" selected from an F1 *V. rupestris* x *M. rotundifolia* population generated by Dr. Olmo in 1988. We have recently discovered that these crosses were largely contaminated by pollen other than the applied *M. rotundifolia* (more details are included in "The genetics of and breeding for Pierce's disease resistant grapes" project summary). The 9621 mapping population is a cross of D8909-15 (*V. rupestris* x *V. arizonica*) x F8909-17 (*V. rupestris* x a *V. arizonica/V. champinii* type). Because of this population's segregation for, and presence of high resistance to, *X. index* and *Xf* it is an extremely valuable asset for map based positional cloning of these resistance genes.

A genetic map of 116 individuals from the 9621 population was created primarily with AFLP markers. The AFLP marker system is very robust, however, dominant inheritance of markers can cause limitations with highly heterozygous crops like grape. We began using more informative markers, such as microsatellites or simple sequence repeats (SSR) for two main reasons. First, a framework genetic map with SSR markers provides essential infrastructure for targeted physical mapping of candidate genes and quantitative trait loci (QTLs). Secondly, SSR markers tightly linked to resistance and phenotypic traits of interest are ideal for marker-assisted selection due to their applicability across different genetic backgrounds. The grape genetic research community formed the International Grape Genome Program (IGGP) to increase coordination and cooperation and to enhance knowledge of the grape genome. Use of the SSR marker system is common among the different research groups so that our mapping efforts can be linked to others. Integrating the 9621 genetic linkage map to other mapping populations will facilitate targeting genomic regions that harbor quantitative trait loci. Comparison to other maps will allow us to identify more markers that are tightly linked to *Xf* resistance and optimize marker-assisted selection strategies in the breeding program (see the "Optimizing marker-aided selection (MAS) for *Xylella fastidiosa* resistance to accelerate the breeding of PD resistant grapes" progress report). It will also more fully support efforts to locate and identify the gene(s) responsible for *Xf* resistance.

OBJECTIVES
1. Increase the core mapping population size from 116 to 188 individuals (more recombinants reduce the distance between markers).
2. Use genomic SSR and ESTP (expressed sequence tag polymorphism) markers as the core marker system and increase the number of SSR markers on the genetic linkage map to 200 (initial efforts were only 100 SSR markers).
3. Screen an additional 100-150 EST derived SSR markers for which functions are known after their comparison to homologues in available EST databases.
4. Develop the core framework map with an average distance of 2-5 cM between markers.

RESULTS AND CONCLUSIONS

Objective 1. The starting material for this project was a complete AFLP marker based genetic map of the 9621 population. This map was initiated several years ago and was based on 116 individuals with 375 AFLP, 32 ISSR, 25 RAPD and 9 SSR markers. We expanded the core set of individuals from the 9621 to 188 individuals and have extracted the complete set of DNA.

Objective 2. The SSR markers used included some that have been previously published and many that were developed by Vitis Microsatellite Consortium. All markers were tested on a small set of 8 DNA samples including both parents and run on 6 % polyacrylamide gels. DNA on the gels was visualized by silver staining with a commercial kit (Promega). Only informative markers were used on the entire set of 188 genotypes. We have tested and used all available informative genomic microsatellite markers for 9621 population. (Table 1).

To develop ESTP markers, sequences of grape cDNA were obtained from Dr. Doug Adams (Department of Viticulture and Enology, UC Davis). Potential PCR primers were designed using the computer program PRIMER 0.5. Primers were selected to have similar properties to facilitate standard conditions for PCR reactions. Primers are 20 to 23 nucleotides long with GC contents of 50-60% and melting temperature ranging from 59-64°C. Amplification and polymorphism for each EST was tested on 2% agarose gels. If length base polymorphism were not revealed, then a set of 10 different restriction enzymes (*Hind*III, *EcoR*I, *Ava* II, *Bst*NI, *Dra*I, H*ae* III, H*inf*1, M*sp* I, *EcoR*V, R*sa* I) were tested to find restriction site based polymorphism among parents D89090-15 and F8909-17. Polymorphic ESTP markers were added on entire progeny of 188 genotypes.

Table 1. Data on number of markers tested and useful for the D8909-15 x F8909-17 mapping population.

Markers	Tested	Amplified	Useful for Map
SSR	310	192	165
EST (D. Adams)	20		14
Total	330		179

We are in the process of developing a collaboration with researchers at INRA (Montpellier, France) to gain access to more SSR markers based on the comparisons with their genetic linkage map of Syrah x Grenache.

Objective 3. There are now a large number of EST derived SSR markers available, in addition to the genomic SSR markers from the Vitis Microsatellite Consortium. The EST derived SSR markers are more valuable if the cDNA sequence from which the EST was derived has a know function as determined by comparisons with homologs from other EST databases. We plan on selecting EST-SSR that show homology to resistance genes to different pathogens and genes that control other important morphological, physiological and agronomic traits. Dr. Doug Cook developed a database with a large number of EST derived SSR markers. Our goal is to screen 100-150 EST-SSR markers with putative known function by June 2004. Thus far, we obtained sequences for 50 markers from the http://cfg.ucdavis.edu/ web site. Twenty-five markers have been screened and 16 were polymorphic in the 9621 population. We are in the process adding them to the genetic linkage map.

Objective 4. Preliminary linkage analysis for each parent was carried out with MAPMAKER 2.0. Each segregating locus was paired with a “dummy” locus, resulting in a doubled data set. Linkage groups obtained from the doubled data set were then divided into two symmetrical sets of groups and one set was chosen for further detailed. The "first order" and "compare" commands were used to determine the probable order of all markers in each linkage group. The integrated linkage analysis to obtain the sex-average map was performed with JOINMAP 2.0. (LOD 5.0 and recombination frequency 0.45). Using the fixed sequence command the order of markers was determined relative to the established order obtained from the initial MAPMAKER analysis. Map units in centimorgans (cM) were derived from the Kosambi (K) mapping function. The integrated consensus map analysis was carried out with JOINMAP 3.0. The consensus linkage map was developed with 156 markers (141 SSR markers, 14 ESTP markers and the Pierce’s disease resistance locus). A total of 153 markers fall in 20 linkage groups and only 3 markers were unlinked. Total map length is 935 cM with average distance between markers of 6.19 cM. All markers were evenly distributed. The largest linkage group was comprised of 13 markers (105cM) and smallest group consisted of 4 markers (17cM). The locus for Pierce’s disease resistance mapped to linkage group 14 with two flanking markers on each side. We continue to add markers - an additional 24 were added to the entire population, but not included in the above analysis. Our goal is to reduce the distance between markers from 6 cM to 2-5 cM; the required distance to initiate the map based positional cloning of genes.

CONCLUSIONS

We are continuing to optimize our genetic linkage map and shorten the distances between markers linked to *Xf* resistance. We are preparing to begin map-based positional cloning. More individuals need to be put on the map and tested for *Xf* resistance to increase the recombination frequency and shorten map distances. We have about 2,000 9621 seedlings in the field to allow these efforts. This project is greatly benefiting our marker-assisted selection project (“Optimizing marker-aided selection (MAS) for *Xylella fastidiosa* resistance to accelerate the breeding of PD resistant grapes”) by fine-tuning makers.

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

THE GENETICS OF AND BREEDING FOR PIERCE'S DISEASE RESISTANT GRAPES

Project Leaders:
Andrew Walker & Alan Tenscher
Department of Viticulture and Enology
University of California
Davis, CA 95616

David Ramming
USDA-ARS
9611 South Riverbend Ave.
Parlier, CA 93648

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ABSTRACT
We continue to make many crosses, produce thousands of seeds and embryos, and about four thousand plants in the field each year. We have been increasing the number of seedlings and high fruit quality selections we test under our greenhouse screen. This screening is very severe, but material that passes the screen is reliably resistant and dramatically restricts *Xylella fastidiosa* movement. We are also co-screening for powdery mildew resistance. The heritability of *Xf* resistance from a range of resistant Southeast US (SEUS) cultivar and species parents is not consistent – some parents produce few resistant offspring, while others produce a large percentage – making careful parental screening very important. We have been able to expand our *Xf* screening this year and are currently testing 178 potential parents (selected backcross progeny, new SEUS parents, and Olmo VR hybrids) and will have resistance results before the 2004 pollination season so that crosses can be optimized. The USDA embryo rescue process has produced a large number of progeny from crosses of resistant males to seedless females including 265 plants from second generation backcrosses. Culture efforts this year (2,702 ovules) produced 484 embryos that are now germinating. Crosses continue to be made with SEUS resistant wine grape selections to *vinifera* wine grapes including Merlot, Syrah, and Olmo selections. Rootstock crosses are also being made and seedlings evaluated and will be used in joint efforts to examine possible inducible tolerance to PD via rootstocks.

INTRODUCTION
This project is a collaborative effort between UC Davis and the USDA/ARS –Parlier, and is focused on breeding new PD resistant cultivars of table, raisin and wine grapes. The project integrates with efforts to develop genetic maps for resistance to *Xylella fastidiosa* (*Xf*) in segregating populations containing resistance from *Vitis arizonica* and a number of southeastern US (SEUS) *Vitis* species and cultivars. The goal of these mapping efforts is the development of strongly linked DNA markers to expedite breeding with the long term goal of characterizing and localizing *Xf* resistance genes leading to genetic transformation efforts.

A noteworthy discovery this year involved our *Vitis rupestris* x *Muscadinia rotundifolia* selections. These selections were produced from crosses made by Dr. H.P. Olmo and the seedlings were raised and evaluated in the Walker lab. The Walker lab has been evaluating these selections for *Xf* and nematode resistance and using progeny from them in mapping efforts for over 13 years. The mapping efforts have focused on a cross of two siblings from Olmo's *V. rupestris* 'A. de Serres' x *M. rotundifolia* 'Cowart' – D8909-15 x F8909-17 which generated the 9621 mapping population. Mapping efforts with this population are the basis of the Walker/Riaz proposal "Map-based Identification and Positional Cloning of *X. fastidiosa* Resistance Genes from Known Sources of Pierce's Disease Resistance in Grape". This year simple sequence repeat (SSR) DNA markers were placed on the map to allow comparisons with other maps being created within the lab and internationally. SSR markers are also ideal for parentage analysis, and once they were applied it became clear that D8909-15 and F8909-17 were not full siblings and that the male parents were incorrect. It is now clear that this cross was contaminated by wind-blown pollen from five male vines in addition to the applied *M. rotundifolia* 'Cowart' pollen. D8909-15 is a cross of *V. rupestris* 'A. de Serres' x *V. arizonica* and F8909-17 is a cross of the same female by what appears to be a hybrid of *V. arizonica* x *V. champinii*. F8909-08 is a true sibling of F8909-17 and the resistance source for a number of crosses in the breeding program.

This discovery has many positive implications for PD breeding. The *Xf* resistance of these *V. arizonica* hybrids is remarkably strong (as good as selections of *M. rotundifolia*) and has been integrated into a large number of *V. vinifera* grapes and is now in the third backcross (BC) generation. The Walker lab has reported on the resistance of these selections to both *Xf* and *Xiphinema index* (Walker and Jin 1998 Acta Hort 473:113-120., Krivanek and Walker 2003 Acta Hort 603:429-432). The horticultural characteristics of *V. arizonica* are far preferable to *M. rotundifolia* and include relatively neutral flavors, normal clusters with fruit that does not abscise when ripe and reasonable rooting ability, although its berry size is small. Most importantly there are no genetic barriers to breeding because *V. arizonica*'s chromosome number is the same as *V. vinifera*, as opposed to *M. rotundifolia*.

OBJECTIVES
1. Develop PD resistant table and raisin grapes by crossing a variety of *Xf* resistance sources with large berried and seedless *V. vinifera* table and raisin grapes.
2. Evaluate existing PD resistant wine-type cultivars for suitability in PD hotspots in California.
3. Breed PD resistant wine-type cultivars by crossing a selected number of *Xf* resistance sources with traditional wine varieties.

4. Investigate the potential of inducing *Xf* resistance by using various *Xf* resistant selections as rootstocks.

RESULTS AND CONCLUSIONS

Resistant Table/Raisin Varieties

We completed the screening of 126 additional genotypes in the last year: 102 were recent crosses of SEUS types backcrossed to advanced *vinifera* table and raisin types; 16 were from the USDA disease resistant table grape program (focused on powdery mildew); and 8 were new SEUS resistant sources added to our collections. In the first group, several resistant genotypes were identified as seedless (small hollow seed traces approaching commercial acceptability). Several others were resistant seeded genotypes with high quality fruit characteristics. Some resistant lines were also powdery mildew resistant. Interestingly, only two of the 16 USDA powdery mildew crosses were resistant to *Xf*. These were both first generation crosses from SEUS *Xf* resistant types, but *Xf* resistance does not seem to be correlated to powdery mildew resistance, which reemphasizes the need pre-screen for *Xf* resistance.

When crossed to *vinifera*, different SEUS resistance sources produce very different ratios of resistant (R) to susceptible (S) progeny. For example, screening data from this year produced the following R:S ratios: Calinda x Daytona 2:3 (n=25), C33-30 x DC1-39 1:3 (n=60), C33-30 x BD5-117 2:1 (n=21). Although n is small, it is clear that resistance is not inherited in the same manner among different parents, which emphasizes the importance of careful progeny testing.

We currently have 178 additional genotypes inoculated in our greenhouse screen. One hundred of these genotypes are progeny from four different resistance sources (*V. champinii*, Roucaneuf, Zehnder, and 0023-019 (D8909-15 x USDA seedless table grape)) backcrossed to USDA advanced seedless types. Early testing is being done so that we can select parents that produce the highest percentage of resistant progeny for next year's crosses. The remaining 55 genotypes in testing are progeny of SEUS-type resistant sources crossed to *vinifera* and that were selected for fruit quality this year. We are also testing 23 genotypes from the Olmo VR (*vinifera* x *rotundifolia*) collection consisting of F1 VR hybrids, VR intercrosses and VR backcrosses to *vinifera*. These have excellent powdery mildew resistance and many are expected to be *Xf* resistant.

This year's increased funding was directed at expanding greenhouse screening efforts as well as increasing the number of crosses, progeny and embryo rescued progeny we generate. It is critical to expand and accelerate screening efforts so that results on seedlings that fruited the year before are available to guide the following year crosses. In addition to the genotypes under testing above, 200 genotypes (split equally from USDA-Parlier and UCD) evaluated for fruit quality are being prepared for testing. Results are due before the breeding season 2004. We plan to test about 1,000 genotypes per year with this increased funding.

At UCD we planted 2070 seedlings from crosses made in 2002. About 32% were second generation BC of the *arizonica* resistance source hybrids to high quality *vinifera*, 17% were intercrosses of first generation resistant types, 35% were first generation *vinifera* crosses to a *champinii* resistance source, and 16% were first generation crosses of *vinifera* to two different SEUS *Xf* resistant selections.

From the crosses made and embryos cultured in 2002 at the USDA, 265 plants were produced from 20 BC1 combinations (D8909-08 resistance source) with table and raisin grape selections (13 table grape crosses = 181 plants in field and 7 raisin crosses = 84 plants in the field). An additional 74 plants were planted in the field from SEUS cultivars and selections crossed with table grape selections. Finally, 70 plants were planted in the field from table grape (1), raisin (1) and wine (2) varieties crossed to a selected VR hybrid. This year a large portion of the seedlings from the 2000 and 2001 seedlings produced fruit at Fresno and were evaluated. It is interesting to note that of the three selections from VR hybrids tested for PD resistance in the greenhouse, one was resistant. An additional 14 seedlings have been selected from VR crosses with improved fruit quality. One mildew resistant selection made from a cross with Suwanee was found to have intermediate *Xf* resistance in greenhouse tests. This selection is a white seedless (med to large trace) selection with 2.6 gram berries and will be used as a parent with table and raisin grapes. In the seedlings from crosses with SEUS cultivars and selections with table and raisin selections, 74 selections were made this year of which 42 were seedless. In crosses from other SEUS breeder's selections by table and raisin selections, 47 selections were made of which 31 were seedless. Many of these selections from SEUS material had seedless berries as large as 13-15/16 inch in diameter with firmness rated 6 (5 = average) and one rated 7 (= firm). The fruit quality in many was also rated good (7).

Twenty different cross combinations were made at UCD in 2003. Eight were second generation BCs of the *arizonica* resistance source to *vinifera*; five involved resistant selections from crosses made in 1999 and 2000; six used two promising SEUS selections (one that has previously shown a high percentage of resistant progeny and another that looks very good in our PD field trial); and one cross with a VR hybrid. One of the 5 crosses with our resistant selections was directed at combining resistance from *shuttleworthii* and *smalliana* with *vinifera*. Four additional crosses were made onto female seedless *vinifera* table grapes involving the same two promising SEUS selections used above. A total of 18 clusters were produced and shipped to USDA-Parlier for embryo rescue.

This year (2003) USDA-Parlier cultured ovules from 8 crosses. Three of these were backcrosses of 0023 seedlings with high quality table and raisin grapes. The rest were SEUS selections crossed to high quality table and raisin grape selections. A total of 2,702 seedless ovules were cultured and resulted in 484 embryos, which are now germinating.

Screening existing Xf resistant wine grape cultivars.
We are currently screening 8 *Xf* resistant wine grape selections from Zehnder, a private breeder in North Carolina. A number of the SEUS field resistant cultivars at our PD field trial in Yountville are wine grapes. As an adjunct to that trial, approximately 90 vines of Blanc du Bois were planted in 2001. The vines were harvested this year for small lot winemaking. USDA-Parlier has crossed Zehnder's selections to Merlot and is evaluating for flavor, pH, and TA. Seedlings of SEUS breeder's selections by wine cultivars were evaluated and 36 selections were made. The cluster sizes ranged from small to large and sugars reached 26°B by the first of October. These selections were also screened for leaf, stem and berry resistance to mildew.

Breeding new Xf resistant wine-types.
In 2000 we made 2 wine grape crosses of the highly *Xf* resistant F8909-08 (*arizonica-champinii* type) onto *vinifera* wine grapes to produce 3,227 seeds. These crosses were repeated this year and about 1,000 of these seedlings will be planted next spring. We also made crosses of SEUS cultivars onto Syrah, producing 2,150 seeds. Marker-assisted selection (see Walker/Krivanek/Riaz project) will be used to pre-screen these seedlings before fruit or *Xf* resistance evaluations.

PD resistant rootstock breeding.
We have about 500 seedlings in the field from crosses of D8909-15 and F8909-8 with 101-14 Mgt and 1616C rootstocks. These crosses were repeated this year to produce about 2,500 seeds. Genetic markers for resistance will be used to pre-screen these plants and their evaluation will be tied to research projects in collaboration with Hong Lin (USDA-Parlier) exploring biochemical resistance mechanisms that might be inducible via a resistant rootstock.

PD Field Trial
We expanded our PD field trail in Yountville this year with assistance from Beringer/Blass. Two additional blocks of plants were added. One contained replicated genotypes of recent crosses with varying degrees of *Xf* resistance as measured in our GH screen as well as some additional SEUS field resistant types. A second block was planted to 18 conventional rootstocks (101-14, 3309C, Schwarzmann, Gravesac, SO4, 5, 420A, 161-49C, St. George, 110R, 1103P, 44-53, 039-16, Riparia Gloire, Fercal, Freedom, Ramsey and Dog Ridge), and is destined for field budding to a field resistant SEUS cultivar to evaluate rootstock vulnerability to PD under California conditions. These grafted plants will be inoculated with *Xf* to evaluate the impact an infected, but resistant, scion has on rootstocks. Both the 2001 and 2003 plantings were inoculated with *Xf* again this year and ELISA and disease symptom expression data were taken in October.

CONCLUSIONS
Collaboration between UCD and the USDA-Parlier is excellent and this program is closely integrated with the other Walker lab projects and those of Lin at the USDA-Parlier. Fruit quality is advancing quickly in many backgrounds (see Figures 1 & 2). Aggressive training is allowing fruit evaluation in year 2 after planting, thus shortening generation times to 3 years. Raisin grape types should be ready for field testing within several generations, as will wine grapes. Table grapes will take longer to achieve large seedless berries with crisp texture, but we are very encouraged with our progress.

FUNDING AGENCIES
Funding for this project was provided by the California Table Grape Commission and the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board. Funding in the past has also been received from the California Raisin Marketing Board and the USDA Animal and Plant Health Inspection Service.

Figure 1. An example of the rapid progress being made breeding *Xf* resistant table grapes. From left to right first generation progeny from *V. vinifera* B90-116 x *V. shuttleworthii* F902: F902, 0070-12, 0070-14, 0070-28 and Redglobe (for size comparison).

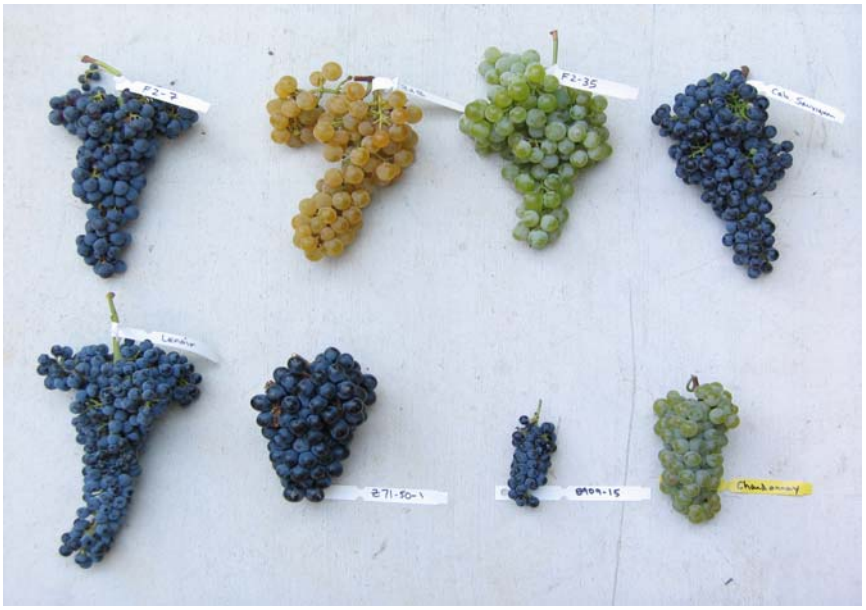

Figure 2. An example of wine grape types being used in crosses. Clockwise from the upper left: F2-7 (Carignane x Cabernet Sauvignon); Blanc du Bois; F2-36 (same cross as F2-7); Cabernet Sauvignon; Chardonnay; D8909-15; Zehnder 71-50-1; Lenoir (Jacquez or Black Spanish).

REAL TIME PCR FOR CLINICAL DETECTION AND DIFFERENTIATION OF *XYLELLA FASTIDOSA* STRAINS

M. Francis
Department of Plant Pathology
University of California
Davis, CA 95616

E.L. Civerolo and H.L. Lin
USDA-ARS-PWA
San Joaquin Valley Agriculture Science Center
Parlier, CA 93648

ABSTRACT

The overall goal of this work is to develop reliable protocols for the clinical detection and identification of *Xylella fastidiosa* (*Xf*) strains. The objectives are to (1) apply PCR-based methods to detect low populations of *Xf* strains causing the Pierce's disease of grapevines infected grape tissue; and (2) to distinguish different *Xf* strains in naturally-occurring single or mixed infections in different hosts, as well as in insect vectors. A major problem is the presence of PCR inhibitors in the grape tissue extracts that result in false negative results. A simplified method for the isolation of grape tissue DNA, using a single tube for grinding and extraction, was developed. Two real time PCR systems were developed for the generic detection of *Xf* strains and the specific detection of the *Xf*-PD strain or pathotype based on the currently available genomic sequences of four *Xf* strains. One system, based on a set of primers, designated HL5/HL6, and a probe labeled with FAM (HL5/HL6-FAM) as a fluorescent dye, detected four *Xf* strains (PD, almond leaf scorch (ALS), oleander leaf scorch (OLS)) and citrus variegated chlorosis (CVC) DNA. The specificity of the primers was tested against several other plant pathogenic bacteria and endophytic bacteria isolated from grape, no amplification products were obtained using 103-104 cells/reaction. The *Xf*-specific amplification product was 221 bp. As few as 5 bacteria per reaction were detected using this system. Standard curves were obtained with intact bacteria in water and in preparations containing grape leaf petiole DNA from the equivalent of 1 mg of fresh grape tissue per reaction. The Ct values ranged from 20 cycles for 105 bacteria per reaction to 36 cycles for 5 bacteria per reaction ($r2 > 0.9$). A second system, based on a set of *Xf*-PD specific primers, designated HL7/HL8, and a probe labeled with TET (HL7/HL8-TET) as a fluorescent dye, was developed. The *Xf*-specific product in this case was 302bp. This set of primers specifically distinguished *Xf*-PD from *Xf*-ALS and *Xf*-OLS, as well as from *Xf*-CVC DNA. The use of these two systems permits the detection of the *Xf* strains and the specific detection of *Xf*-PD, and could be used to detect as few as 5 bacteria per reaction.

USE OF FLUORESCENT RECOMBINANT ANTIBODIES FOR IDENTIFICATION OF *XYLELLA FASTIDIOSA*

S.E.M Silva-Valdés
Universidad Autónoma de Baja California, México
Km 103 carretera Tijuana-Ensenada
Ensenada, B.C. México

J.L. Stephano-Hornedo
Universidad Autónoma de Baja California, México
Km 103 carretera Tijuana-Ensenada
Ensenada, B.C. México

A. Portillo-López
Universidad Autónoma de Baja California, México
Km 103 carretera Tijuana-Ensenada
Ensenada, B.C. México

M. Gould
Universidad Autónoma de Baja California, México
Km 103 carretera Tijuana-Ensenada
Ensenada, B.C. México

ABSTRACT

A library of recombinant antibodies ScFv was produced by phage display following immunization of a chicken with *Xylella fastidiosa*. Using ELISA, a clone in the vector pComb3X was selected from this library. The clone produces soluble antibody also carries 6His and HA epitope tags. Currently we are trying to develop a fluorescent immunocytochemical method with this antibody that will be able to detect *Xylella* in a plant or insect.

Section 2A: Xylella fastidiosa Genetics and Eco-Physiology

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

Project Leader:
Jianchi Chen
USDA-ARS-PWA
San Joaquin Valley Agricultural Sciences Center
Exotic and Invasive Diseases and Pests Research
Parlier, CA 93648

Cooperator:
Edwin L. Civerolo
USDA-ARS-PWA
San Joaquin Valley Agricultural Sciences Center
Exotic and Invasive Diseases and Pests Research
Parlier, CA 93648

Reporting Period: Anticipated to begin January 2004**.**

ABSTRACT
This project is to develop and evaluate a microarray-PCR-based system for accurate and quick identification of *Xylella fastidiosa* strains from *in vitro* culture. Attempts will also be made to use this system to detect *X. fastidiosa* strains from their hosts. A particular emphasis is on strains of grape Pierce's disease, almond leaf scorch disease and oleander leaf scorch disease, which are currently important in California.

INTRODUCTION
Traditionally, *Xylella fastidiosa* is identified by cultivation on complex media, serological tests and pathogenicity tests. From the genetic standpoint, these traditional methodologies target the expressions and regulations of multiple genes characteristic to the pathogen. However, there are many drawbacks associated with the traditional multi-locus test methodology. The nutritional fastidiousness of *X. fastidiosa* poses the major challenge for the use of media-based identification scheme. A direct implication from this challenge is the difficulty in establish a taxonomy system under the species level, leading to, in many cases, the uncertainty of a newly isolated *X. fastidiosa* strain. This further affects the control of *Xylella* diseases. Serological tests have an advantage of being simple and fast. They, however, suffer from cross antibody-antigen reactions from different pathotypes. Pathgenicity tests are of high economical value, but such tests are laborious and time-consuming. Current pathogen regulation policy also hinders the large scale usage of this method.

Theoretically, PCR allows the amplification of characteristic gene or DNA sequences from a single DNA molecule. For this reason, PCR has recently been the most common technique for *X. fastidiosa* identification. There are, however, technical problems limiting the application of PCR. Specific primers may fail to amplify DNA from a particular isolate if there is a spontaneous mutation(s) in the primer-binding site, leading to a false negative result. Non-specific amplicons may occur which complicate the interpretation of the electroporesis data and may result in a false positive conclusion. The sensitivity and specificity of PCR amplification tend to be inversely related.

A combination of PCR with DNA-DNA hybridization can improve the detection specificity and maintain high sensitivity. While traditional DNA-DNA hybridization assays are laborious and time-consuming, microarray technology provides a solution. DNA microarrays have been widely used for gene expression studies. This technology is also a powerful tool in pathogen identification and detection. Unlike other hybridization tools, such as microplates or dot blots for DNA-DNA hybridization with membrane-bound probes, miniature glass microarrays are capable of containing DNA probes specific to thousands of individual target DNAs. Potentially, microarray technology allows the rapid determination of thousands of genetic characters of a microorganism in one hybridization experiment. This mimics the multi-genic methodology, but microarray is superior in that the genes or DNA sequences in test can be manipulated, testing time is much shorter, and test accuracy and efficiency are much greater.

OBJECTIVES
The primary objective of this project is to develop and evaluate a microarray-PCR-based system for accurate and quick identification of *Xylella fastidiosa* strains from *in vitro* culture. Attempts will also be made to use this system to detect *X. fastidiosa* strains from their hosts. A particular emphasis is on strains of grape Pierce's disease, almond leaf scorch disease and oleander leaf scorch disease, which are currently important in California. Two specific objectives are: 1) using the complete and annotated genome sequence of *X. fastidiosa* Temecula strain as a guide, select appropriate DNA sequences and evaluate their potential for pathotype / genotype identification. Design and construct a DNA microarray with PCR amplicons; and 2) evaluate the effectiveness of the constructed microarray through hybridization experiment. Using the microarray as a reference, analyze genomic variation of different pathotypes with multiple strains collected from broad

geographical areas and hosts. Attempt will be made to develop a genovar system to substitute the pathotype or pathovar system which is by far un-standardized and difficult to test.

RESULTS AND CONCLUSIONS

We believe that the proposed project has a high probability of success. There have been two reports regarding the use of microarray analysis focused on the transcriptome of CVC strain of *X. fastidiosa* (Costa de Oliveira et al., 2002; Nune et al., 2003). This proposed project will explore the application perspective of microarray in *X. fastidiosa* identification. Objective 1 of this proposal addresses the utilization of genome DNA sequence information. Complete genome sequences of four *X. fastidiosa* strains are publicly available. Unitization of the bioinformation implied by these sequences is the goal of genome sequencing and we have just begun this effort. Objective 2 links the *X. fastidiosa* genome research from laboratory to the field. A large number of field isolates will be incorporated into genome comparisons, leading to a better and faster characterization of this nutritionally fastidious prokaryote. Efforts to extensively collect strains of *X. fastidiosa* are currently underway.

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FUNDING AGENCIES

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

Project Leader:
Donald A. Cooksey
Department of Plant Pathology
University of California
Riverside, CA 92521

Cooperator:
Ching-Hong Yang
Department of Biology
University of Wisconsin-Milwaukee

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

ABSTRACT
The identification of genetic factors that enable *Xylella fastidiosa* to express Pierce's disease symptoms is essential to the further development of several disease control strategies. One such control strategy is to use non-pathogenic derivatives of the Pierce's disease pathogen itself to competitively exclude pathogenic strains in grapevines. We have made progress in the development of genetic tools for this purpose and in identifying several putative virulence genes through comparative genome and mutational analyses. However, with over 50% of the *X. fastidiosa* genome consisting of genes with no known function, a more comprehensive approach is needed to identify genes that are important for virulence of the Pierce's disease strain of *X. fastidiosa*. This new project is using a DNA microarray approach for this purpose.

INTRODUCTION
The DNA microarray approach to identifying bacterial virulence genes follows the hypothesis that many virulence genes are differentially expressed (up-regulated or down-regulated) during infection of the host. This hypothesis has been proven over and over for numerous virulence genes in both plant and animal pathogens (Handfield and Levesque, 1999). With the full genome sequence now available (Bhattacharyya, A., et al. 2002; Simpson et al., 2000; Van Sluys et al., 2003), a complete DNA microarray of the genome of the Pierce's disease strain of *Xylella fastidiosa* is feasible to produce. The major advantage of this approach is that, unlike conventional reporter gene or hybridization strategies, microarrays can simultaneously produce relative expression data for thousands of genes in the target organism, and no prior knowledge of the genes and their function or regulation is required. Microarrays therefore represent an appealing approach to identifying bacterial gene sets that are up-regulated or down-regulated by growth in specific environments, and are attractive for studying gene expression by pathogenic bacteria in their hosts because of the large number of genes involved.

We previously conducted a preliminary study with a DNA macroarray of about 100 genes from the *Xyella fastidiosa* genome arrayed on a filter (Hernandez-Martinez et al., 2002). This study showed strong evidence of differential expression of several genes that were identified as possible virulence factors from the original genome sequence annotation. Using techniques similar to our work on the functional genomics of another plant pathogen, *Erwinia chrysanthemi* (Okinaka et al., 2002; Yang et al., 2003), we have just begun conducting a comprehensive DNA microarray/mutational approach to identify genes that are important for virulence of the Pierce's disease strain of *Xylella fastidiosa*.

OBJECTIVES
1. Conduct DNA microarray analysis of gene expression patterns in *Xylella fastidiosa* during infection of plants vs. growth in other conditions.
2. Mutate putative virulence genes and characterize virulence defects.

RESULTS AND CONCLUSIONS
Results
Research during the first three months of this project have been directed toward the development of effective RNA extraction methods for infected grapevines and in designing a full-genome DNA microarray.

Conclusions
This work will contribute significantly to fundamental information on the genetics and pathogenicity of *Xylella fastidiosa* and will benefit researchers pursuing various strategies of management of Pierce's disease. As mentioned above, this information is essential for our continuation of the strategy to use non-pathogenic strains for biological control of Pierce's disease. Identification of virulence genes can also lead to recognition of new unforeseen targets for management strategies. In addition, the construction of a DNA microarray for this pathogen, and identification of genes differentially expressed during infection, will complement work by others on differential expression of grapevine genes during infection. This will open the door to "interactive genomic" studies that will enhance our understanding of the bacterial-plant interaction that leads to Pierce's disease, and in the future, studies of interactions with its insect vectors.

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FUNDING AGENCIES

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UNDERSTANDING *XYLELLA FASTIDIOSA* COLONIZATION AND COMMUNICATION IN XYLEM LUMINA

Project Leaders:
Harvey C. Hoch
Thomas J. Burr
Department of Plant Pathology
Cornell University
NYSAES
Geneva, New York 14456

Post-doctoral Researcher:
Yizhi Meng
Department of Plant Pathology
Cornell University
NYSAES
Geneva, New York 14456

Cooperator:
James N. Turner
New York State Department of Health
Wadsworth Center
Albany, New York

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

ABSTRACT
Flow cells that emulate xylem vessels have been microfabricated in silicon and in polydimethylsiloxane. *Xylella* cells in these artificial vessels are being studied for colonization and biofilm development.

INTRODUCTION
Pierce's disease of grape is caused by *Xylella fastidiosa*. Symptoms are generally recognized as being caused by restricted sap flow and resultant water stress due to plugging of xylem elements by live bacterial aggregates and associated mucilage (Goodwin et al., 1988; Purcell and Hopkins, 1996; Mollenhauer and Hopkins, 1974). It is not clear whether the extracellular polymeric mucilage is of bacterial and/or plant origin. Based on the analysis of the complete genome sequence of *X. fastidiosa*, gums produced by the *X. fastidiosa* are similar to the 'xanthan gums' produced by *Xanthomonas campestris* pv *campestris*, although they may be less viscous (Simpson et al., 2000). In addition, tylose development in xylem vessels in response to the presence of the bacterium further restricts sap flow (Mollenhauer and Hopkins, 1976). These general concepts *X. fastidiosa* pathogenicity are readily recognized, although it is not understood how the bacterium becomes established in the turbulent habitat of a 'fluid conduit' i.e., xylem vessels and tracheae. Bacterial spread through xylem elements is also poorly understood, albeit enzymatic degradation of pit membranes is thought to be involved (Mollenhauer and Hopkins, 1976). Colony formation is likely to be influenced by the physical constraints of the xylem element surface much like the formation of bacterial biofilms is influenced by surface characteristics (microtopography, chemistry, etc.) in other aqueous and fluid environments such as medical stints and prostheses, food handling equipment, and water supply systems (Ridgway and Olson, 1981; LeChevallier et al., 1987; Caldwell and Lawrence, 1988; Sternberg et al., 1999). Surface microtopography of these environments influence the temporal and spatial aspects of bacterial colonization (Bremer et al., 1992; Gorman et al., 1993; Korber et al., 1997; Arnold, 1999). Surfaces become colonized as cells (in this case bacteria) attach initially via physio-chemical forces, and ultimately with extracellular polysaccharides or ligand-mediated interactions. The end result is the establishment of biofilms consisting of bacteria in a polysaccharide matrix that provide a protective habitat that is conducive for continued cell growth and colony formation. The recently completed sequencing of the *X. fastidiosa* genome has revealed several open reading frames with putative functions that may be associated with bacterial colonization of xylem vessels and disease (Simpson et al., 2000). For example, at least one ORF with homology to the *luxR* family of transcriptional regulators has been identified (GenBank accession AAF83782). Such genes encode proteins (LuxR homologs) that when bound by acyl-homoserine lactone autoinducer molecules (AI), regulate transcription of diverse types of genes (Fuqua et al., 1996). Autoinducers are synthesized by enzymes that are encoded by *luxI* homologs or other synthase genes. The *luxI – luxR* regulatory system was first discovered in the marine bacterium *Vibrio fischeri*, however now related systems have been discovered in diverse species of bacteria including plant and animal pathogens (Cha et al., 1998). Autoinducers diffuse bi-directionally across bacterial membranes and reach concentrations for efficient activation of LuxR regulators in environments of high bacterial density. Thus the ability of AI to activate the LuxR regulators is a cell density-dependent response referred to as quorum-sensing or autoinduction. The discovery of *luxR* homologs in *X. fastidiosa* suggests that the bacterium produces AI or related signal molecules that may regulate genes that are associated with biofilm production in a density-dependent manner. This finding is intriguing because it suggests that a quorum-sensing regulatory system may be functioning in *X. fastidiosa* biofilm communities in xylem vessels.

OBJECTIVES

1. To understand how the physical parameters of xylem tracheae and vessels influence *Xylella fastidiosa* colonization. Toward this, we will evaluate colony formation, mucilage production, biofilm development, and evaluate flow rate during and following colonization. Our approach will be to use microfabricated 'artificial' vessels that mimic topologies and chemistries of 'real' *in planta* vessels.
2. Determine whether *X. fastidiosa* produces acyl-homoserine lactone autoinducer molecules that are involved in regulation of genes associated with ability to cause Pierce's Disease.

RESULTS AND CONCLUSIONS

Results

Development of artificial xylem vessels (flow chambers). Flow chambers of four different size dimensions have been designed and microfabricated. Positive masters for the chambers were either fabricated directly in semiconductor grade silicon wafers using photolithography and reactive ion etching procedures or as negative masters on similar silicon wafers

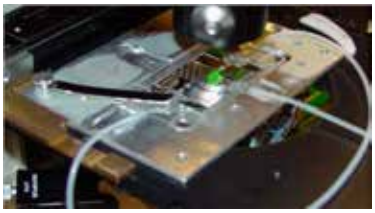

Figure 1. PDMS 'flow chamber' mounted on microscope stage with connected media supply tubing.

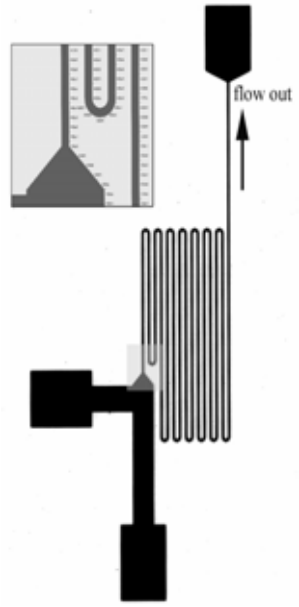

Figure .2. Schematic of 14 cm long flow chamber. Channel is 100 □m wide.

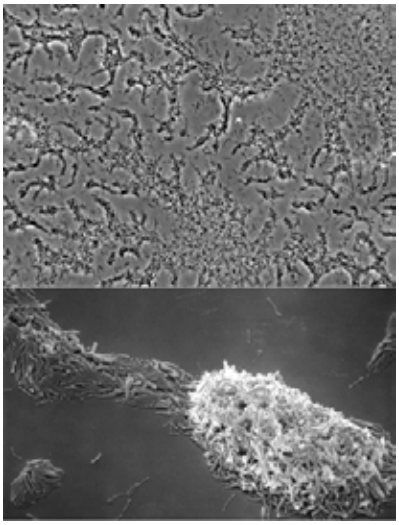

Figure 3. Xylella cells colonizing PDMS (LM and SEM images)

with patterned SU-8 photoresist providing the relief pattern from which subsequent chambers were fabricated. In either instance, the final chambers were made of polydimethylsiloxane (PDMS) which when sealed with a cover glass after plasma etching constituted a complete flow chamber (Figs. 1, 2). Chamber dimensions varied from 4-14 cm in length, 100 μm deep, and 50, 100, and 1000 μm in width. Introduction of media and *Xylella* cells into the chambers was controlled with a syringe pump. Once cell attachment to the pre-chamber surface was complete, non-attached cells were flushed from the chamber and sterile media was pulled continuously through to emulate plant xylem fluid flow. Temporal and spatial changes in *Xylella* cell distribution, colony development, and morphology are being assessed.

Adhesion and attachment of Xylella to inert surfaces. A range of surface treatments were examined for adhesion and attachment of *Xylella* cells in preparation for use in the xylem flow chambers. Glass cover slips were treated with various silanes to create surface chemistries of specific affinities (Table 1). In addition PDMS and cellulose were also examined. *Xylella* cells adhered to and colonized best to diphenyldichlorosilane, and dimethyldichlorosilane. PDMS was also very efficiently colonized (Fig. 3). Temporally, attachment of *Xylella* cells occurred nearly as soon as contact with the surfaces were made. Attachment was either at the polar ends or along the length of the cells appeared, in the short term at least, to be secure and irreversible. Highest concentrations of cells appeared at the air-liquid interface, although a significant number of cells attached submerged in the growth media as well.

Table 1. Attachment of *Xylella* cells to various substrata

Substratum	Attached cells
polydimethylsiloxane	++++
muffled glass	+
dialysis membrane	+
bis(2-hydroxyethyl)-3-aminopropyltriethoxysilane	+
3-aminopropyltriethoxysilane	+ ½
diphenyldichlorosilane	++
n-octadecyltrichlorosilane	+++
dimethyldichlorosilane	+++

Biofilm development dependency on initial cell concentration. Colonization and biofilm development (as determined by crystal violet staining) occurred on inert polyproplylene and polystyrene surfaces over a period of one week. Biofilms density was affected by the initial concentration of cells that were added to the medium. For example, cultures initiated from a cell suspension of OD_{600}= 0.1 had a significantly denser biofilm than from those initiated from OD_{600} = 0.7.

Assays to select adhesion-deficient mutants are currently being done.

Conclusions

The results of this investigation will result in understanding temporal and spatial aspects of *Xylella* colonization and movement to new sites.

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THE *XYLELLA FASTIDIOSA* CELL SURFACE

Project Leader:
Michele M. Igo
Section of Microbiology
Division of Biological Sciences
University of California, Davis
Davis, CA 95616

Cooperators:
Bruce Kirkpatrick
Department of Plant Pathology
University of California, Davis
Davis, CA 95616

M. Andrew Walker
Department of Viticulture and Enology
University of California, Davis
Davis, CA 95616

Reporting period: The results reported here are from work conducted from October 1, 2002 to September 30, 2003.

ABSTRACT
The Gram-negative bacterium *Xylella fastidiosa* is the causative agent of Pierce's disease of grapevines. Many of the strategies proposed to control the spread of this bacterium are dependent on the ability of a particular compound to get into the cell by crossing the outer membrane. The goal of our project is to identify the outer membrane proteins of *X. fastidiosa* and to examine how different physiological and environmental signals influence the protein composition of the outer membrane. Our strategy has been to isolate the outer membrane fraction and to analyze the proteins in this fraction using one- and two-dimensional gel electrophoresis. Comparisons of the outer membrane protein profiles for cells grown under different environmental and physiological conditions will increase our understanding of the *X. fastidiosa* cell surface as well as provide important information concerning the role of the outer membrane proteins of *X. fastidiosa* in the development of Pierce's disease.

INTRODUCTION
The causative agent of Pierce's disease is the Gram-negative bacterium *Xylella fastidiosa*, which is a member of the gamma subgroup of the Proteobacteria and is phylogenically related to the *Xanthomonads* (for a review, see Hopkins and Purcell, 2002). *X. fastidiosa* is highly specialized and is capable of multiplying in both the foregut of xylem-feeding insects, such as the glassy-winged sharpshooter and in the xylem system of the host plant. The ability of *X. fastidiosa* to thrive in both the insect foregut and the xylem suggests that the bacterium has evolved regulatory mechanisms that help it to cope with the unique stresses experienced in these two very different ecological niches.

A common response of Gram-negative bacteria to any stress is to change the composition of their cell surface, particularly the protein composition of their outer membrane. The outer membrane is the outermost continuous structure on the bacterial cell surface and serves as a selective barrier between the cell and the external environment. Changes in the protein composition of the outer membrane are known to have a profound effect on the sensitivity of Gram-negative bacteria to detergents, antibiotics, and bacteriophages. Therefore, in order to develop effective methods for controlling the spread of *X. fastidiosa*, it is important to obtain information concerning the protein composition of the *X. fastidiosa* outer membrane and how the composition of this membrane changes in response to environmental signals.

OBJECTIVES
The overall goal of this proposal is to analyze the outer membrane proteome of *X. fastidiosa* and to determine how the proteome profile changes in response to various physiological and environmental conditions. This project will focus on the following two objectives:

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

RESULTS
Identifying the major outer membrane proteins of *X. fastidiosa*.
Our strategy for analyzing the *X. fastidiosa* outer membrane is similar to the strategy that was successfully used to analyze the outer membrane proteome of *Caulobacter crescentus* (Phadke et al. 2001). For this analysis, we used two strains: the strain Temecula 1 provided by B. Kirkpatrick and the strain Stags Leap provided by A. Walker. Both strains were still virulent based on assays performed in these laboratories. We grew the two strains in 1.0 liter of PD3 media for 7-10 days at 28^{o}C. The cells were harvested and broken using a French pressure cell. The outer membrane fractions were isolated by sucrose density gradient centrifugation and then the proteins in this fraction were analyzed using one- and two-dimensional gel electrophoresis.

In our initial experiments, the outer membrane fractions were analyzed using 1-D SDS-polyacrylamide gels. These gels allowed us to quantitate the amount of the different proteins in the outer membrane for the two strains and to predict the sizes of the proteins based on their migration in the gels. These experiments revealed that there were between 8-10 major proteins and 16-18 minor proteins in the *X. fastidiosa* outer membrane. The sizes of these proteins range from 130 kD to 18 kD. (Proteins smaller than 18 kD would not have been detected in this series of experiments.) Our comparison of the outer membrane profiles of the Temecula and Stags Leap strains indicated that the protein composition of their outer membranes is very similar. We did observe slight differences in the amounts of some of the minor outer membrane proteins for the two strains. This may reflect slight variations in how these two strains respond to environmental conditions. However, a more detailed analysis is needed before we can draw any conclusions about these differences.

Although our initial analysis of the outer membrane fractions using 1-D gels provided valuable information about the *X. fastidiosa* outer membrane, it was not possible to completely separate all of the proteins using this method. Recently, we have begun to analyze our outer membrane fractions using 2-D gel electrophoresis. This technique separates proteins based on their isoelectric points (p*I*) and their apparent molecular weights. In our first series of experiments, we identified over 40 well-separated spots. We are currently in the process of analyzing our 2-D gels using Phoretix proteome analysis software. This software will allow us to make a tentative assignment of molecular weights and isoelectric points to the predominant proteins. The 2-D gels will also allow us to determine the relative abundance of each of the outer membrane proteins. Finally, these gels will provide us with an outer membrane protein map for *X. fastidiosa* Temecula 1 that can be compared to the published whole-cell protein map for *X. fastidiosa* CVC (Smolka et al. 2003).

Assigning the outer membrane proteins to specific genes on the X .fastidiosa chromosome

Our analysis of the proteins present in the outer membrane fraction has provided us with important information concerning the molecular weights and isoelectric points of these proteins. The next step has been to assign these proteins to specific genes on the *X. fastidiosa* chromosome. This step has been greatly facilitated by the recent sequencing and annotation of the *X. fastidiosa* Temecula 1 genome (Van Sluys et al., 2003) and by the availability of the published whole-cell protein map for *X. fastidiosa* CVC (Smolka et al., 2003). The use of these resources has allowed us to tentatively assign many of our outer membrane proteins to specific genes on the *X. fastidiosa* Temecula 1 chromosome.

To confirm the identification of some of the ambiguous spots, we are using mass spectrometry. Using this technique, we have successfully assigned one of our outer membrane proteins to a specific gene on the *X. fastidiosa* chromosome. For this analysis, we ran the outer membrane fraction on a preparative SDS-polyacrylamide gel and excised one of the distinct bands from the gel. The protein in this band was then subjected to trypsin digestion and the resulting fragments were analyzed by MALDI-TOF-MS at the UC Davis Molecular Structure Facility. Based on this analysis, we were able to assign this protein to a specific gene on the *X. fastidiosa* chromosome. Given our success with this protein, it should be possible for us to begin assigning other proteins to specific genes.

Determining how the protein composition of the X. fastidiosa outer membrane is influenced by different environmental and physiological signals

We have also begun to look at the outer membrane profiles of *X. fastidiosa* grown under different environmental and physiological conditions in the laboratory. To date, we have used 1-D gels to compare the outer membrane profiles when the strains were grown: (1) in rich versus defined medium, (2) in liquid medium versus on plates, (3) to exponential phase versus stationary phase. Our results indicate that the abundance of some outer membrane proteins does not change. This is particularly true of proteins that are predicted to have a structural role in maintaining cell surface integrity. However, the abundance of other proteins is more sensitive to changes in the growth conditions. It is highly likely that some of these changes will have profound effect on cell permeability and on the sensitivity of *X. fastidiosa* to detergents, antibiotics, and bacteriophages.

CONCLUSIONS

Proteins on the bacterial cell surface play an important role in the ability of pathogenic bacteria to induce the disease state. During the past year, we have focused on developing methods for studying the protein composition of the *X.fastidiosa* outer membrane and have begun to examine how different physiological and environmental signals affect the relative abundance of these proteins. We are now in the position to examine how changes in the abundance of specific proteins correlate to changes in virulence. This information should provide insights into the role of the outer membrane proteins in *X. fastidiosa* virulence and identify potential new targets that may help in the development of effective strategies for controlling the spread of Pierce's disease.

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ANALYSIS OF *XYLELLA FASTIDIOSA* TRANSPOSON MUTANTS AND DEVELOPMENT OF AN *EN PLANTA XYLELLA FASTIDIOSA* PLASMID VECTOR

Project Leader:
Bruce Kirkpatrick
Department of Plant Pathology
University of California, Davis
Davis, CA 95616

Cooperator:
Magalie Guilhabert
Department of Plant Pathology
University of California, Davis
Davis, CA 95616

ABSTRACT
We have developed an autonomously replicating *Xylella fastidiosa* (*Xf)/E. coli* plasmid that efficiently transforms *Xf*; unfortunately this plasmid was not stably maintained in *Xf* cells *en planta* or without antibiotic selection *in vitro*. Another plasmid, containing an *Xf* native plasmid, a Kan^R cassette and cloned in pUC18 was also constructed and shown to be unstable without antibiotic selection. 1000 random Tn5 mutants were again screened for virulence in grapevines growing in the greenhouse. An expectedly high percentage (35%) did not develop typical pierce's disease (PD) symptoms; the insertion sites of the Tn5 in these apparently avirulent mutants are being sequenced. Approximately 3% of the random mutants were hypervirulent as compared to the wild type parental strain. Insertion sites of Tn5 in these mutants showed 2 were in putative LPS genes and 1 was in a hemagglutinin-like gene. The phenotype of these putative hypervirulent mutants is being confirmed in a second round of grapevine inoculations.

INTRODUCTION
During the past 4 years one of the objectives of our research on Pierce's disease (PD) has involved the development of transformation and transposon mutagenesis systems for the bacterium that causes PD, *Xylella fastidiosa* (*Xf*). We developed a random transposon based mutagenesis system for *Xf* in 2001 (Guilhabert et. al., 2001). Recently, we developed an *E.coli/Xf* plasmid shuttle vector based on the plasmid RSF1010 that replicates autonomously in *Xf* (Guilhabert and Kirkpatrick, 2003), however this plasmid is only stably maintained in *Xf* cells that are kept under selection using the antibiotic, kanamycin. Therefore, this shuttle vector will be useful for *in vitro* studies of *Xf* gene function; however it cannot be used to study the function of *Xf* genes in the plant host. We continue to evaluate other plasmids that can be stably maintained in *Xf* cells inoculated into plant hosts.

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

OBJECTIVES
1. Develop a *Xylella fastidiosa* (*Xf*)/*E. coli* plasmid shuttle vector that is stable *en planta.*
2. Screen a library of *Xf* transposon mutants for *Xf* mutants with altered pathogenicity, movement or attachment properties**.**

RESULTS AND CONCLUSION
<u>Objective 1</u>
We are cloning the RSF1010 origin of replication into pUC18, creating a plasmid harboring a polylinker, a LacZ selection and expressing the pUC18 antibiotic cassette, carbenicillin that was showed to be expressed in an *Xf* background (Qin and Hartung, 2001). Such vector will allow selection for plasmid maintenance using an antibiotic that is different from the kanamycin resistance gene carried on the transposome that we use to create Tn5 *Xf* mutants and provide the necessary tool to complement and prove the function of a Tn-tagged *Xf* gene.

Another approach to create an *Xf* plasmid shuttle vector is to modify native *Xf* plasmids with a selectable marker. Such a strategy was successful in developing a stable plasmid vector for citrus (CVC) infecting strains of *Xf* (Qin and Hartung, 2001) however this plasmid (pER10) did not replicate in grape strains of *Xf* (Guilhabert and Kirkpatrick, 2003). A 1.3 kb size plasmid from a PD *Xf* strain was cloned in pUC18 and sequenced. Nucleotide and amino acid sequence analysis revealed conserved sequences that are typical of initiator (Rep) proteins involved in rolling-circle type DNA replication as well as a

putative origin of replication (Guilhabert and Kirkpatrick 2000). The kanamycin resistance cassette carried by the transposome was cloned into the multiple cloning site of the pUC18, creating plasmid pXF001. Plasmid DNA was electroporated in *Xf* in the presence of a TypeOne™ Inhibitor (Epicentre Technologies, Madison, WI) and *Xf* transformants were obtained using the Fetzer, Traver and Temecula strains. Plasmid DNAs were extracted from Kan^R *Xf* colonies and no plasmid DNAs were visualized on a gel after electrophoresis on a gel. The plasmid DNA extracted from Kan^R *Xf* cells was used to transform *E. coli* cells. Plasmid DNAs of same size as the original pXF001 were visualized on a gel (Figure1). These results indicate the ability of the pXF001 to replicate autonomously in *Xf*. Maintenance of pXF001 in *Xf* was measured in absence of antibiotic selection. As showed in Table 1, pXF001 is not stably maintained in Fetzer, Temecula or Traver strains without antibiotic selection. However, stability in *Xf* strains harboring other plasmids (Traver) seems higher than in the strain harboring no plasmid (Fetzer). We are currently evaluating the UCLA strain harboring 4 plasmids (Hendson et al., 2001) for transformation with pXF001 and maintenance without antibiotic selection.

Objective 2

Using the transposome technology previously described, we obtained 2000+ *Xf* Tn5 mutants, which should represent fairly random mutagenesis events throughout the *Xf* genome. During the spring and summer 2002, we inoculated 1,000 chardonnay plants growing in pots in this greenhouse with the *Xf* Tn5 mutants. Four month after inoculation, we noticed an unexpectedly high percentage (35%) of inoculated vines that did not develop typical PD symptoms. One might have expected no more than 5% or so of the mutants to be non pathogenic. We will sequence the *Xf* DNA that flanks the Tn5 element in order to determine the specific location of the Tn5 insertion in each mutant that we plan to further characterize. Insertions in open reading frames (ORFs) that code for proteins that have possible roles in plant colonization or ORFs with no known function will be further characterized, while Tn5 insertions in known "house-keeping" genes will not be immediately screened further.

Three percent of the inoculated vines showed hypervirulence when compared to the vines inoculated with wild type *Xf* cells. The insertion sites of 9 of 13 mutants were sequenced and the result is summarized in Table 2. The hypervirulent mutants will be further characterized for bacterial populations in grapevines and *in vitro* attachment to glass, cellulose and chitin substrates.

Table 1. Maintenance of pXF001 plasmid in *Xf* strains under non-selective conditions of growth[a]

Xf strains	*Xylella fastidiosa*	
Number of passages	1	3
Fetzer	1	0
Traver	66	ND[b]

[a]Expressed as the percentage of Kan resistant clones after 1 or 3 generations
[b]Not done

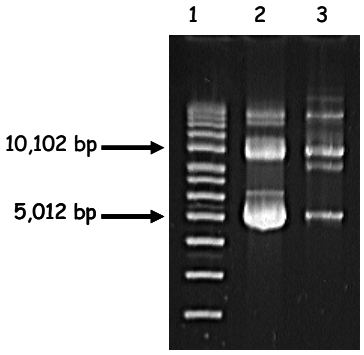

Figure 1. Gel electrophoresis of pXf001 DNA isolated from *E. coli.* and *Xf.*

Table 2. Sequence analysis & putative function of *Xf* DNA flanking Tn5 transposon insertions in hypervirulent *Xf* mutants.

Tn5 Clone	ORF designation[a]	Map location of *Xf* PD strain[b]	Putative gene function[c]
02.18. B3	1771.2	267360-267914	O-antigen lipopolysaccharides synthesis
02.18. F7	13981.2	1788832-1787882	O-antigen lipopolysaccharides synthesis
04.03. C4	19301.2	2512329-2501959	hemagglutinin-like secreted protein
03.06A C8	6371.2	852374-854482	dipeptidyl aminopeptidase
02.30. G4	11011.2	1394890-1397307	ferric enterobacyin receptor
02.30 C9	22291.2	1087319-1085869	hypothetical protein
03.06A B10	23541.2	1446631-1446398	hypothetical protein
03.06A B4	-[d]	-[d]	hypothetical protein in CVC
02.08 E10	-[d]	-[d]	hypothetical protein in CVC
02.15 B1	ND	ND	ND
03.06A E3	ND	ND	ND
03.06A G2	ND	ND	ND
02.30 B7	ND	ND	ND

[a] Identification number of open reading frame (ORF) in PD strain of *Xf*
[b] Numbers indicate the position of the Tn5 transposon in the genomic sequence of PD strain of *X*
[c] Putative function of ORF based on homology with other gene sequences
[d] No corresponding ORF in the PD strain. However, ORFs were found in the CVC strain

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

Project Leader:
Steven E. Lindow
Department of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Cooperators:
Karyn Lynn Newman
Department of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Alexander Purcell
Rodrigo Almeida
Department of Environmental Science Policy and Management
University of California
Berkeley, CA 94720

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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 probably shares a similar means of coordinating gene expression in a community (2, 7). Using the *Xcc* paradigm as a guide, we have investigated 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 *Xf* produces a cell-cell signal and that the *rpfF* gene is necessary and sufficient for signal synthesis. We compared *rpfF* mutants to the wild type and found they 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. We are in the process of investigating the mechanism of hypervirulence. To further elucidate the behavior of *Xf in planta*, we created a green fluorescent strain of *Xf* and used confocal laser scanning microscopy to observe this strain of *Xf* within the xylem of plants. We found that vessel plugging is the colonization feature most tightly correlated with disease symptom expression, providing strong evidence that vessel plugging causes disease. We screened several collections of bacterial strains isolated from plants and identified bacterial strains that can interfere with *Xf* signaling. We are in the process of testing how these strains interact with *Xf* in the xylem, identifying to which species they belong and isolating the genes responsible for signal interference activity.

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, sending out "scouts" to colonize new areas within the host. 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 investigate cell-cell communication in *Xf* to determine its role 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). 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). Thus *Xf* likely employs a cell-cell signaling apparatus similar to that of *Xcc*. Based on our knowledge of density-dependent gene regulation in other species, we predict the targets of Rpf regulation would be genes necessary for colonizing the xylem and spreading from vessel to vessel. For example, expression of extracellular polysaccharides, cellulases, proteases and pectinases might be induced by the signal. 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.

It is conceivable that cell-cell signal interference may be used by other organisms to interfere with density-dependent behaviors, such as pathogenicity or spreading through the habitat. Several recent studies indicate that other organisms can disrupt or manipulate the cell-cell signaling system of bacteria (4, 5). Examination of *Xf* population size in plants where *Xf* lives as an endophyte versus those in which *Xf* causes the xylem blockage symptoms of Pierce's disease demonstrates a positive relationship between population size and symptom development (3). We hypothesize that an interaction between *Xf*

and other organisms, such as another endophyte or the host plant itself, may modulate density-dependent behaviors in *Xf* by interfering with cell-cell signaling.

OBJECTIVES

1. Characterize cell-cell signaling factors in *Xf.*
2. Determine role of signaling factors on virulence and transmissibility of *Xf.*
3. Identify degraders of signaling factors of *Xf.*
4. Identify inhibitory analogs of signaling factors of *Xf.*

RESULTS

Objective 1. We have constructed "signal-sensing" strains of *Xcc* to determine whether *Xf* uses the same butyrolactone signal as *Xcc.* These strains carry a green fluorescent protein (gfp) gene under the control of a promoter that is up-regulated in response to the signal. We have successfully detected a signal from *Xf* using this system (Figure 1a,b), however the response is much weaker than that of *Xcc.* We conclude that *Xf* may make high concentrations of the signal only under specific conditions, such as *in planta.* A second possibility is that the *Xf* signal differs slightly from the *Xcc* signal and cannot fully activate the *Xcc* signal sensor except at high concentrations. To further investigate the ability of *Xf* to make DSF, we cloned the *Xf rpfF* gene, which is predicted to function as the signal synthase, into an *Xcc rpfF* mutant and determined that it could restore DSF production to the *Xcc* strain (Figure 1h). Interestingly, the *Xf* gene could not achieve the same level of rescue as the *Xcc* gene (Figure 1g), further suggesting that the *Xf* signal may have a slightly different structure that the *Xcc* signal.

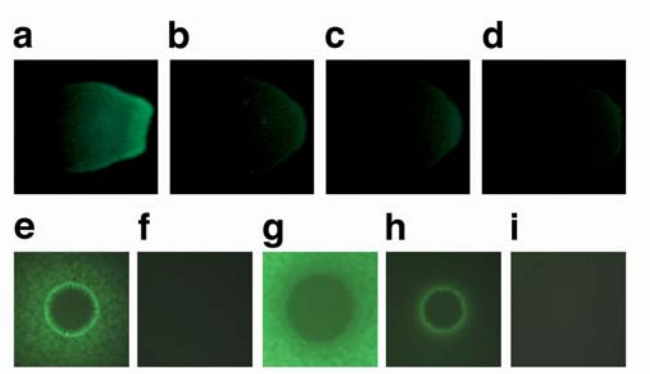

Figure 1. Detection of DSF from *X. fastidiosa* and *Xanthomonas* strains. (a-d) DSF-reporter strain grown to the left of concentrated *X. fastidiosa* culture extracts of the wild-type strain Temecula (a), sterile medium (b), or rpfF mutant strains KLN61 (c) and KLN62 (d). (e-i) DSF-reporter strain sprayed over colonies of *Xanthomonas* wild type (e), *Xanthomonas* rpfF mutant (f), *Xanthomonas* rpfF rescue strain (g), *X. fastidiosa* rpfF rescue strain (h), empty vector control strain (i). Green fluorescence indicates detection of DSF that has diffused from the culture extract or colony.

Objective 2. We have constructed strains of *Xf* Temecula in which the *rpfB* and *rpfF* genes, which are each required for production of the signal in *Xcc*, are knocked out. These mutants were constructed using exchange of the wild-type allele for a deleted copy carrying an antibiotic resistance gene on a suicide plasmid. We tested *Xf rpfB* and *rpfF* mutants for DSF production. Although *rpfB* mutants are still able to make DSF (data not shown), *rpfF* mutants can no longer make the signal (Figure 1b,c). *rpfB* and *rpfF* mutant strains were tested for their ability to infect and move within host plants and to cause Pierce's disease symptoms. Neither of these genes is strictly required for virulence as mutant strains cause symptoms similarly to the wild type. However, 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 differences if they existed. We observed colonies *in planta* via scanning electron microscopy and again found no obvious differences. We hypothesize that *rpfF* mutants may be causing increased vessel blockage in the grapevine, leading to increased symptom expression. We are in the process of creating a green fluorescent *rpfF* mutant to investigate the pattern of colonization by the mutant and compare it to that of the wild type.

We have tested transmissibility of the mutant strains by an insect vector. While the *rpfB* mutant was transmitted with equal frequency as the wild type by blue-green sharpshooter leafhoppers, the *rpfF* mutant was virtually untransmissible. This defect in transmissibility by the signaling-deficient mutant was unexpected and 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 of the mutant whereas the wild type is never cleared from leafhoppers without molting (6). Scanning electron micrographs of leafhopper foreguts revealed that the *rpfF* mutants are unable to form the characteristic polar biofilm in the precibarium (Figure 2).

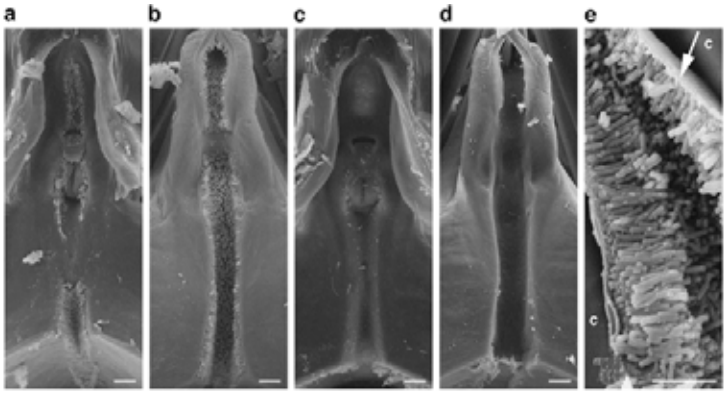

Figure 2. Formation of polar biofilm in insect foreguts. Scanning electron micrographs of the precibarium epipharynx (a,c) and hypopharynx (b,d) of blue-green sharpshooter leafhoppers fed on grapevines infected with the wild-type strain Temecula (a,b,e) or the rpfF mutant KLN61 (c,d). Xylem sap enters the precibarium from the top and runs through the canal, which is coated with a biofilm by wild-type cells (a,b) but not rpfF mutant cells (c,d). (e) High magnification of polar biofilm that has slightly detached from cuticle (c) during fixation revealing a mat-like structure at the attachment site (arrow). Bar = 10 μ (a-d) and 5 μ (e).

To better direct our analyses, we have constructed a strain of *Xf* that constitutively expresses Gfp in order to bring the *in planta* growth habit of *Xf* during symptom formation into sharper focus. By observing differences in colonization between symptomatic and asymptomatic samples we have developed a clearer image of the mechanism of symptom development and the best strategies for preventing it. We found that symptomatic leaves had a forty-fold higher frequency of plugged vessels that asymptomatic leaves and that vessel plugging was the colonization feature most highly correlated with symptom expression (Figure 3a, b, d). By contrast, the frequency of colonized vessels was only five-fold higher in symptomatic leaves than asymptomatic leaves. (Figure 3c) This observation suggests that it is unlikely that bacterial toxins or plant-initiated vessel failure lead to disease symptoms. We also found evidence for *Xf* movement into adjacent xylem vessels via the bordered pits (Figure 3e), supporting the current hypothesis that this bacterium uses extracellular enzymes to digest through the pit membranes and gain access to new habitat.

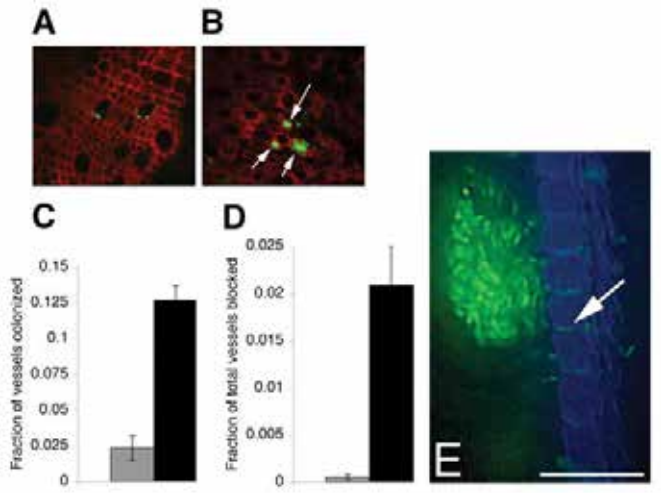

Figure 3. Analysis of xylem colonization using a gfp-marked strain. Confocal laser scanning micrograph of representative xylem bundles from asymptomatic (a) and symptomatic (b) leaf petioles. Arrows mark plugged vessels. Histograms comparing the frequency of vessels colonized (c) or plugged (d) in a ~0.9 micron section of asymptomatic leaves (grey bars) versus symptomatic leaves (black bars). Deconvolution micrograph of *Xf* cells gaining access to a new vessel (right side) from an adjacent vessel (left side) through the bordered pits (e.g., arrow).

Objectives 3 and 4. We have collected 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. We have recovered bacteria from these samples to generate a comprehensive collection of bacterial strains that grew in contact with the signal molecule. These strains have been tested for the ability to interfere with cell-cell signaling in *Xf* in an assay using the signal-sensing strains from Objective 1. We have isolated several strains that inhibit or activate cell-cell signaling. We have introduced these strains, along with *Xf*, into greenhouse-grown grapevines to monitor their effect on Pierce's disease development. We are sequencing 16S rRNA gene sequences from these strains to determine their species identity. Candidates from the interfering strains are being chosen for mutational analysis of the interfering activity. 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 timing and severity of disease is currently being monitored.

CONCLUSIONS

We have investigated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease. In this vein we have found that *Xf* indeed produces a cell-cell signal. We have shown that the *rpfF* gene is necessary and sufficient for synthesis of *Xf*'s cell-cell signal. We found that *Xf* strains that cannot signal are also not transmissible by an efficient insect vector. We show that this lack of transmissibility stems from the *rpfF* mutant being unable to form a biofilm in the insect foregut. 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. While we are still in the process of investigating the mechanism of hypervirulence, this finding suggests that cell-cell signaling is used by *Xf* to control virulence to the plant. If such a hypothesis is correct, it may 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. To that end, we characterized the behavior of *Xf in planta* using confocal laser scanning microscopy to observe a green fluorescent strain of *Xf* within the xylem of grapevines. Our analysis showed tight correlation between vessel plugging and symptom development, providing strong evidence that vessel plugging causes disease. This finding will be helpful in designing strategies to reduce disease by indicating, for example, that treatments that reduce vessel plugging may diminish disease symptoms. We have identified bacterial strains that can interfere with *Xf* signaling. These strains may be useful as protective agents for grapevines exposed to *Xf*. Alternatively, when we isolate the gene conferring the interfering activity, we may be able to directly introduce the protective trait into the plant itself.

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

Project Leader:
Steven E. Lindow
University of California
Department of Plant and Microbial Biology
Berkeley, CA 94720-3112

Cooperator:
Alexander H. Purcell
University of California
Division of Insect Biology
Berkeley, CA 94720-3112

Reporting Period: The results reported here are from research conducted from December 15, 2002 to October 15, 2003.

ABSTRACT

Xylella fastidiosa causes Pierce's disease, a serious disease of grape, citrus variegated chlorosis, almond and oleander leaf scorches, and many other similar diseases. Although the complete genome sequences of several strains of this organism are now available, the function of most genes in this organism, especially those conferring virulence, is lacking. We are elucidating the role of fimbriae and adhesins in the pathogenicity of *X. fastidiosa* to grape, in the attachment to grape or to insect mouthparts, and in the transmissibility of the bacteria to grape via insects. We are also investigating the role of the non-fimbrial adhesins (HecA, B, C, and XadA) in the attachment process because we believe that these genes are important in the early steps of adhesion in bacterial cell-host cell's surface attachment. We were successful in producing XadA- mutants of *X. fastidiosa* strains 'Temecula' and 'STL' and conducted various adhesion assays comparing wild-type to XadA- mutant cells' phenotypes. Polymerase chain reaction and southern blot analyses of the mutants indicated that a double crossover event had occurred exclusively within the *xadA* gene, replacing the chromosomal gene with the disrupted gene and abolishing production of the corresponding protein, XadA. Scanning electron microscopy revealed that attachment to glass was inhibited for the XadA- mutants of *X. fastidiosa* when compared with the parental strain. XadA- mutants of *X. fastidiosa* remained pathogenic to grapevines, but further characterization of virulence and insect transmissibility are underway.

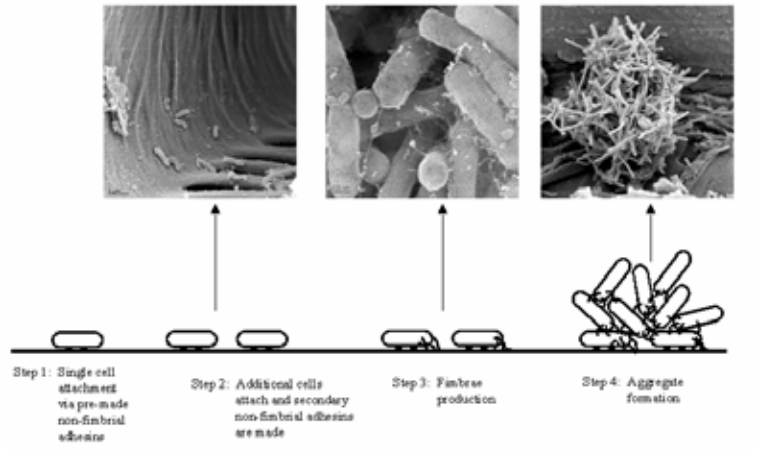
INTRODUCTION

Adhesion is a well-known strategy for phytobacteria to begin colonizing their plant hosts and a precursor step to invasion (Romantschuk et al. 1994). Electron micrograph studies described *X. fastidiosa* attached to grape xylem vessels or to the cuticle lining the foregut in insect vectors (Purcell et al. 1979). Within the grape xylem vessels, *X. fastidiosa* also appeared to be embedded in a filamentous matrix (H. Feil *unpublished*). The fibril-like structures were said to be analogous to fimbriae or pili in other bacteria (Hopkins 1977). *Xylella fastidiosa* possesses many genes involved in attachment or adhesion. Simpson et al. (2000) identified 26 genes encoding proteins involved in the biogenesis and function of Type 4 fimbriae filaments (*pilA, B, C*…). We have focused on the fimbrial operon, which is composed of 6 genes (*fimA, ecdD, fimC, D, E,* and *F*). It was shown that *X. fastidiosa* contains only one chaperone/usher-dependent fimbrial operon compared with enteric bacteria that contain 10 to 15 such operons (Bhattacharyya et al. 2002). Even though the fimbrial mutants remained virulent to grape, we observed phenotypic differences in vitro between mutants and wild-type cells. Scanning electron microscopy (SEM) revealed that fimbriae size and number and cell aggregation were reduced for the $FimA^-$ or $FimF^-$ mutants of *X. fastidiosa* when compared to the parental strain suggesting that fimbriae probably play an essential role in self-aggregation of *X. fastidiosa* cells. Several questions remained to be answered:

1. How do the fimbrial mutants of *X. fastidiosa* behave in grape?
2. Are the fimbrial mutant cells still transmissible to grape via the insect vector?
3. If fimbriae are key components of the self-aggregation of *X. fastidiosa* cells, what genes contribute to the initial attachment of *X. fastidiosa* to grape vessels?

One reason that could explain why the fimbrial mutants remained virulent is that since the mutant cells don't aggregate so well they are freer to move within the xylem vessels of grape and colonize other vessels more rapidly than the wild-type cells; such cells might prove to be hypervirulent. We hypothesized that the lack of fimbriae in the mutants may reduce the adhesion capacity of the cells to plant tissue and/or to insect mouthpart thereby diminishing the transmissibility of the bacteria to plants via the insect. We propose to further investigate the attachment of the mutants to grape and to insect by conducting inoculation and acquisition/transmission experiments with the BGSS, the mutant and wild-type *X. fastidiosa.*

Even though the fimbrial mutant cells had less fimbriae than the wild type cells as seen in scanning electron micrographs, the cells seemed to still be able to attach to surface by another mechanism (Feil et al. 2003). This suggests that fimbriae are more important in cell-to-cell adhesion than in cell to surface adhesion. We hypothesized that the afimbrial adhesins are responsible for early attachment of *X. fastidiosa* to grape xylem vessels. The cartoon below depicts our proposed diagram of the steps in the adhesion of *X. fastidiosa* to xylem vessels:



In step 1, upon first contact to the host cell's surface, the *X. fastidiosa* cells stick to the surface via non-fimbrial adhesins. These adhesins allow the bacteria to stick to the surface by binding the xylem vessel's surface. Step 2 represents the secondary contact via more adhesins. These adhesins form a matrix around the cells allowing for more binding between the bacterial surface and the host cell's surface. In step 3, fimbriae are formed between bacterial cells to enhance cell-to-cell attachment. Finally, step 4 depicts bacterial cells aggregated to one another via fimbriae and fibrils to form a mass of cells within the xylem vessel. Fimbriae appear to be more important for cell-to-cell aggregation and therefore enter late in the adhesion process.

Because we think the early steps in adhesion are crucial to successful colonization of grape xylem vessels by *Xylella fastidiosa*, we are directing our research towards the investigation of the role of other adhesin genes, which have been shown to reduce virulence in other bacterial system. Different strains of *X. fastidiosa* were shown to have different afimbrial adhesins suggesting a role for these genes in host specificity (Bhattacharyya et al. 2002). Other host specific adhesin may include the *hia* gene, homolog of the major adhesin of *Haemophilus influenzae*. Other adhesins present in the *X. fastidiosa* genome are HecA, B, and C. *hecA*, a hemagglutinin homolog in *Erwinia chrysanthemi*, the causal agent of soft-rot disease of chrysanthemum, played a role in the attachment, aggregation, cell killing, and virulence of this organism to tobacco seedlings (Rojas et al. 2002). Epifluorescence and confocal laser-scanning microscopy revealed that this mutant was reduced in its ability to attach, to form aggregates and to kill epidermal plant cells. The genome of *X. fastidiosa* has three hemaglutinins (*hecA, B,* and *C*) genes with similarity to *hecA* and to a *Neisseria meningitidis* secreted protein (Tettelin et al. 2000). These genes are large (over 10 Kb each) and distributed within the genome at least 400 Kb apart. They share high homology especially in the upstream sequence of the gene. The downstream third of their sequence has less identity to the other two sequences. This suggests that their respective specificity resides in the expression of the 3' end of their sequences. We are targeting the 3' end of each of these genes in producing mutants to study their role in attachment and pathogenicity.

Another adhesin, XadA, an outer membrane protein found in *Xanthomonas oryzae* pv *oryzae* and in *Xanthomonas campestris* pv. *vesicatoria*, has been implicated in virulence for these two organisms. XdaA-deficient mutants of the rice pathogen, *Xanthomonas oryzae* pv *oryzae* are less virulent (i.e. cause smaller lesion on rice than that of the wild-type cells) and altered colony morphology (Suvendra et al. 2002). Recently *xadA* has been found in *Xanthomonas campestris* pv. *campestris* and in *Xanthomonas axonopodisis* pv. *citri* (Da Silva et al. 2002). The genome of *X. fastidiosa* also has a *xadA*-homolog suggesting that this conserved gene may have a general importance in pathogenicity for the plant pathogens. We disrupted this gene using the same site-directed method used for the fimbrial mutant and are determining the role of this gene in attachment and pathogenicity.

We have investigated the role of several fimbrial genes in pathogenicity (Feil et al. 2003) and propose to further elucidate the role of fimbriae and adhesins in the pathogenicity of *X. fastidiosa* to grape, in the attachment to grape or to insect mouthparts, and in the transmissibility of the bacteria to grape via insects. However since fimbriae are important in cell-to-cell attachment, which is probably relatively late in the adhesion process, we also investigated the role of the non-fimbrial adhesins (HecA, B, C, and XadA) in the attachment process. We believe that these genes are important in the early steps of adhesion in bacterial cell-host cell's surface attachment. We also chose these particular non-fimbrial adhesins because they were found to play a significant role in virulence for other plant bacterial pathogens. A better understanding of this important aspect of the biology of Pierce's disease should allow potential new ways to control this serious plant pathogen to be developed and will elucidate the processes that occur during colonization of both grape as well as sharpshooter vectors.

OBJECTIVES

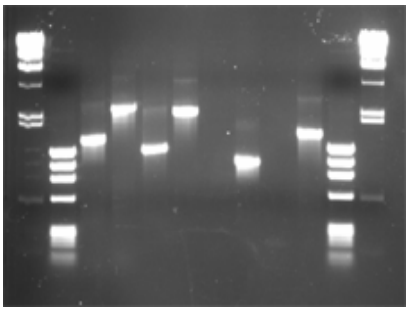
1. To further characterize the behavior of the fimbrial mutants of *Xylella fastidiosa* in grape.
2. To determine if these mutants can attach to the insect vector and be transmitted to grape.
3. To determine the role of adhesins other than those found in the fimbrial operon, in particular of the hemagglutinins and the adhesin XadA in the attachment and virulence of *X. fastidiosa* in grape.

RESULTS AND CONCLUSIONS

Objective 1: We have infected grape tissue with mutants of FimA, FimF, and XadA or wild-type cells of the 'Temecula' grape strain. Sampling for presence of bacteria in sections at several point in time following inoculation are underway. Disease symptoms are being followed over time to ascertain the hyper or decreased virulence of these mutants. Microscopic observation of these tissue sections are being done to visualize *X. fastidiosa* in plants and to compare the extent of colonization between mutant and wild *X. fastidiosa* strains.

Objective 2: With a similar approach than for objective 1, we are determining the role of the *fimA*, *fimF*, and xadA genes in attachment to insects (blue-green sharpshooter and glassy-winged sharpshooter). Plants infected with the FimA, FimF, and XadA mutants are being used as source plants for insect transmission. The efficiency of transmission of these mutants will be compared with that of the wild-type strain in greenhouse feeding studies.

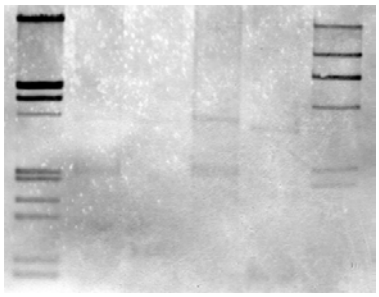
Objective 3: We constructed vectors to disrupt the hemagglutinin genes (*hecA*, *B*, and *C*) and the adhesin gene (*xadA*) of *X. fastidiosa*. We were successful in producing XadA- mutants of the 'Temecula' and 'STL' strains of *X. fastidiosa*. We are in the process of making hemagglutinin mutants. We further characterized the Xada- mutants by PCR (Figure1), southern blot (Figure 2), and sequencing.



ladders 1 2 3 4 5 6 7 8 ladders

Figure 1. PCR of wild-type vs. XadA- DNA

1. Wild-type (WT) DNA/primers outside *xadA*
2. XadA- mutant DNA/primers outside *xadA*
3. Wild-type (WT) DNA/ *xadA* primers
4. XadA- mutant DNA/ *xadA* primers
5. Wild-type (WT) DNA/ *kan* primers
6. XadA- mutant DNA/ *kan* primers
7. Wild-type (WT) DNA/*xadA* forward-*kan* reverse primers
8. XadA- mutant DNA/ *xadA* forward-*kan* reverse primers



ladder XadA WT XadA WT ladder

Figure 2. Southern blot of wild-type vs. XadA DNA. The probe was the xadA gene. The larger size of the XadA digest indicates insertion of the *kan* gene within *xadA*

We tested how different the adhesion of the Xada- mutants was as compared to the adhesion of wild-type cells on various substrates (balsa wood, glass, silicon chip). We also tested if adhesion of the mutant or wild-type cells was affected by various media (PW broth, Fructose broth, xylem sap). We observed that a thick ring of cells formed around the glass flasks for wild-type cells whereas no ring was detected using the Xada- cells (Figure 3).

Figure 3. Wild-type (WT) vs. XadA- mutant cells growing in flasks in fructose broth for 10 days. A thick ring can be seen around the flask with the wild-type cells whereas no ring is observed for the XadA- cells.

This is the first phenotypic difference observed between wild-type and XadA- cells and it suggests that adhesion to host surfaces may be impaired in the XadA- cells.

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FUNDING AGENCIES

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DETERMINATION OF GENES CONFERRING HOST SPECIFICITY IN GRAPE STRAINS OF *XYLELLA* FASTIDIOSA USING WHOLE-GENOMIC DNA MICROARRAYS

Project Leaders:

Steven E. Lindow
Department of Plant and Microbial Biology
111 Koshland Hall
University of California
Berkeley, CA 94720

Paul Richardson
Head-Genomic Technologies
DOE, Joint Genome Institute
2800 Mitchell Drive, B400
Walnut Creek, CA 94598

Reporting Period: The results reported here are from research conducted from July 1, 2003 to October 15, 2003.

ABSTRACT

The genomic sequence of several *Xylella fastidiosa* (*Xf*) strains, including one pathogenic to grape (strain 'Temecula1') have been determined. While these strains share most genes, the difference in host range that they exhibit is presumably due at least in part to unique traits that each pathovar possesses. Some of these strains originating from host plants other than grape do not sustain viable populations or are not virulent in grape. Using microarray technology and *Xf* 'Temecula1' as the reference strain, those genes present in the grape strain but not in strains that are unable to grow in grape can be determined through subtractive analysis. A 70-mer-oligonucleotide microarray representing 2543 of the 2574 predicted ORFs of *Xf* 'Temecula1' was designed. *Xf* 'Temecula1' genes identified as putative host-specificity genes of will be subjected to further analysis. Identifying host-specific genes of the grape strain will allow us to target those genes in gene-knockout studies and determine their contribution to virulence in grape.

INTRODUCTION

Some strains of *Xylella fastidiosa* originating from host plants other than grape do not sustain viable populations or are not virulent in grape. In particular, many of the almond strains of *X. fastidiosa* do not infect grape (Almeida and Purcell, *unpublished*). Cross inoculation greenhouse studies showed that the OLS and PD strains of *X. fastidiosa* were not pathogenic to citrus and that the ALS strain was not pathogenic to oleander (Feil et al. *unpublished*). Other cross-inoculation studies provide evidence for host specificity variation among strains of *X. fastidiosa*. Recently, the CVC strain of *X. fastidiosa* was found to be pathogenic to coffee and grape (Li et al. 2001) and these authors are now inoculating citrus with the coffee leaf scorch strain to determine if it is pathogenic to citrus.

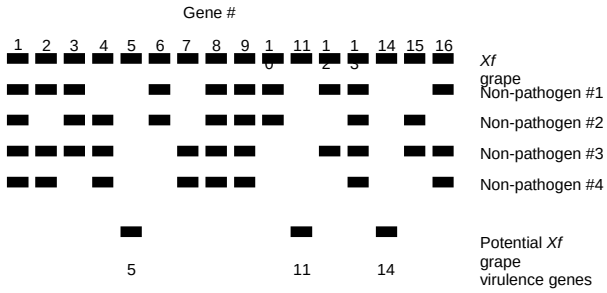
Other studies provide evidence for host specificity among the *X. fastidiosa* strains. Grape strains of *Xylella fastidiosa* were found to cluster together away from oak, plum, mulberry, and periwinkle strains using RFLP data (Chen et al. 1992, Chen et al. 1995). Pooler and Hartung (1995) divided the *X. fastidiosa* into 5 groups (citrus, plum, grape-ragweed, almond, and mulberry) based on RAPD-PCR data. Most almond strains clustered away from the grape strains but a few clustered within the grape strains group whereas oleander, peach, and oak strains were distinct from other strains using RAPD-PCR, CHEF gel electrophoresis, and 16S-23S rRNA sequence analysis (Hendson et al. 2001). In Brazil, CVC strains were found to cluster together away from the coffee and grape strains of *X. fastidiosa* using RAPD (Lacava et al. 2001). At best, the previous studies were able to group the relative strains into groups. The limitations of these earlier studies were that only a small portion of the genome was sampled, and therefore many of the virulence determinants were likely to be missed. The loci they studied may or may not have been related to virulence. In this study we are looking at all of the genes of the genome.

We believe that microarray technology is a good technique to study host specificity between different strains of the same bacteria. The main reason is that the entire genome is represented on a chip to give an immediate global comparison tool. Many studies have shown that microarray technology can differentiate bacterial strains. Microarray analysis of 22 strains of *Salmonella* revealed that approximately 400 genes (out of 4,596) were unique to the reference strain (Porwollick et al. 2002). Another microarray study showed that one serovar of *Salmonella* (serovar Arizona) had a more distant relationship to the subspecies I serovars (Chan et al. 2003). Microarray analysis of 50 strains of *Listeria monocytogenes* relative to a reference array made with sheared DNA from 10 different strains revealed that a number of the polymorphic probes identified in the study are important in the virulence of their respective strain (Call et al. 2003). Strain-specific virulence genes were identified for *Helicobacter pylori* using whole-genome microarray technology (Salama et al. 2000). These authors found that 22% of the *H. pylori* genome was dispensable in other strains and they defined a minimal functional core of 1281 genes but more importantly a differentiating section of the genome referred to as pathogenicity island made of 362 strain-specific genes. Similarly, we will use a *X. fastidiosa* 70-mer-oligonucleotides microarray using the grape strain to do global comparison between the grape strain relative to other strains non-pathogenic to grape.

Comparative analysis of curated pathways and their components present in the finished sequence of the citrus strain of *X. fastidiosa* genome relative to the draft-genomes of the almond and oleander strains of *X. fastidiosa* were described (Bhattacharyya et al. 2002). Based on this analysis, we estimate that ca. 4% of the whole genome of the oleander was unique to that strain. We hypothesized that the grape strain also possesses ca. 4% of unique genes in comparison to other strains that do not infect grape (the CVC strain does infect grape and is thus not helpful in finding host-specificity genes). This means that approximately 100-200 genes are unique to the grape strain. However probably not all 100 to 200 genes will be of

interest and related to virulence, and we can expect that only 20 to 30 will be virulence determinants. To identify these genes, we will use the grape strain microarray as a reference and perform pairwise comparison via hybridization experiments using each *X. fastidiosa* strain that is non-pathogenic to grape.

The cartoon below depicts the procedure:



The three fragments on the bottom of the cartoon represent the unique genes found in the grape strain but not in the other four non-pathogenic strains. The more non-grape pathogens and grape pathogens that can be compared in this process, the more selective such a "filter' will be for those genes that are in common only to grape pathogens. In this process we will eliminate the many housekeeping genes and genes involved in general virulence attributes that all xylem colonists would have, and identify specifically those genes that distinguish grape pathogens from all others. High-density arrays will be generated using 70-mer oligonucleotides that will be designed based on the coding sequence of the >2500 annotated genes from the grape *X. fastidiosa* genome 'Temecula1' (http://integratedgenomics.com). DNA extraction, labeling, hybridization and data analysis will be conducted as described (Salama et al. 2000). Using the subtractive method described above we will identify the unique virulence genes for the *X. fastidiosa* grape strain 'Temecula1' and we will perform site-directed mutagenesis and grape inoculation experiments to investigate their pathogenicity.

OBJECTIVES

1. To identify host-specific virulence determinants of the *X. fastidiosa* grape strain 'Temecula1'.
2. To investigate the role of host specificity genes identified in objective 1 in virulence.

RESULTS

Oligonucleotide and Array Design. 70-mer oligodeoxynucleotides were designed using 'ArrayOligoSelector' software (http://arrayoligosel.sourceforge.net) based on the coding sequence of 2543 of the 2574 predicted ORFs of *Xf* 'Temecula1'. These oligos were generated with a 5' amino linker that will allow for covalent binding to aldehyde or epoxy coated slides. Oligos are in the process of being printed onto glass slides using a GMS 417 Arrayer. The total number of genes represented by gene-specific oligodeoxynucleotides on the arrays will be 2555 including negative and positive controls.

ArrayOligoSelector did not design oligos for the 48 ORFs listed in Table 1. The list includes 19 ORFs which were less than 70 bp in length, and 12 ORFs that were greater than 70 bp but less than 300 bp. The remaining ORFs were from duplicated regions, and the program could not determine a unique sequence for those ORFs. We will manually design 70-mer oligodeoxynucleotides for ORFs greater than 300 bp in length, but only one oligo will be used to represent duplicated ORFs. The remaining oligos that will be included on the array will include 8 negative and 4 positive controls. We are now optimizing the experimental conditions for the printing and hybridization steps of the microarray.

CONCLUSION

We are comparing the genomes of several strains of *X. fastidiosa* that do not sustain viable colonies or are non-pathogenic to grape (i.e several almond, oleander, oak, peach, etc…) to the genome of the grape strain 'Temecula1' by performing hybridization experiments using a microarray representing the whole genome of the grape reference strain 'Temecula1'. In a process of elimination those genes that are in common to all grape strains but missing from other pathovars will be identified as putative host-specificity genes. Their contribution to virulence to grape will be verified by using site-specific gene knockouts of each of the candidate genes in inoculation trials. We are confident that by comparing the genome of the grape strain to many other strains that are non-virulent to grape, we will identify genes unique to the grape strain. The list of putative host-specificity genes will be examined for their contribution to pathogenicity and virulence in subsequent mutagenesis experiments in objective 2. Methodologies for gene knock-outs are working well in our lab, and we expect no problem in being able to disrupt candidate genes as they are identified in objective 1.

Table 1. List of ORFs for which oligonucleotides were not designed by ArrayOligoSelector.

ORF #	Annotation	Length (bp)
RXFZ00021	Hypothetical	192
RXFZ00036	Hypothetical	69
RXFZ00075	Hypothetical membrane spanning protein	840
RXFZ00178	Hypothetical	178
RXFZ00179	Formamidopyrimidine-DNA glycosylase	813
RXFZ00282	Hypothetical	57
RXFZ00404	Hypothetical	60
RXFZ00413	Hypothetical	57
RXFZ00415	Hypothetical	54
RXFZ00595	Hypothetical	282
RXFZ00627	hypothetical	57
RXFZ00643	hypothetical	63
RXFZ00724	hypothetical	60
RXFZ00815	hypothetical	57
RXFZ01099	hypothetical	63
RXFZ01239	hypothetical	222
RXFZ01318	hypothetical	75
RXFZ01429	Tail protein I	567
RXFZ01430	Baseplate assembly protein J	891
RXFZ01438	hypothetical	519
RXFZ01439	hypothetical	324
RXFZ01440	hypothetical	258
RXFZ01476	Phage-related protein	564
RXFZ01480	Phage-related protein	387
RXFZ01532	Phage-related protein	558
RXFZ01537	Phage-related protein	639
RXFZ01538	Phage-related protein	828
RXFZ01539	Hypothetical	315
RXFZ01557	Phage-related DNA helicase	1416
RXFZ01558	Hypothetical	168
RXFZ01590	Hypothetical	201
RXFZ01812	Hypothetical	60
RXFZ01834	Hypothetical	60
RXFZ02159	Hypothetical	57
RXFZ02169	Hypothetical	96
RXFZ02255	Hypothetical	60
RXFZ02281	Hypothetical	195
RXFZ02282	Hypothetical cytosolic protein	258
RXFZ02283	Phage-related protein	1275
RXFZ02285	Antirepressor	1599
RXFZ02286	AhpD protein	2178
RXFZ02287	Phage-related protein	276
RXFZ02289	Phage-related DNA helicase	1416
RXFZ02297	Hypothetical	69
RXFZ02461	Hypothetical	63
RXFZ02612	Hypothetical	60
RXFZ02696	Hypothetical	66
RXFZ02698	Hypothetical	60

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SURROGATE GENETICS FOR *XYLELLA FASTIDIOSA*

Project Leader:
Valley Stewart
Section of Microbiology
Division of Biological Sciences
University of California
Davis, CA 95616

Cooperators:

Michele Igo
Section of Microbiology
Division of Biological Sciences
University of California
Davis, CA 95616

Bruce Kirkpatrick
Department of Plant Pathology
College of Agricultural & Environmental Sciences
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted from December 15, 2002 to October 15, 2003.

ABSTRACT
Our long-term goal is to understand the specificity determinants that govern transcription initiation in *Xylella fastidiosa (X. fastidiosa)*. Our approach is to use *Escherichia coli* as a surrogate host for reconstituting regulated transcriptional control circuits. This allows us to identify and focus on *X. fastidiosa*-specific features. Our initial studies are aimed at understanding the transcriptional regulation of type IV pilus synthesis.

INTRODUCTION
Xylella fastidiosa presents a formidable challenge to the molecular geneticist. Methods for the basic operations of genetic exchange, mutant isolation, and complementation are in early stages of development. The slow generation time and poor plating efficiency are further complications. Surrogate genetics (Maloy & Zahrt, 2000) provides a means to at least partially bypass these challenges. Here, one creates a hybrid organism, transplanting genes of interest from the poorly-studied species (e.g., *X. fastidiosa*) into a well-studied surrogate host (e.g., *E. coli*). Given sufficiently related hosts, one expects the transplanted genes to function in the surrogate essentially as they do in the original. One may then exploit the advantageous properties of the surrogate to perform a large number of experiments, making and discarding hypotheses to define various aspects of gene function. Once gene function in the surrogate has been thoroughly explored, one can perform a focused set of experiments, informed by the results from the surrogate, to examine function in the native host. The use of *E. coli* as a surrogate host for studying gene regulation would open a range of experimental approaches that are currently unavailable in *X. fastidiosa*, and lead to more rapid advances in understanding the control of key pathogenicity determinants. We are analyzing the transcriptional regulation determinants for genes whose products may be involved in pathogenesis (e.g., type IV pili) as well as "housekeeping" genes involved in central metabolism (e.g., glutamine synthetase, involved in amino acid biosynthesis and nitrogen assimilation).

OBJECTIVES
1. Reconstitute regulated Φ(*glnA-lacZ*) expression in *E. coli.*
2. Reconstitute regulated Φ(*pilA-lacZ*) expression in *E. coli.*
3. Begin analyzing housekeeping gene promoter structure in *X. fastidiosa.*

RESULTS
We chose first to study the regulation of *pil* gene expression from *X. fastidiosa* Temecula (Van Sluys et al., 2003). These genes control the formation of type IV pili in a variety of organisms, where they are required for gliding motility, adhesion, transformation and pathogenesis (Winther-Larsen & Koomey, 2002; Shi & Sun, 2002). Expression of *pilA* structural genes requires a specialized RNA polymerase specificity determinant (σ^{54}), which recognizes a strongly conserved -12/-24 nucleotide sequence. One of two *pilA* homologs (XF2542) in *X. fastidiosa* contains a σ^{54}-dependent promoter. We constructed a Φ(*pilA-lacZ*) operon fusion in *E. coli*, and observed that it expressed detectable levels of LacZ enzyme. We also cloned the regulatory *pilSR* genes (XF2546 and XF2545) from *X. fastidiosa*. However, we have not yet observed a *pilR*-dependent increase in LacZ synthesis, indicating that the PilSR regulators may not function well in *E. coli*.

Recent work of others indicates that a σ^{54}-dependent activator from another species does not function well with *E. coli* RNA polymerase (Richard et al., 2003). To approach this question directly, we are currently studying expression of the *glnA* gene encoding glutamine synthetase (XF1842). This is the best-studied σ^{54}-dependent gene in *E. coli*, and the *X. fastidiosa glnA* upstream regulatory region is similar to that of *E. coli*. Furthermore, *X. fastidiosa* encodes the NtrB-NtrC sensor-regulator system for controlling *glnA* gene expression (XF1849 and XF1848). Because *E. coli* also encodes NtrB-NtrC, we are able to evaluate *glnA* expression in response to both the *X. fastidiosa* and the *E. coli* regulatory proteins. We found that the *X. fastidiosa glnA* promoter functions well in *E. coli* when activated by the *E. coli* NtrB-NtrC proteins. This confirms the

identity of this promoter, and establishes its mode of regulation. However, the *X. fastidiosa* NtrB-NtrC proteins did not activate this promoter in *E. coli*. We hypothesize that the NtrC protein must make a species-specific contact with RNA polymerase, perhaps via the σ^{54}-subunit. Experiments to evaluate this idea are under way.

CONCLUSIONS

We know little about the specificity determinants for regulated transcription initiation in *X. fastidiosa*. RNA polymerase interactions with transcriptional activators such as the NtrC and PilR proteins may involve species-specific protein-protein interactions. Furthermore, the structure of general "housekeeping gene" promoters is not known. Our studies to define these specificity determinants will allow more insightful predictions of gene expression and function.

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Section 3A: Xylella fastidiosa Epidemiology and Biological Control

ENVIRONMENTAL FATE OF A GENETICALLY MARKED ENDOPHYTE IN GRAPEVINES AND THE GLASSY-WINGED SHARPSHOOTER

Project Leader:
Blake Bextine
Department of Entomology
University of California, Riverside, 92521

Consultant:
Frank Richards
Yale University
New Haven, CT 06520

Project Director:
Thomas A. Miller
Department of Entomology
University of California, Riverside, 92521

Collaborators:
Carol Lauzon
Department of Biological Sciences
California State University, Hayward
Hayward, CA 94542

David Lampe
Department of Biological Sciences
Duquesne University
Pittsburgh, PA 19219

Reporting Period: The results reported here are from work conducted from July 1, 2003 to October 31, 2003.

ABSTRACT
A permit was obtained from EPA in late spring to conduct field trials in covered grapevines at three commercial vineyards and one experiment station in California. *Alcaligenes xylosoxidans denitrificans* (*Axd*) bacteria, modified to produce a fluorescent protein, were applied to grapevines by needle inoculation, foliar spray application, and soil drench. The plants were covered with insect-free screening, to exclude arthropods from test plants. Samples were taken throughout the growing season and are currently being processed. Grapevines at the Riverside field site were exposed to glassy-winged sharpshooters to test the affect of insect feeding on the translocation of *Axd* in grapevines. Samples from these plots are being analyzed. The results are too preliminary to report at this meeting.

INTRODUCTION
Paratransgenesis 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. *Alcaligenes xylosoxidans denitrificans* (*Axd*) was selected for further study and genetically altered with a fluorescent marker. We propose to follow the movement of *Axd* in grapevines and in the vector insect, the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*.

Regulatory and industry confidence in this approach require knowing the fate of *Axd* in various locations, various plant types and the spread at different locations and in plants at different times of the year. Our current detection methods employ PCR (polymerase chain reaction) and fluorescence microscopy. RT-PCR provides a quantitative measure of bacteria in the samples, which is missing from existing methods. This is important because it relates to determining optimum dose and timing for application and expression of the anti-*Xylella* compound.

Fluorescent protein gene markers are now commonly used in genetics and are not considered an environmental danger since they are based on natural compounds. The bacterial transformation cassette was inserted with so-called jumping genes (mobile or transposable elements) originally identified in *Drosophila mauritiana* and called mariners. The mariner elements have had their jump mechanism removed (so the inserted gene will not be mobilized) and all antibiotic genes used for selection have been removed (so no antibiotic factors can be moved inadvertently to other bacteria). Our results confirm that the transgenic strains are very stable and grow readily in culture.

Since the marker genes were placed next to an open reading site that is designed to contain the future anti-*Xylella* compound, the bacteria we are using now are nearly complete. In other words it is as close to the final product as we can get without actually using the compound itself. Thus, we can study the biology of the vehicle bacterium, *Axd*, and its behavior in the vineyard ecosystem.

We prefer to do this in vineyards because we feel that laboratory experiments will not be subject to natural forces that will be present in actual crops, and therefore might not be fully indicative of natural fate. It will also be important to choose widely separate locations across the grape growing regions in California for similar reasons. We need to determine now if the transgenic endophyte will travel to the fruit of the grape plant and to learn of its fate during harvesting operations. Our intention is to design a control method that will be considered safe to consumers. Greenhouse experiments will not provide rigorous enough conditions. Therefore it is necessary to obtain field data.

OBJECTIVES

1. Track the movement of *Alcaligenes xylosoxidans* (*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 AND CONCLUSIONS

In July, field sites were established at four locations in the state of California: Napa, Bakersfield, Temecula, and Riverside. At the Napa, Bakersfield, and Temecula sites, *Axd* was applied to grapevines using three 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 are currently being processed. Grapevines at the Riverside field site were needle inoculated with *Axd* 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 *Axd* in grapevines. We collected mature grapes and plant parts for analysis from grapevines at all four field sites. We are analyzing whole grapes as well as are dissecting out and surface sterilizing different tissues of the grapes to determine if *Axd* colonizes these tissues. Preliminary results suggest the *Axd* is not present in the fruit of the grape, but samples have not been fully analyzed yet.

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FUNDING AGENCIES

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BIOLOGICAL CONTROL OF PIERCE'S DISEASE WITH NON-PATHOGENIC STRAINS OF *XYLELLA FASTIDIOSA*

Project Leader:
Donald A. Cooksey
Department of Plant Pathology
University of California,
Riverside 92521

Reporting Period: The results reported here are for the completed project from July 1, 2002 to June 30, 2003.

ABSTRACT
This project is to construct and test nonpathogenic strains of *X. fastidiosa* derived from a pathogenic Pierce's disease strain for competitive exclusion of the pathogen in grapevines. Potential virulence genes were selected from comparative genome sequence analyses as well as DNA macroarray studies of differential gene expression. A more comprehensive analysis of differential gene expression with a DNA microarray approach is an outcome of this project and is funded under a new grant. Disruption of xanthan gum production by mutations in gumD and gumH resulted in less of a slime layer and fewer adhering cells than the wild-type strain on plastic and wood surfaces. However, when biofilm formation was quantitatively measured in polystyrene microtiter plates, both gumD and gumH mutants formed significantly more biofilm than the wild-type *X. fastidiosa*. In addition, the disruption of rsmA resulted in significantly more biofilm that the parent strain. Virulence assays in grapevine are still in progress, but gum mutants showed fewer symptoms than the parent strain in an alternative host assay. The analysis of potential virulence genes has also had side benefits for epidemiological work on *Xylella* by providing new primer sets for differentiate certain host strains of *X. fastidiosa*.

INTRODUCTION
A general approach that is being considered to manage Pierce's disease is biological control of the bacterial pathogen. Specific biological control approaches include the use of antagonistic endophytic bacteria isolated from the xylem of grape, bacteriophages, and interference with bacterial intercellular signaling. Another approach is the possible use of a nonpathogenic strain of *X. fastidiosa* derived from a pathogenic Pierce's disease strain for competitive exclusion of the pathogen in grapevines. This concept has certain advantages over other biological control approaches and considerable precedent in bacterial, fungal, and viral systems, including the biological control of xylem-inhabiting bacterial pathogens.

Colonization and protection of plants with less virulent or completely nonpathogenic strains of plant pathogens has been demonstrated in a number of bacterial (Wilson and Lindow, 1993), fungal (Sneh, 1998), and viral systems (Fulton, 1986). Some studies relied on naturally occurring avirulent strains, while other researchers have developed defined nonpathogenic mutants of pathogenic strains for this purpose, following the expectation that they would have the same ecological requirements for growth and are therefore ideal competitors (Wilson and Lindow, 1993). Another advantage of this approach is the specificity of the interaction, which reduces or eliminates possible deleterious effects on non-target organisms (Cook et al., 1996). This concept has also been demonstrated for the xylem-inhabiting vascular wilt pathogen, Ralstonia (Pseudomonas) solanacearum (Frey et al., 1994). Nonpathogenic mutants of this pathogen still colonized vascular tissues and resulted in high protection rates against the pathogenic strain. Our goal is to test this concept of competitive exclusion with nonpathogenic, or reduced virulence mutants, of the xylem-inhabiting *Xylella fastidiosa* for biological control of Pierce's disease.

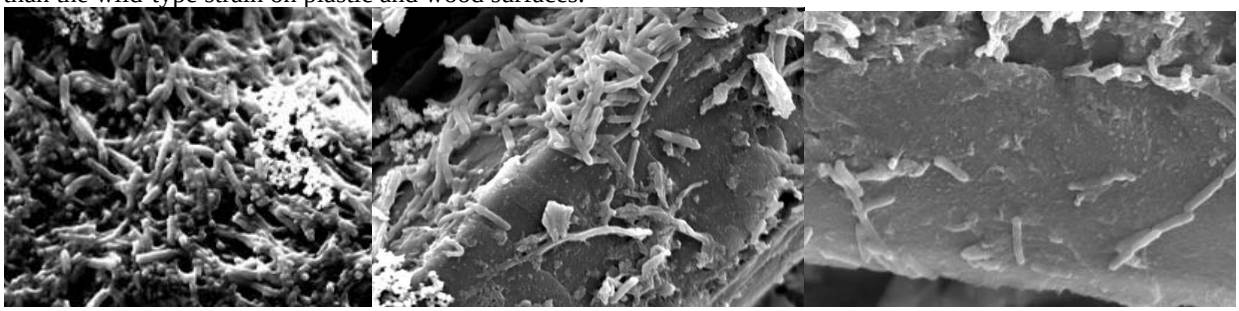
OBJECTIVES
1. Construct deletion mutations in putative virulence genes of
2. Test mutant strains for virulence in grapevines.
3. Test mutant strains for biological control of pathogenic strains in grapevines.

RESULTS AND CONCLUSIONS
Results
Selection of candidate virulence genes
We have utilized the full genome sequences of *Xylella fastidiosa* strains (Bhattacharyya, A., et al. 2002; Simpson et al., 2000; Van Sluys et al., 2003) to select open reading frames specifying putative pathogenicity and virulence factors. In addition, we constructed a DNA macroarray with about 100 of these genes to analyze their expression in different *Xylella* strains *in planta* and *in vitro* (Hernandez-Martinez et al., 2002). This work follows the hypothesis that many genes important in virulence and symptom will be differentially expressed in the bacterium grown in culture vs. during infection of plants. We have shown that these genes are expressed to varying degrees ranging from none to very high. However, since over 50% of the *Xylella fastidiosa* genome consists of genes with no known function, a more comprehensive approach toward the identification of virulence genes is necessary. We have recently obtained funding from the CDFA in a separate project to continue this work through the use of full genome microarrays.

Mutational analysis of virulence genes

Construction of several virulence gene mutants of has been done using the EZTN transposon or by insertional cloning of antibiotic resistance cassettes to create disruptive insertions into cloned genes that were amplified by PCR based on genomic sequence. The mutated clones have been subcloned into pUC129 and gene knockouts were constructed through homologous recombination in *Xylella fastidiosa*. Among virulence genes included in mutational studies are those of the *gum* operon, for which we have recently constructed successful knockout mutations.in *Xylella*. Other genes we are manipulating include a number of regulatory genes that likely control other virulence factors, such as RsmA.

Disruption of xanthan gum production by mutations in *gumD* and *gumH* resulted in less of a slime layer and fewer adhering cells than the wild-type strain on plastic and wood surfaces.



Wild-type on wood surface *gumD* mutant on wood *gumH* mutant on

However, when biofilm formation was quantitatively measured in polystyrene microtiter plates (Espinosa-Urgel et al., 2000; O'Toole and Kolter, 1998), both *gumD* and *gumH* mutants formed significantly more biofilm than the wild-type *X. fastidiosa*. In addition, the disruption of *rsmA* resulted in significantly more biofilm that the parent strain.

Virulence assays on grapevine have not been consistent in our laboratory, so assessment of the effects of these mutations on virulence of *Xylella* is still underway. We are also working to develop more rapid virulence assays with other hosts. Chorotic and necrotic symptoms were obtained by infiltration of Chenopodium leaves with wild-type Pierce's disease strains of *X. fastidiosa*, but the gum mutants appeared to cause fewer or no symptoms on this host. The *rsmA* mutant caused similar symptoms to the wild-type strain. Since RsmA is a global regulator, the effects of the *rsmA* mutation are being studied at a broader level using macroarray/microarrays to determine the expression profiles of other genes in *Xylella* in the *rsmA* mutant vs. wild type.

Additional findings from the project. The analysis of potential virulence genes has also had side benefits for epidemiological work on *Xylella*. We previously reported that while the *gumB* gene was conserved in all *Xylella* strains tested, we could digest the PCR product of this gene with frequent-cutting restriction endonucleases and differentiate certain host strains of *X. fastidiosa*. This was particularly important in our project on assessing inoculum sources of the grape strain in southern California, where we needed to differentiate grape strains from the oleander strain that is also present but not a threat to vineyards. We subsequently found that a number of our primer sets that we had designed for the macroarray project above were able to differentially amplify DNA from different host strains. We now have the ability to rapidly differentiate strains by PCR amplification with differential primers from cultures or often directly from infected tissues. We also used these primers to detect whether individual glassy-winged sharpshooters can carry and transmit multiple strains of *X. fasidiosa*.

CONCLUSIONS

We have not yet achieved the original goal of this project, to produce nonpathogenic mutants and test them for biological control of Pierce's disease. However, we have made progress in identifying potential virulence genes and in genetically modifying this difficult bacterium. Xanthan gum genes appear to play a role in colonization of abiotic surfaces and affected virulence in at least one plant assay. Our new project that involves a much comprehensive approach toward the identification of virulence genes using a DNA microarray approach will greatly enhance our ability to achieve the goals of this biological control project. Mutants generated in that study will continue to be assessed for virulence and tested for the ability to reduce Pierce's disease symptoms through competitive exclusion.

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FUNDING AGENCIES

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EPIDEMIOLOGY OF PIERCE'S DISEASE IN SOUTHERN CALIFORNIA: IDENTIFYING INOCULUM SOURCES AND TRANSMISSION PATHWAYS

Project Leader:
Donald A. Cooksey
Department of Plant Pathology
University of California,
Riverside 92521

Heather S. Costa
Department of Entomology
University of California,
Riverside 92521

Reporting Period: The results reported here are for the completed project from May 1, 2000 to July .31, 2003.

ABSTRACT
Xylella fastidiosa can cause a number of plant diseases in a variety of plant hosts including Pierce's disease of grapevines, almond leaf scorch disease, alfalfa dwarf, citrus variegated chlorosis, leaf scorch of live oak, pear leaf scorch, and oleander leaf scorch. In Southern California, the primary insect vector of concern is the glassy-winged sharpshooter, *Homalodisca coagulata* (Say). Previous studies of *Xylella fastidiosa*-induced diseases have described systems dealing with different primary vector species and different alternate host plants than those that are found in the Southern California systems. In this project, a variety of plant species found near a severe outbreak of Pierce's disease in vineyards in the Temecula valley of California were tested to identify potential sources of inoculum in the area. Plants were tested though three summer seasons using ELISA, culture on specialized media, and PCR methods to monitor for the presence of the pathogen. Plant species from the field that consistently tested positive for a grape strain of *X. fastidiosa* were the previously known hosts grape and almond, and two new hosts, Spanish broom, *Spartium junceum* and wild mustard, *Brassica spp.* Samples of oleander, *Nerium oleander*, also tested positive, however the strain of *X. fastidiosa* that infects oleander differs from the grape strain and does not appear to infect grape plants. Greenhouse transmission studies indicate that the glassy-winged sharpshooter was able to transmit a grape strain of the pathogen to *Spartium junceum, Brassica nigra* and other hosts.

INTRODUCTION
Diseases caused by *Xylella fastidiosa* threaten some of California's largest agricultural commodities, including the grape and wine industries, nursery, almond, and alfalfa production (Hopkins and Purcell 2002). Thus far, strains of *X. fastidiosa* that cause Pierce's disease of grapevines , almond leaf scorch, alfalfa dwarf, and oleander leaf scorch have been identified in California. The Pierce's disease (PD) strain and oleander leaf scorch (OLS) strain have caused devastating losses of grapevines and oleander plants respectively in California (Blua et al. 1999, Perring et al. 2001, Feil and Purcell, 2001). These two strains are genetically distinct (Hendson et al. 2001), and the strain of the pathogen that infects oleander does not infect grape, and vice versa (Purcell et al. 1999). The grape strain appears to have a broader host range than the oleander strain. However, the complete host range of each strain is not completely known (Hopkins and Purcell 2002).

In Southern California, the primary insect vector is the glassy-winged sharpshooter, *Homalodisca coagulata* (Say), a recent introduction from the southeastern U.S. (Sorensen and Gill, 1996; Blua et al., 1999). This insect feeds on xylem tissue, and is reported to feed on over 75 species of plants in 35 families (Turner and Pollard, 1959). The feeding habits and host range of GWSS differ from other vector species in California that have previously been associated with this pathogen. Studies in Northern California suggest that the PD pathogen is primarily spread by leafhopper vectors that move into vineyards from outside habitats (Purcell, 1981; Purcell and Saunders, 1999). Thus, studies of alternative plant hosts and inoculum sources of *X. fastidiosa* conducted in Northern California concentrated on plants in riparian habitats surrounding vineyards (Purcell and Saunders, 1999). However, in grape- growing regions of Southern California, the habitats and plant hosts that surround the vineyards are much different than those found in Northern California. In addition to naturally occurring vegetation, vineyards in Southern California are frequently adjacent to citrus groves and suburban landscapes. Because *X. fastidiosa* has a broad host range and in some cases can be present in plant tissue without causing noticeable symptoms (Purcell and Saunders 1999), it is not always easy to identify plants that serve as alternate hosts and potential sources of inoculum.

Knowledge of the source of disease inoculum is essential to the development of effective disease management strategies. The objective of this study was to determine which plant species in Southern California are hosts of *Xylella fastidiosa* and serve as potential sources of inoculum for PD infection of grapevine. In addition, because more than one strain of *X. fastidiosa* is could be present in our sampling area, it was necessary to identify the strain of the pathogen that was present in positive samples to determine if they were PD strains that would be considered a threat to grapevines in the area. For example, a plant that tested positive for the oleander strain of *X. fastidiosa* would not be a threat to vineyards, since it does not infect grape.

OBJECTIVES
1. Determine which plant species near vineyards harbor *Xylella fastidiosa* and serve as potential reservoirs of inoculum for the spread of Pierce's disease to grapes.
2. Measure the ability of the glassy-winged sharpshooter to acquire and transmit *X. fastidiosa* to and from grape, citrus, almond, and other plant species identified as potential hosts and sources of inoculum for the spread of Pierce's disease.

3. Comparison of the sensitivity and specificity of various methods to screen large numbers of plant and insect samples for the presence of Pierce's disease.

RESULTS AND CONCLUSIONS

Results

Field Samples

Over 60 species of plants, and more than 5000 samples were processed. Of the species tested in the Temecula valley, only grape, (*Vitis vinifera*), almond (*Prunus* spp.), Spanish broom (*Spartium junceum*), wild mustard (*Brassica spp*.) and oleander (*Nerium oleander*) consistently tested positive by two or more methods. Of these, wild mustard and Spanish broom were not previously reported as hosts for *Xylella fastidiosa*. *Brassica* plants tested positive by both ELISA and PCR, but we were unable to culture *X. fastidiosa* isolates from field samples for unknown reasons. We were, however, able to culture *X. fastidiosa* from *B. nigra* plants used in greenhouse transmission studies. Coyote brush, elderberry, citrus, and a few other samples occasionally tested positive with ELISA, however we were not able confirm those results with PCR or culture. Coyote brush and elderberry have previous been reported as hosts of *X. fastidiosa* when inoculated by insects under greenhouse conditions but tested negatively in field experiments (Purcell and Saunders 1999).

We were unable to detect the pathogen in grape and almond using ELISA in early spring. However, some samples of Spanish broom and oleander tested positive all year. Wild mustard plants tested positive in mid summer, and were generally not present in the field during the winter months.

Comparison of ELISA and IC/PCR for early season detection

n the first collection of grape samples in Spring (May), 15/25 samples from symptomatic field grapes, and 0/10 of the non-symptomatic plants tested positive by ELISA. Immunocapture PCR (IC/PCR) of the same plants did not detect any infected grape at that time. Both methods did detect infected oleander plants used as positive controls. In samples collected five weeks later (June), 5/21 symptomatic grape plants positive with ELISA, and these same plants also tested positive with IC-PCR. In this case, analysis of samples with IC/PCR early in the season did not help to detect any additional infected plants. Because ELISA was easier, less expensive and less time consuming, it was the best method we had for screening for the presence of *Xylella fastidiosa* in numerous plant samples. Samples testing positive could subsequently be confirmed with PCR.

Transmission Studies

Studies testing the ability of the GWSS to transmit *X. fastidiosa* from grape to 30 species of host plants found transmission of a PD strain to grape, black mustard, Spanish broom, almond, black sage, and Mexican elderberry. Previous studies have demonstrated that transmission of the grape strain to oleander did not occur (Purcell and Saunders 1999; Purcell et al. 1999). Similarly, in our studies no transmission from infected grape to oleander was observed.

Conclusions

Overall, few plant species could be documented as alternate host plants for the PD strain of *X. fastidiosa* in the Temecula valley. In the Temecula valley, it appears that infected grapevines, almond trees, Spanish broom, and wild mustard likely serve as major sources of PD inoculum. Thus, in addition to removing infected grapevines and almond trees, growers were advised to remove Spanish broom and wild mustard from areas surrounding the vineyards.

Citrus is a favored host of the GWSS, and is the most important year-long reproductive host in the Temecula valley (Perring et al. 2001, Hix et al. 2002). Although citrus plants were repeatedly sampled in the field, and exposed to infected insects in experimental transmission tests, we could never document infection in citrus in the field or in transmission test plants. Although citrus samples collected from the field occasionally tested weakly positive by ELISA, they could never be confirmed with PCR or culture. It is not clear if this is the result of occasional low levels of infection in citrus that are difficult to detect, or falsely positive ELISA results.

In our studies, we found that ELISA testing of plant samples appeared to be just as effective in detecting the pathogen in plant tissue as other method we used (PCR, media culture, IC/PCR). Thus, this method of analysis will continue to be extremely important when screening large numbers of plant samples. However, additional analysis of positive plants samples with other methods was necessary to eliminate the possibility of false positives with ELISA, and to identify the strain of the pathogen that was present. The strain-specific primers used were effective in giving a preliminary identification of *X. fastidiosa* strains. Comparative analysis using the 16-23s spacer region sequence was consistent with PCR results using the strain-specific primers. Additional strain-specific primer pairs could be designed to differentiate other strains of this pathogen that may be present in different geographic areas.

Some species of plants that tested positive for *X. fastidiosa* in field surveys and greenhouse transmission experiments in northern California (Raju et al 1983, Purcell and Saunders 1999) were never confirmed positive in samples collected in the Temecula valley even though they were present near infected grape plants. This could be a reflection of differences in the species of insect vectors present, the types of plant materials that dominate, or differences in host range of the *X. fastidiosa* isolates present in each location. Previous examination of strains of *X. fastidiosa* strains revealed genetic difference between northern and southern California strains of *X. fastidiosa* from grape (Hendson et al. 2001). More detailed testing is

being done to better characterize the PD strains of *X. fastidiosa* present in the host plants identified in the Temecula valley to determine if they differ from those found in other geographic areas.

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

Project Leader:

Abhaya M. Dandekar
Department of Pomology
University of California
Davis, CA 95616

Nam Goutam Gupta
B-1, MS M888,LANL
Los Alamos, NM87545

Elizabeth Hong-Geller
B-1, MS M888, LANL
Los Alamos, NM 87545

Karen McDonald
Chem Engg & Material Sciences
University of California
Davis, CA 95616

Cooperators:

George Bruening
Department of Plant Pathology
University of California
Davis, CA 95616

Edwin L. Civerolo
SJVASC
Parlier, CA 93468

Patrick R. Shiflett
MS M888,B Division, LANL
Los Alamos, NM 87545

Pat J. Unkefer
B-3, MS E529, LANL
Los Alamos, NM 87545

Cliff J. Unkefer
B-3, MS G758, LANL
Los Alamos, NM 87545

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INTRODUCTION

Globally, one-fifth of potential crop yields is lost due to plant diseases primarily of bacterial origin. *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 (Cohn et al., 2001; Magor and Magor, 2001). 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 the pathogen recognition element (SRD) and the pathogen killing element (defensin) in the chimeric molecule is a novel concept and has several short and long term impacts.

ABSTRACT

Xylella fastidiosa, a gram-negative xylem-limited bacterium is a causative agent of Pierce's disease (PD) in California grapevines. During very early stages of *Xylella fastidiosa* (*Xf*) infection, specific proteins/carbohydrates/lipids on the outer membrane of *Xf* interact with plant cells and are important for virulence (Pieters, 2001). Design of a protein inhibitor that interrupts this step of the plant-*Xf* interaction will be useful in combating virulence and controlling PD. Traditionally, antibiotics are prescribed as a preferred therapy. Antibiotics target the enzymes involved in the biogenesis of the bacterial outer membrane and diminish pathogen viability. However, a pathogen often develops antibiotic resistance without the corresponding loss of virulence and pathogenicity (Baquero and Blazquez, 1997). In this UC/LANL project, we offer a novel countermeasure against *Xf* as a viable alternative to antibiotic therapy. We propose to develop chimeric anti-microbial proteins with two functional domains. One domain (referred hereafter as the surface recognition domain or SRD) will be designed to target either a protein or a carbohydrate moiety on the outer membrane whereas the other (a defensin molecule) will be designed to insert and lyse the *Xf* lipid membrane. For protein binding, the SRD will contain either an elastase or a single chain full-length variable region (scFv) antibody targeted against the newly discovered *Xf* outer membrane protein, mopB (Bruening et al., 2002). For carbohydrate binding, the SRD will contain the carbohydrate recognition domain (CRD) of a lectin (Barre et al, 2001; Feinberg et al., 2001; Sharma and Surolia, 1997) to specifically bind to the carbohydrate on the lipid head or on the X*ylella* surface. The defensin molecule will be chosen from group IV plant defensins that exhibit strong anti-bacterial activity (Segura et al., 1998).

OBJECTIVES

1. Design of SRDs (15-20KDa) and defensins (5 KDa) by utilizing the literature data on *Xylella* lipids, surface carbohydrates, or outer membrane proteins.
2. Expression of SRDs and defensins in insect and plant cells.
3. *In vitro* testing of *Xylella* binding by SRD and anti-*Xylella* activity by defensins.

RESULTS AND CONCLUSIONS

The focus of our experiments will be objectives 1, 2 and 3 respectively. We will begin by examining the potential of the 218 amino acid residue long Leukocyte elastase a human neutrophil granular protein in order to target surface proteins (Sinha et al., 1987). Neutrophils contain a variety of proteins that enable the cells to migrate toward and eliminate microbial pathogens (Elsbach and Weiss, 1988). Until 1991, no specific antibacterial activity had been ascribed to Elastase (Wasiluk et al., 1991). However recent research has established that Elastase is the only human neutrophil protein, which is capable of individually killing *Borrelia burgdorferi*, the causative agent of Lyme disease (Garcia et al., 1998; Lusitani et al., 2002). Furthermore, it is known that Elastase can augment the cidal properties of other active proteins. This has been proven to be the case for *C. sputigena*, where very high concentrations of Azurocidin, an antimicrobal granule protein, became cidal upon addition of Elastase (Miyasaki and Bodeau, 1991). Currently, we are studying the efficacy of Leuckocyte Elastase and antibacterial peptides Cecropin and Defensin HNP-2 in killing *X. fastidiosa*. Their killing capability will be tested by establishing kill-curves, which show the number of colony forming units remaining after bacterium was exposed to these proteins individually or in combination. We will also define the protein on the bacterial surface that is a target for elastase activity. We will begin culturing grape embryo cultures as well as callus cultures expressing PGIP using membrane bioreactors CELLine 350 (Integra Biosciences, Inc.). In this bioreactor the plant cells are contained in a relatively low volume, rectangular chamber (5 mL) bounded by an oxygen-permeable membrane on one side and a protein-impermeable, 10kD molecular weight cut-off membrane on the other side that separates the cell compartment from a much larger nutrient medium compartment (350 mL). The use of a bioreactor will serve several important purposes. First, it will enable us to develop and optimize strategies for growing transgenic grape cell cultures. Second, it will allow us to monitor the total protein concentration in the cell chamber as a function of time and to characterize the proteins secreted into the medium in the cell chamber using SDS-PAGE. Third, we will be able to replace the entire contents of the medium compartment under sterile conditions. We will be using these bioreactors to express SRDs and Defensins. and to study the effects of environmental conditions (nutrient medium composition, temperature, pH, oxygen, light etc) on biomass growth, sucrose consumption, total protein concentration in the extracellular medium and recombinant protein production.

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FUNDING AGENCIES

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ROLE OF TYPE I SECRETION IN PIERCE'S DISEASE

Project Leader:
Dean W. Gabriel
Department of Plant Pathology
University of Florida
Gainesville, FL 32611

Cooperator:
Richard Lee
Citrus Research and Education Center
University of Florida
Lake Alfred, FL

Reporting Period: The results reported here are from work conducted from November 8, 2002 to October 31, 2003.

ABSTRACT
In an effort to make knockout mutations of a TolC homologue, marker interruption was attempted using two different vectors, one of them newly constructed for the purpose. Although these vectors worked well in *Xanthomonas*, repeated efforts to obtain such mutants in *X. fastidiosa* failed. In an attempt to utilize splice-overlap PCR to obtain marker eviction and generate marker-free exconjugants, a marker-eviction vector was constructed. Again, although the vector and method worked well in *Xanthomonas*, the method failed in *X. fastidiosa.* Marker exchange experiments are currently underway.

INTRODUCTION
Xylella fastidiosa (*Xf*) is a xylem-inhabiting Gram-negative bacterium that causes serious diseases in a wide range of plant species (Purcell and Hopkins, 1996). Two of the most serious of these are Pierce's Disease (PD) of grape and Citrus Variegated Chlorosis (CVC). The entire genomes of both PD and CVC have been sequenced (Simpson et al., 2000). Availability of the complete genomic DNA sequence of both a PD and a CVC strain of *Xf* should allow rapid determination of the roles played by genes suspected of conditioning pathogenicity of CVC and/or PD. For example, analyses of the CVC and PD genomes showed that there was no type III secretion system, but there were at least two complete type I secretion systems present, together with multiple genes encoding type I effectors in the RTX (repeats in toxin) family of protein toxins, including bacteriocins and hemolysins. RTX proteins form pores in lipid bilayers of many prokaryotic and eukaryotic species and cell types; at least one is associated with pathogenicity in plants. However, lack of useful DNA cloning vectors and/or techniques for working with either CVC or PD strains have impeded progress in functional genomics analyses.

Last year we described the transformation of two *X.f* PD strains using the small, stable, broad host range shuttle vector, pUFR047 (De Feyter et al., 1993). Both the vector and the transformed PD strains are available upon request. The vector is relatively stable in both PD strains.

This year we have focused on attempts to perform marker-interruption in the PD strains using various suicide vectors and techniques. Although marker-interruption using suicide vectors is normally an efficient, single crossover event in many bacteria, repeated marker-interruption attempts with *X. fastidiosa* in our lab and in others have failed (Feil et al., 2003; Monteiro et al., 2001; Guilhabert et al., 2001). We report here that attempts to use three different suicide plasmid constructs that we have successfully used for marker interruption or marker-eviction in *Xanthomonas* have all failed, despite repeated attempts. Since marker-exchange has now been reported to be successful with *X. fastidiosa* (Feil et al., 2003), we recently turned to this method.

OBJECTIVES
This is a two year proposal with three objectives:
1. Develop an effective functional genomics tool kit for efficient transformation and gene knock-out experiments in a PD strain (Year 1).
2. Determine culture conditions for activation of type I secretion (Year 2).
3. Determine the effect of type I secretion gene knockout experiments on pathogenicity of a PD strain on grape (Year 2).

RESULTS AND CONCLUSIONS
PD strains of *X. fastidiosa*, PD-A (Hopkins, 1985) and Temecula (Guilhabert, 2001), were grown in PD3 (Davis et al., 1981) medium supplemented with MOPS (3-4[morphomino] propane sulfonic acid; (Gabriel et al., 1989). Both strains were confirmed to be pathogenic on Madagascar periwinkle. Symptoms appeared after 3 months. Because of reports that others had problems with electroporation of pUFR047 into PD strains, we again confirmed transfer from *E. coli* DH5∀ to the spontaneous Rif resistant PD-1R strain by electroporation.

We attempted knockout mutagenesis on TolC, indicated below from CVC as XF2586, and also found in the PD strain as PD1964. We constructed marker-interruption plasmids using an internal fragment of PD1964 cloned by PCR in pUFR012,

which we had used for exactly the same purposes in *Xanthomonas* (Kingsley et al., 1993). Initial experiments failed. We then tried to knockout *fimA* using the same method. Marker-exchange had been reported for *fimA* at last year's symposium (Feil et al., 2003). These experiments also failed. We thought it might have to do with our level of kanamycin resistance, so we obtained the same *nptII* gene as had been used by Feil et al (2003) and created pAC3 (see Figures 1-3 below). pAC3 differs from pUFR012 principally by having the *nptII* gene transcribed in the same direction as *lacZ* (thus giving somewhat higher levels of resistance to kanamycin).

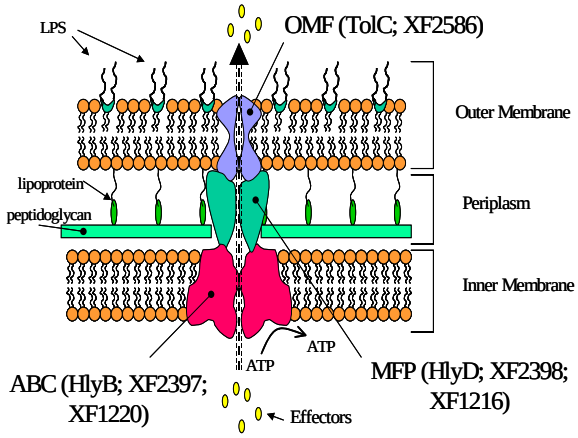

Figure 1. Type I Secretion system, including ABC (ATP binding cassette), MFP (membrane fusion protein) and OMF (outer membrane factor).

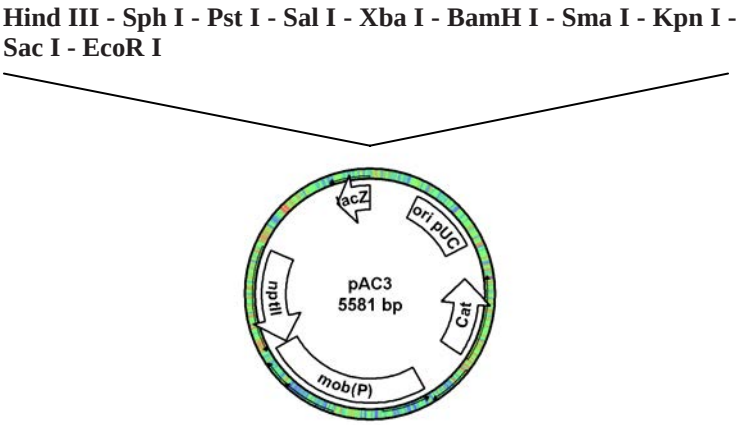

Figure 2. Suicide vector pAC3, with enhanced kanamycin resistance.

Again, we could not obtain marker-interruption mutants of either gene target. Simultaneously, we created another vector in order to attempt splice-overlap PCR deletions (marker eviction) of the target. This method involves constructing a gene deletion *in vitro* using splice-overlap extension PCR to generate and then fusing two ~500 bp flanking DNA fragments (Horton, 1995), and then site-specifically inserting the deletion into the genome in place of the wild-type gene using a two-step, SacB-assisted, marker-eviction mutagenesis (Hoang et al., 1998; Reyrat, 1998). We constructed pUFR080 for the purpose. Again, although the method works well in our hands in several different xanthomonads (unpublished), the method requires initial formation of a cis-merodiploid, and it failed in the PD strains we used.

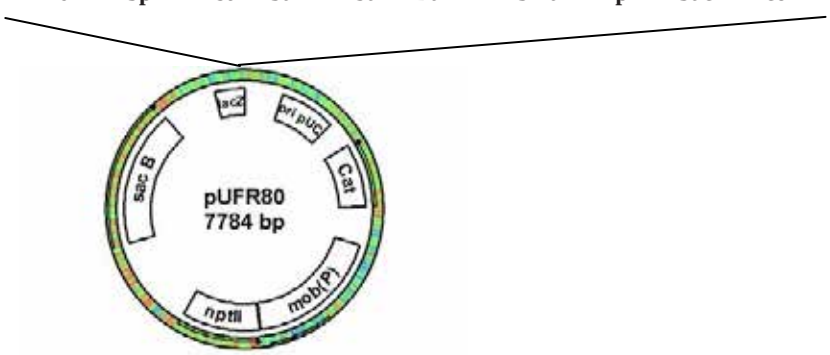

Figure 3. Suicide vector pUFR080, with enhanced kanamycin resistance and levan sucrase gene, used for marker eviction mutagenesis.

The reasons for the failure of marker-interruption are unknown, but speculation centers on the possibility that the origin of replication (pUC) of the suicide vectors may interfere with chromosomal replication (Feil et al, 2003). We have since turned to marker-exchange mutagenesis, and have obtained a no cost extension on the project to complete the objectives.

CONCLUSIONS

Despite our expectations to the contrary, our repeated efforts to obtain marker interruption and marker eviction mutants failed. Both methods required the formation of cis-mero diploids using a pUC based vector, and confirm recently published observations of others. We have repeated electroporation of our repW based vector, pUFR047, and have a relatively stable vector that should useful for complementation.

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FUNDING AGENCIES

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

Project Leaders:
Russell Groves
Jianchi Chen
USDA-ARS-SJVASC
Parlier, CA 93648

Cooperators:
Kent Daane
Division of Insect Biology
University of California
Berkeley, CA 94720

Marshall Johnson
Department of Entomology
University of California
Riverside, CA 92521

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

ABSTRACT
The overall goal of this project is enhance our present understanding of the epidemiology of Pierce's disease (PD) in the central San Joaquin Valley (SJV) of California by elucidating factors that influence its geographical distribution and movement. The objective of this research will be to characterize the seasonal abundance and dispersal biology of the glassy-winged sharpshooter (GWSS), a primary vector of *Xylella fastidiosa (Xf*), to identify where the vector(s) acquire the pathogen, to determine when vectors move into vineyards and transmit the pathogen to grapes, and to genetically characterize the populations of *Xf* isolated from GWSS collected in different perennial cultivated and non-cultivated plant species compared to strains present in PD- affected vineyards. Based on preliminary results of seasonal plant utilization by GWSS, we conclude that host plant species can significantly influence GWSS population biology. GWSS adult, nymph, and egg mass densities varied among perennial, cultivated crop plant species and non-cultivated weed species examined in this study including sweet cherry, navel, lemon, olive, avocado, plum, pomegranate, pistachio, and grape. Temporal patterns of GWSS capture, representing dispersal activity of both overwintered and first generation adult GWSS, varied among the perennial crop species examined. Moreover, patterns of adult GWSS capture among the distances sampled along linear transects extending into perennial crops were dissimilar among perennial crops.

INTRODUCTION
The glassy winged sharpshooter (GWSS, *Homalodisca coagulata*) was introduced into Southern California around 1990 and first identified in 1994 (1). This sharpshooter has continued to expand its range in the state and is expected to affect the overall increase in plant diseases caused by *Xylella fastidiosa* (*Xf*) (5). *Xylella fastidiosa* has an extensive and diverse host range including many common cultivated crop and ornamental plant species as well as numerous, non-cultivated, wild host species. *Xylella fastidiosa* strains have a complex pathogenic relationship with a diverse host range including monocots and dicots (7). Analyses of the genetic diversity of *Xf* have elucidated differences between many of the strains and a conclusion emerging from some studies is that strains of *Xylella* cluster within groups based upon host association. Knowledge of the genetic diversity of strains that comprise the population of *Xf* in the central San Joaquin Valley (SJV) of California will help in devising effective strategies for managing Pierce's disease (PD), as well as other diseases caused by this bacterium.

X. fastidiosa is transmitted by xylem feeding sharpshooters (Cicadellidae) and spittlebugs (Cercopidae) (4). In California, there are at least 20 species capable of transmitting the pathogen (2), although only four species are considered to be epidemiologically important for PD in grapes (6). Knowledge of which vector species transmit *Xf* in the central SJV, where they acquire the pathogen, when they move into vineyards, and when they spread the pathogen to grapes is critical to understanding and managing the spread of PD in this area. This overall goal of this project is to further our understanding of the epidemiology of PD in the central SJV of California with a focus on the identification of factors that influence its geographical distribution and movement.

OBJECTIVES
1. Identify and characterize the seasonal abundance and dispersal biology of GWSS, a primary vector of *Xylella fastidiosa*, and their patterns of host plant utilization within and among perennial, cultivated and non-cultivated plant species in agricultural production systems.
2. Compare the genetic structure of *Xylella fastidiosa* strains isolated from GWSS in different perennial, cultivated and non-cultivated plant species to those strains present in PD-affected vineyards.

RESULTS
The seasonal plant host utilization of GWSS within and among a variety of perennial, cultivated crop plants including sweet cherry, navel, lemon, olive, avocado, plum, pomegranate, pistachio, and grape are being sampled in GWSS-infested areas at each of three locations for each crop type in Tulare County in central California. Additionally, non-cultivated annual and perennial weed species occurring within and surrounding GWSS-infested perennial tree crops were sampled for GWSS

populations. Host utilization was assessed monthly at each of three locations for each crop type based on sweep/beat-net sampling for adult and immature GWSS and visual inspections for GWSS egg masses through the interval May to October, 2003. Based on preliminary results, host plant species can influence GWSS population biology. The largest mean number of adult GWSS (Figure 1a) were collected from citrus (navel and lemon), pomegranate, olive, avocado, and non-crop weed species whereas mean nymphal population densities (Figure 1b) were greatest from citrus and pomegranate with fewer nymphs collected from cherry, plum, and non-crop weed species. Non-crop plant species upon which adult GWSS were collected included red-root pigweed, prickly lettuce, annual sowthistle, little mallow, lambsquarters, field bindweed, blue morning glory, curly dock, evening primrose, johnsongrass, and ground cherry. The greatest mean number of GWSS egg masses (Figure 1c) was collected from citrus, pomegranate, and cherry whereas no egg masses were collected from non-crop weed species. The absence of detectable GWSS life stages in grapes and pistachio are not a true reflection of host utilization in these studies, as the only available experimental locations for these crops were just outside of the active GWSS-infested area.

Seasonal dispersal of adult GWSS was monitored within and among the variety of previously indicated perennial crop plant species. Beginning March, 2003, yellow sticky traps (approx.15 X 30 cm) were suspended 2 m above the ground between tree canopies along 4 linear transects (20 per crop type) at distances of 0, 5, 10, 50, and 100 m at each of 3 experimental locations for each crop sampled. All traps were collected and replaced weekly to capture adults dispersing within and moving among different crop types and the surrounding vegetation. Through October, 2003, a total of 27,264 adult GWSS, 18 green sharpshooters (GSS, *Draeculacephala minerva*), and 588 unidentified spittlebugs (Cercopidae) were captured on yellow sticky cards. Temporal patterns of GWSS capture, represented by plotting mean proportions of adult GWSS captured over time, were similar in citrus and pomegranate (Figure 2a) throughout the sampling interval (March – August) representing dispersal of both overwintered and first generation adult GWSS. Seasonal patterns of GWSS capture in olive, avocado, and plum were dissimilar to that of either citrus or pomegranate with only minor dispersal of overwintered adult GWSS in the early season and a delay of approximately 8-12 days in the periods of peak capture of first generation adult GWSS (Figure 2b). Patterns of adult GWSS capture among the distances sampled along linear transects also varied among the crop types examined throughout the emergence of first generation adult GWSS. Specifically, uniform mean trap captures across all distances were observed within GWSS-reproductive hosts navel and lemon compared to avocado and olive where highest mean trap captures were detected along only the margins of crops and declined with distance into the field.

In addition, to maximize our ability to identify actual vectors of *Xf*, the presence of the pathogen in a subsample of vectors captured on yellow cards from perennial and non-crop species will be determined initially through PCR (3). Based on genomic information, strain specific primers will subsequently be used to investigate the pathotype profile. Research has yet to begin on this objective to identify the incidence and pathotype profile of *Xf* infectious GWSS collected at different times throughout the season and from different specific habitats or perennial crop plant types.

CONCLUSIONS

We believe that this project will generate significant new information regarding the epidemiology of Pierce's disease in the central SJV of California and in providing practical guidance towards management of this pathosystem. This information may be useful in understanding the epidemiology of other economically important diseases caused by *Xf*. Both objectives address gaps in our present understanding that must be filled in order to develop PD and GWSS management strategies. This research will expand on previous work by documenting the relative importance of potential vector species that transmit *Xf* in the central SJV, where they acquire the pathogen, when they move into vineyards, and when they spread the pathogen to susceptible crops in the agricultural landscape of the central San Joaquin Valley of California. Knowledge of the genetic diversity of strains that comprise the population of *Xf* in detected from potentially infectious GWSS will help in devising effective strategies for managing Pierce's disease , as well as other diseases caused by this bacterium.

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FUNDING AGENCIES

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

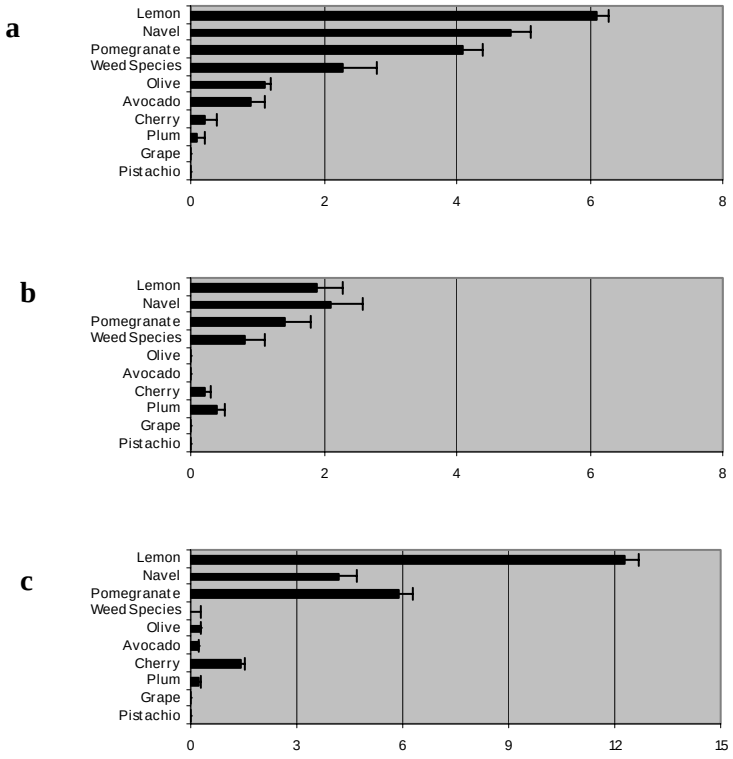


Figure 1. Mean (±SEM) number of adult (a), nymphal (b), and egg mass lifestages (c)of GWSS collected through sweep and beat-net sampling through the sampling interval May-September, 2003 at experimental locations in Tulare County, California.

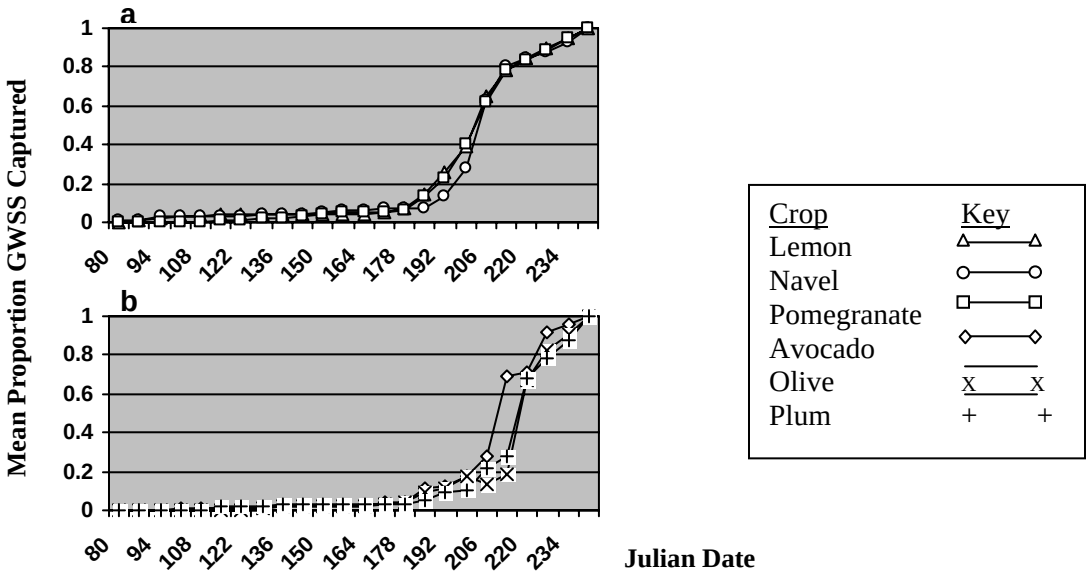


Figure 2. Mean proportion of adult GWSS captured on yellow sticky cards March-August, 2003 in navel, lemon, and pomegranate (a) as well as avocado, olive, and plum (b). Temporal patterns of capture represent both early-season dispersal of overwintering adults and 1st generation adult GWSS.

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

Project Leaders:
Jennifer Hashim
UC Cooperative Extension
Bakersfield, CA 93307

Barry L. Hill
Calif. Dept. of Food & Agriculture
Sacramento, CA 95814

Cooperators:
Maggi Kelly & David Shari
Center for the Assessment and Monitoring of Forest and Environmental Resources
University of California
Berkeley, CA 94720

Alexander H. Purcell
Department of. Environmental Science Policy and Management
University of California
Berkeley, CA 94720

Reporting period: The results in this report are from work conducted from April 2001 to October 2003.

ABSTRACT
Vineyards in seven grape production areas of Kern County's area wide management project for 2002-2003 were surveyed for PD. Incidence of Pierce's disease (PD) in the highly affected areas (General Beale and North) peaked in 2002, and declined in 2003. Treatments to reduce glassy-winged sharpshooter (GWSS) and identify and remove PD infected vines each year were associated with these dramatic reductions. A representative General Beale vineyard is mapped for years 2001 – 2003. An epidemiology data processing center was established at Center for the Assessment and Monitoring of Forest and Environmental Resources (CAMFER) at University of California, Berkeley.

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.

The epidemiology of Pierce's disease (PD) changed dramatically in California with the arrival of the glassy-winged sharpshooter (GWSS) about 15 years ago. Before that time the disease caused losses, but the damage accumulated gradually resulting in the loss of a small percentage of vines. With the arrival of the GWSS, however, PD spread has increased logarithmically, such that entire vineyards were destroyed in as little as 3 to 5 years. In Kern County where the disease was previously inconsequential, PD may now threaten more than 88,000 acres of grape production. To cope with this development there have been extensive field studies to determine effective methods to control the insect vector, the GWSS. However, our understanding of how to control the disease (goal of project 1) and the characterization of the changes in the epidemiology of PD when the causal bacterium is transmitted by GWSS (goal of project 2) has been based on limited field data.

The cooperative area-wide pest management of the GWSS project has defined seven distinct grape growing areas in Kern County. These areas represent various "stages" in the PD epidemic, ranging from the General Beale area--where GWSS was first observed in 1997 and where the epidemic occurred first and has been most severe--to the Highway 65-Delano area where GWSS was first observed in 2002 and where there is still very little PD. This variation among growing areas in combination with the significant accumulation of field data about these areas makes Kern County an ideal area to locate epidemiological projects. Extensive data have been obtained about GWSS populations and the effectiveness of various treatments in controlling GWSS. These two projects are obtaining data about the incidence of PD over time in each area, and the control measures and possible epidemiological factors that may affect the epidemic.

OBJECTIVES
Project 1: Monitoring and Control Measures For Pierce's Disease In Kern County.

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.

Project 2: Epidemiological assessments of Pierce's Disease.
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.
Create a central data processing facility at the Center for the Assessment and Monitoring of Forest and Environmental Resources (CAMFER) on the University of California, Berkeley campus to compile the data from this project in a GIS format. Share the resulting data, maps, and information with collaborating plant pathologists, statistical analysts, agricultural economists, and other legitimate researchers.

RESULTS AND CONCLUSIONS

Vineyards were monitored by visually inspecting each vine for PD symptoms and collecting and testing (by ELISA) samples from symptomatic vines. Tables 2 and 3 summarize the results for the 7 grape growing areas in Kern County. About 5% of Kern county's grape production acreage was monitored. The General Beale, north, central, south, and west areas have had GWSS since about 1997. In the General Beale and north areas the GWSS populations reached very high numbers in 2000-2001 (see Figure. 1), and the south, central, and west areas have had much lower but persistent populations. GWSS was detected in 2002 in the Hwy 65-Delano area. More than 10 vineyards in the General Beale area and more than two vineyards in the north area were severely impacted by PD in 2001 and 2002, with infection rates between 2% to more than 50%. Many of these were not included in this survey because sampling and testing the high number of infected vines would require more resources than was available. However Figure. 2 presents the progression of the epidemic in a representative Redglobe vineyard. The high infection rate in 2002 probably represents infections that were established in 2000 and 2001. The dramatic reduction apparent in 2003 is associated with a management program of area-wide GWSS reduction combined with roguing PD vines and replanting. This project also demonstrated that monitoring vineyards for PD, testing, removing infected vines, and replanting is very inexpensive when PD incidence is low, in the order of less than $5 per acre per year.

All PD survey data from this project has been compiled in GIS and database formats by CAMFER at University of California, Berkeley. This is the second in a projected five year project. In addition to the vineyards shown in tables 2 and 3, about 3000 additional acres in Kern and Tulare counties have been monitored. The data and the information from GWSS trapping surveys are being added to the data set at CAMFER. The resulting data, maps, and information will be made available to other scientists, government, and industry people involved in the management of PD in California.

A profile was created for each vineyard and the variables recorded include: GPS coordinates, cultivar, vine age/plant date, row and vine spacing, pruning and trellising system, weed index, proximity to other host crops of GWSS, and confirmed presence of *Xylella fastidiosa*, pesticide use information when available, and presence and population levels of GWSS. Fifteen cultivars of varying ages were examined during the project to correlate respective tolerances to PD (Table 1.). Analysis of temporal and spatial PD patterns and comparisons among the vineyards over time should lead to better models of PD epidemiology, a with quantitative estimates of how epidemiological variables, such as the incidence of PD combined with sampled populations of GWSS affect the further spread of PD. This understanding should lead to better control and management practices.

Table 1. Cultivars monitored in 2002-2003 for Pierce's disease.
Vine susceptibility: 1=most tolerant, 2=less susceptible, 3=most susceptible, NA=unknown.

Green		Red		Purple/Black	
Calmeria	3	Christmas Rose	NA	Autumn Royal	NA
French Colombard	2	Crimson Seedless	2	Black Emerald	NA
Jade Seedless	3	Flame Seedless	2	Fantasy Seedless	NA
Muscat	NA	Redglobe	3		
Perlette	NA	Ruby Seedless	2		
Thompson Seedless	1				
Superior Seedless	NA				

Table 2. Summary of the Monitoring for Pierce's disease in 2002.

Areas surveyed for PD	Number of vineyards	Number of acres/ Number of vines	Number of vines tested	Number of PD + vines Number PD+ vines per 1000
General Beale Pilot Area	41	849 ac 450991v.	2095	1238 PD+ * 2.75 +v./1000
North: Edison/Bena	7	159 ac 80769v.	159	116 1.44
South A: Arvin	21	304 ac 154208v.	46	9 0.058
South B: Arvin	28	261 ac 131247v.	74	7 0.053
Central: Arvin	5	55 ac 32631v.	5	0 0.0
West: Hwy 166	32	797 ac 375671v.	57	6 0.016
Hwy 65 and Delano	83	1636ac 790181v.	243	0 0
Total	216	4060ac 2015698v.	2543	1376 0.68

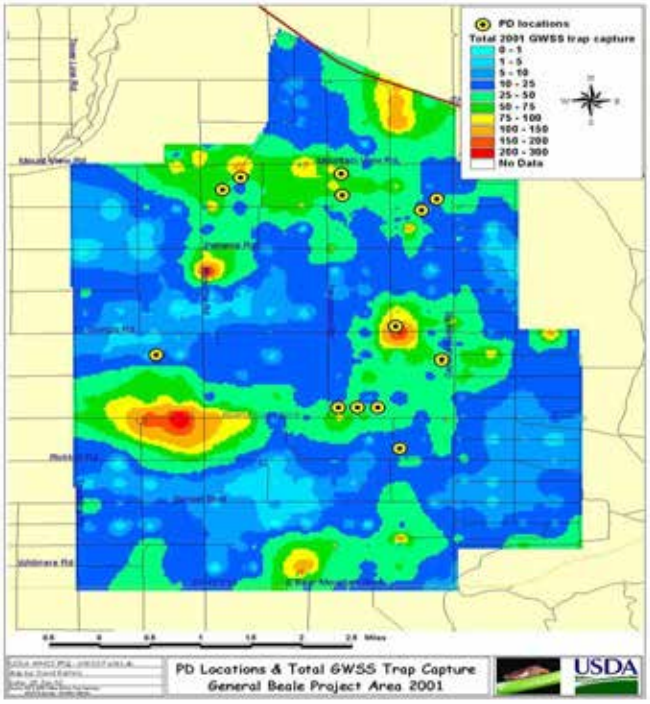
* 98.8% (1224 of 1238) of the PD positive vines in the General Beale area were in 2 out of 6 varieties, Redglobe and Crimson, on 113.4 acres and 40 acres respectively.

Table 3. Summary of the Pierce's disease survey effort in Kern County in 2003.

Areas surveyed for PD	Number of vineyards	Number of acres	Number of vines tested	Number of PD + vines Number PD+ vines per 1000
General Beale Pilot Area	41	849	326	188 PD+* 0.42 +v./1000
North: Edison/Bena	7	159	108	82 1.03
South A: Arvin	21	304	28	2 0.013
South B: Arvin	28	261	36	9 0.069
Central: Arvin	5	55	5	0 0.0
West: Hwy 166	32	797	99	22 0.065
Hwy 65 and Delano	83	1636	127	3 0.0038
Total	208	3958.63	729	306 0.152

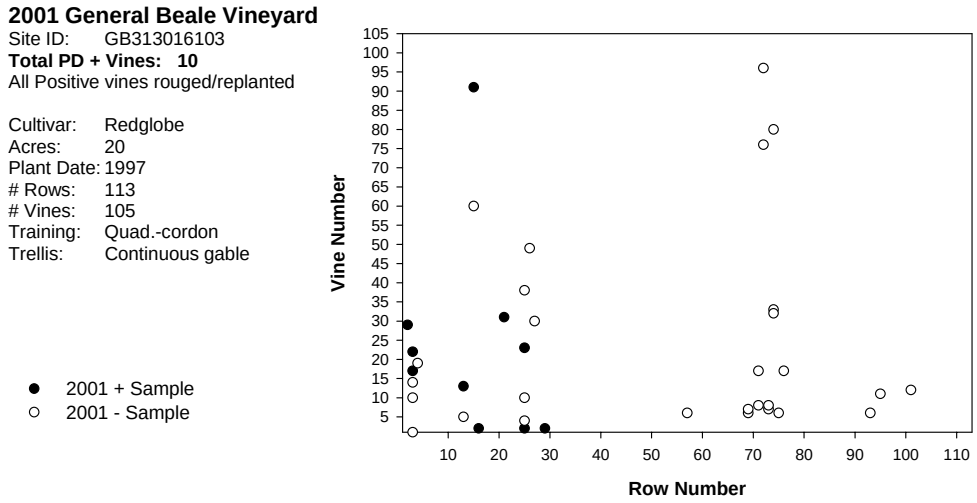
* 96.8% (182 of 188) of the PD positive vines in the General Beale area were in the same 153.4 acres of Redglobe and Crimson as in 2002.

Figure 1. PD locations* and total GWSS trap captures in the General Beale Pilot Project area in 2001.



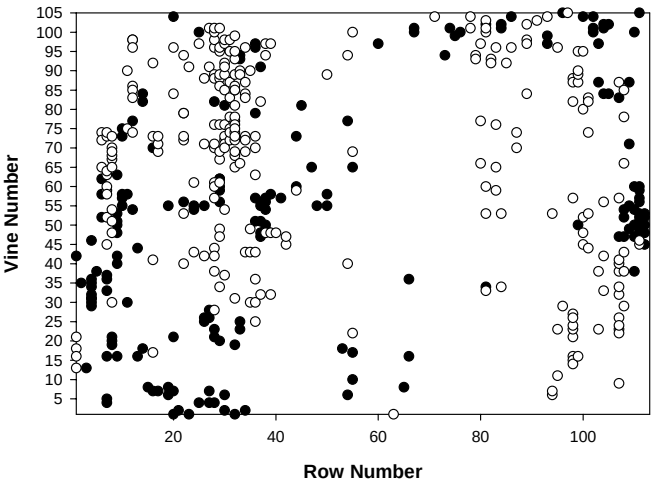
* PD locations are where PD vines were observed but not in all cases mapped nor the incidence quantified.

Figure 2. Three years results of vineyard survey in General Beale area.



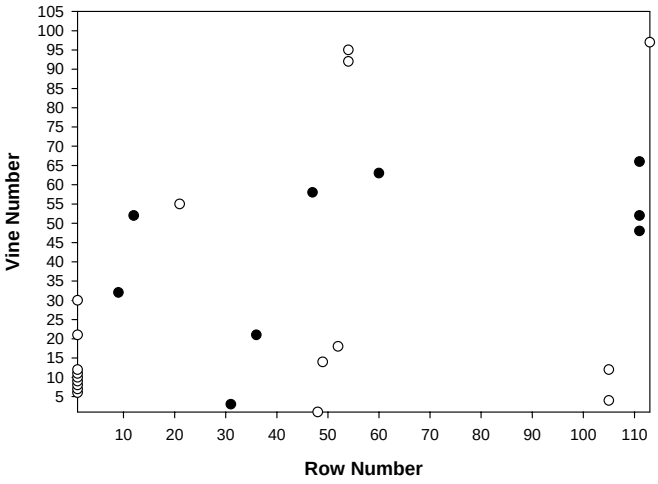
2002 General Beale Vineyard
Site ID: GB313016103
Total PD + Vines: 184
All Positive vines rogued/replanted

Cultivar: Redglobe
Acres: 20
Plant Date: 1997
Rows: 113
Vines: 105



2003 General Beale Vineyard
Site ID: GB313016103
Total PD + Vines: 9
All Positive vines rogued/replanted

Cultivar: Redglobe
Acres: 20
Plant Date: 1997
Rows: 113
Vines: 105



FUNDING AGENCIES
Funding for these projects was provided by the University of California Pierce's Disease Grant Program, and the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board.

CHARACTERIZE AND ASSESS THE BIOCONTROL POTENTIAL OF BACTERIAL ENDOPHYTES OF GRAPEVINES IN CALIFORNIA

Project Leader:
Bruce Kirkpatrick,
Department of Plant Pathology
University of California
Davis, CA 95616

Cooperators:

Dawna-Darjean Jones
Department of Plant Pathology
University of California, Davis
Davis, CA 95616

Edwin Civerolo
USDA-ARS-PWA
SJV Agriculture Science Center
Parlier, CA

A.H. Purcell
Division of Insect Biology
University of California
Berkeley, CA 94720

Reporting Period: The results reported here are from the worked conducted from January 2003 to November 2003.

ABSTRACT

Over 1000 putative bacterial endophytes were isolated from healthy, PD-affected and "escape" (apparently healthy grapevines growing in a vineyard with high incidence of Pierces disease) grapevines growing in Napa and Davis, California from 2000 to 2002. There were no differences in total populations of endophytes isolated in Napa versus Davis, however higher populations of endophytes were recovered from PD-affected versus healthy vines. The endophytes were identified by RFLP and sequence analysis of a portion of the 16S rDNA. Sixty six RFLP groups were determined and sequence analysis showed a diversity of bacterial genera were recovered from grapevines; the most predominant genus was *Bacillus*. Nineteen of the isolates completely inhibited the growth of *Xylella fastidiosa* (*Xf)* in an *in vitro* plate assay. Approximately 80 of 138 isolates that were tested were classified as grapevine colonists because they could be recovered in high populations more that 6cm from the point where they were inoculated into grapevines. Thirteen of the endophytes were classified as both *Xf*-antagonists and grapevine colonizers. Five of these isolates were pin-prick inoculated into grapevines growing in the greenhouse. These vines were then challenged by *Xf*-infectious sharpshooters in the insectary at University of California, Berkeley. None of the endophyte-inoculated vines prevented infection by *Xf* or decreased the severity of PD that developed in these vines.

INTRODUCTION

Many higher plants contain internal populations of bacteria that apparently cause no damage to the plant. Some of these endophytic bacteria can colonize the xylem of plants; the same unique niche that *Xylella fastidiosa (Xf*)occupies. There has only been one published study on bacterial endophytes of grapevines and this study was undertaken in Nova Scotia, a climate significantly different from California. We undertook this study to determine the diversity and relative seasonal abundance of bacterial grapevines endophytes in California. We also wanted to determine whether any of these bacteria exhibited any antagonism towards *Xf* growing on petri dishes in the lab and whether isolates that exhibited *Xf* antagonism *in vitro* might colonize and protect grapevines from *Xf* infection. This effort comprised the PhD. research of Dawna Darjean-Jones who is now in the processing of writing her thesis. We will present a very brief overview of her results here.

OBJECTIVES

1. Isolation and identification of endophytes colonizing grapevines in California:
   i. Determine quantitative and qualitative differences in endophytic populations of grapevines between areas that support natural infections of PD (Napa) and areas where no natural infection of PD has been observed (Davis).
   ii. Determine whether endophytic populations vary quantitatively and qualitatively due to seasonal changes.
   iii. Compare endophytic populations of healthy grapevines with grapevines infected by *X. fastidiosa*
   iv. Compare endophytic populations of "escaped" grapevines (healthy grapevines growing among many infected vines that appear to have escaped disease) with that of healthy grapevines.
2. Determine if any grapevine isolates are antagonistic to or can prevent infection by *X. fastidiosa*.

RESULTS AND CONCLUSIONS

Isolation and identification of endophytes colonizing grapevines in California

Healthy established grapevines from two vineyards in the Davis area and two vineyards in the Napa area, as well as diseased and PD-escape vines from the Napa area were sampled periodically in 1999-2000. These same vines (when possible; diseased vines were sometimes removed without notification) were sampled continuously every other month beginning in September 2001 through December 2002. Xylem sap was expressed from shoots using a pressure chamber. Aliquots of xylem fluid were plated onto 3 microbiological media. Colonies were quantified, streaked to purity, then frozen at -80 ^{O}C until they identified. Using universal primers for the 16S rRNA gene, a PCR product was generated for each isolate.The PCR product was double-digested with restriction enzymes to generate a restriction fragment length polymorphism (RFLP) pattern for each strain. To reduce the redundancy in the identification process, the RFLP patterns of the strains were

subjected to computer analysis using the GelCompar program to avoid sequencing numerous isolates with the same RFLP pattern. 16S PCR products of representatives of each unique RFLP group were sequenced to identify the unknown bacteria.

A total of 1018 endophytes were collected from healthy and PD-affected grapevines during the 3-year study. The total numbers of endophytes, both qualitatively and quantitatively, were similar in Davis and Napa healthy vines. Population of endophytes in PD-affected vines were slightly higher than healthy vines growing in Napa or Davis and more endophytes were isolated in the spring and fall than during the summer. Analysis using the GelCompar program has identified 66 RFLP groups of strains, thus reducing the number that had to be sequenced For examples, several *Bacillus* spp. have been collected from vines classified as healthy, diseased or escapes. Endophytes identified thus far include species from the following genera: *Bacillus, Pseudomonas, Agrobacterium, Erwinia, Streptomyces, Cellulomonas, Pantoea, and Paenibacillus.*

Identification of grapevine endophytes that are natural antagonists to *Xf* and capable of systemic movement in grapevine
A total of 138 endophyte isolates from 50 of the 66 RFLP groups were pinprick inoculated into greenhouse grown grapevines to assess potential movement within grapevine. Each endophyte was inoculated into two shoots from the same vine**.**

After 4 weeks sterilized razor blades were used to divide the surface sterilized stem into seven sections. Each sample was ground in 2 mL of buffer and 100 µl of ground plant suspension was plated onto isolation medium. Resulting colonies were visually compared to the original isolate, sub-cultured, PCR-amplified and the RFLP pattern was compared to the database pattern for the endophyte. Endophytes moving 6 or more cm from the point of inoculation were considered to be potential systemic colonizers of grapevines.
Thirty six of 138 isolates were not recovered from the point of inoculation or any area of the plant sampled. 80 of of the isolates multiplied and were recovered in numbers of at least $7X10^4$ / gram of tissue, i.e. more than 1000/100 ul of sap. About 58 of endophytes were recovered 6cm or more from the point of inoculation and were provisionally classified as systemic colonizers of grapevines.

Bacterial suspensions of *Xf* were spread plated onto solid PD3 medium to form a "lawn." Cultures were incubated for 3 days at 28^oC. Three, 5 µl droplets of overnight endophyte culture were placed onto the previously inoculated *Xf* plates and allowed to dry. Cultures were returned to the incubator for an additional 7 days. Growth of *Xf* and the endophyte were scored. Endophytes inhibiting the growth of *Xf* within a 1-3 mm zone were considered weakly antagonistic. Those inhibiting growth more than 3 mm were considered antagonistic.

Of the 125 grapevine endophytes that were tested for *in vitro* antagonism to the growth of *Xf,* 24 exhibited positive inhibitory activity and 19 isolates completely inhibited the growth of *Xf*. Of the 24 that were inhibitory, 13 isolates were classified as systemic colonizers of grapevines as described previously. Among the antagonists/colonizers were members of 6 *Bacillus* RFLP groups, a *Cellulomonas* sp., a *Rahnella* sp. and a bacterium belonging to the genus *Streptomyces*.

Five of the 13 antagonist/colonizer endophytes were pin-prick inoculated into each of 10 grapevines growing in pots in the greenhouse and allowed to colonize the plants for 1 month. With the generous assistance of the Purcell lab at UC Berkeley, the 50 endophyte-inoculated plants, plus a set of 10 non-inoculated control vines, were exposed to *Xf*-infectious blue green sharpshooters (BGSS) in the insectary. The vines were exposed to 5 BGSS for 48 hours and the insects were then removed. The vines were monitored for symptoms of PD for 5 months following exposure to BGSS. The vines were then rated on a scale of 0 (healthy) to 4 (dead vine) and all vines were tested for *Xf* by IC-PCR.

At least 60% of all the endophyte-inoculated vines and the non-inoculated controls developed symptoms of PD and tested positive for *Xf* using IC-PCR. There was no apparent reduction in the severity of disease in the endophyte-inoculated vines. Thus it appears that none of these 5 isolates has the ability to prevent *Xf* infection of grapevines, however this assay will be repeated and next time the endophytes will be given 2 months to colonize the vines before being challenged with *Xf*. The 8 other antagonist/colonizing endophytes will also be evaluated. We hope to identify xylem-colonizing bacteria that will decrease the ability of *Xf* to multiply to high populations and cause Pierce's disease.

FUNDING AGENCIES
Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and the University of California Pierce's Disease Grant Program.

EVALUATION OF BACTERICIDES AND MODES OF DELIVERY FOR MANAGING PIERCE'S DISEASE

Project Leader:
Bruce Kirkpatrick
Department of Plant Pathology
University of California
Davis, CA 95616

Cooperators:
Chad Vargas
Dawna-Darjean Jones
Dept. of Plant Pathology
University of California
Davis, CA 95616

Edwin Civerolo
USDA-ARS
Parlier, CA

A.H. Purcell
Division of Insect Biology
University of California
Berkeley, CA

Peter Andersen
University of Florida
Quincy, FL 32351

Ed Weber
UC Cooperative Extension
Napa, CA

Rhonda Smith
UC Cooperative Extension
Sonoma, CA

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ABSTRACT

For the past four years we have been evaluating plant micronutrients and inducers of systemic acquired resistance as prophylactic agents to protect grapevines against infection by *Xylella fastidiosa (Xf)* or as therapeutic agents to cure Pierce's disease (PD) affected grapevines. To date**,** none of the plant micronutrients containing zinc, copper or manganese or compounds that induce systemic acquired resistance in plants such as Actiguard^{R}, Messenger^{R}, or Resist^{R} showed efficacy when used as prophylactic agents to prevent infection of *Xf* in healthy grapevines, both in field trials and, for some materials, greenhouse evaluations of materials applied to potted grapevines that were then exposed to *Xf*-infectious sharpshooter vectors.

Evaluation of plant micronutrients and two antibiotics as therapeutic agents for curing, or at least causing a remission of symptoms in PD-affected grapevines, were more encouraging. Many of the PD-affected Merlot grapevines that were injected with zinc or streptomycin in Fall, 1999 and Spring, 2000 remained free of PD symptoms 3 years following treatment, whereas nearly all of the untreated control vines were dead. However, inspection and evaluation performed in October 2003 revealed that some of the vines that were symptomless in 2002 have now developed some early symptoms of PD. These symptoms may be the result of residual *Xf* populations that were not killed by the bactericide treatment finally multiplying to levels where they induced disease symptoms or these early infections may be the result of recent inoculation of treated vines by infectious-PD vectors. Field evaluation of a citrus terpene-based bactericide in Fall, 2002 did not reduce *Xf* populations in treated vines and all of these treated vines had progressed symptoms of PD in Fall, 2003.

INTRODUCTION

Bactericides such as fixed coppers, zinc, and antibiotics have been used with some degree of success in preventing some plant diseases. However, except for some phloem-limited pathogens such as phytoplasmas, all of the bacterial diseases in which bactericides have been of some value involve bacterial pathogens that reside on the surface of plants. Pierce's disease is caused by a xylem-limited bacterium, *Xylella fastidiosa* (*Xf)* which, to date, has not been successfully managed with bactericides. Although *Xf* is quite sensitive to a number of common bactericides that have been used on agricultural crops (Jones and Kirkpatrick, 2000) the major impediment to treating grapevines prophylactically to prevent infection by *Xf* or therapeutically to cure PD-affected vines has been the inability to introduce bactericides into grapevine xylem. If methods could be developed to effectively deliver prophylactic or therapeutic bactericides into grapevines, this could provide a comparatively straightforward solution to a very complex disease problem. Although genetic resistance to PD is probably the ultimate solution the ability to produce PD-resistant, commercially acceptable, multiple varieties and clones of *Vitis vinifera* will be a challenge. The development of a successful bactericide solution, particularly a prophylactic approach that would prevent infection of healthy vines, could be used as a viable interim solution to PD until more desirable approaches are developed to manage or prevent PD.

OBJECTIVES

1. Determine the efficacy of plant micronutrients and antibiotics as potential therapeutic bactericides for eliminating *Xylella fastidiosa* (*Xf*) in grapevines.
2. Determine the efficacy of plant micronutrients and systemic acquired resistance inducers to provide prophylactic protection against *Xf* infection of grapevines.

RESULTS AND CONCLUSIONS

Prophylactic Trials

Previously established prophylactic trials in Napa, Temecula, and Santa Cruz received three consecutive treatment applications this spring as opposed to the two treatments applied in previous years. Numbers of treated and control vines that subsequently developed Pierce's disease (PD) were still low in most of the trials; subsequently, these trials have not provided useful information at this time. However, two of the prophylactic trials, a White Riesling and a Merlot vineyard in Napa

experienced higher levels of infection and provided some insight into the potential efficacy of some treatments, especially those that appeared to offer little or no protection against *Xf* infection (Table 1).

Therapeutic Trials

Disease rating data from two Merlot trials in Napa was evaluated for efficacy following the final disease rating taken on September 11, 2002. Data for treatments that provided statistically significant benefit for suppressing PD symptoms, compared to the untreated controls for two Merlot trials, one vineyard rated 3 years after treatment and the second vineyard rated 2 years after treatment, is presented in Tables 2a, 2b. The results suggest that the most effective method for delivering the bactericides was the drill-through agar injection method and that zinc and streptomycin were two materials that provided significant positive results in both vineyards. The 2003 results were similar to rating data obtained in 2002. The 2001 results lead to the establishment of a third therapeutic trial in Fall, 2001 which tested the five best therapeutic injection treatments and one new treatment, phosphorous acid, in one of the previously used Merlot vineyards. It is too early to differentiate the beneficial effects of severe pruning, which was used in combination with all of the bactericide treatments, from the any effect of the applied bactericide in this plot. In Fall, 2002 two new therapeutic trials were established, one trial in Napa and one in Sonoma. A Sauvignon Blanc vineyard with mild to severe symptomatic vines was chosen in Napa, and four agarose injection treatments including copper sulfate, zinc sulfate, streptomycin, and phosphorous acid were applied. Each treatment was replicated on 15 vines and 15 untreated control vines were marked. The same treatment regime was repeated in the Sonoma Chardonnay vineyard with equally symptomatic vines. All bactericide treatments were applied in October of 2002.

A new root drench treatment was also tested in these two vineyards. The root drench product containing citrus terpenes was applied to 90 diseased vines in Napa at a rate of 1 gallon per vine with a second application one week after the first application. In addition, 20 diseased vines received a single 2-gallon dose with no additional applications. Untreated diseased vines were established as controls. A similar design was established in Sonoma using only the 1X rate. Petioles from treated vines were processed and evaluated for viable *Xf* cells. All of the treated vines still contained viable *Xf* cells at populations that were similar to untreated, diseased vines. This result was confirmed by a colleague in Georgia. The treated vines also had delayed shoot growth that was typical of a PD-infected vine and in October 2003 all of the terpene treated vines in Napa and Sonoma had typical symptoms of PD. These results indicate that the terpene had no beneficial effect in treated *Xf*-infected vines in Northern California.

Table 1. Percentage of vines in each disease-rating category for two prophylactic trials located in a White Riesling and a Merlot vineyard located in Napa, CC. Data reflects total amount of disease that occurred in 2001 through 2003. Disease Ratings = 0) healthy, 1) 1 or a few PD canes, 2) symptomatic canes throughout canopy, 3) cordon dieback, 4) dead vine.

			Percentage of healthy and PD vines as per 10/20/2003				
Vineyard	**Treatment**	**Reps**	**0**	**1**	**2**	**3**	**4**
White Riesling	ZincAA	20	60	10	5		25
	MnAA	20	65	5			30
	Rezist 2% + Stabilizer 2%	20	45				55
	Actigard	20	60				40
	Messenger	20	65	10			25
	Manganese Carbonate (4X)	20	70	5	5		20
	Zinc 50 (4X)	20	70				30
	Zn sulfate –soil applied	20	60				40
	Untreated Control	**20**	**65**	**10**			**25**
Merlot	ZnAA	20	60	15			25
	MnAA	20	75	5			20
	Rezist 2% + Stabilizer 2%	20	60	15	10		15
	Actigard 1X	20	80	10			10
	Messenger	20	65	5			30
	Manganese Carbonate (4X)	20	60	25			15
	Zinc 50 (4X)	20	65	5	10		20
	Zn sulfate – soil applied	20	50	5	5		40
	Untreated Control	20	70	10	5		15

Table 2a. Results of Cumulative Logit Model for disease rating in Merlot Vineyard A THREE years after initial treatment. Only treatments that were positively significant at 90% level are presented.

Treatment	Application Method	p	Parameter Estimate	Std. Error	Odds Ratio
Manganese	Injection	0.01	2.28	1.00	9.77
Streptomycin	Injection	0.004	2.86	1.08	17.54
Zinc sulfate	Injection	0.002	2.91	1.07	18.27
Zn/Mn	Foliar spray	0.01	2.88	1.3	17.81
Zn sulfate	Foliar spray	0.02	2.71	1.26	15.04

Table 2b. Results of Cumulative Logit Model for disease rating in Merlot Vineyard B TWO years after initial treatment. Only treatments that were positively significant at 90% level are presented.

Treatment	Application Method	p	Parameter Estimate	Std. Error	Odds Ratio
Zinc sulfate	Injection	0.06	1.45	0.79	4.26
Streptomycin	Injection	0.01	1.25	0.78	3.5
Tetracycline	Injection	0.01	1.25	0.78	3.5
Zn sulfate	Soil applied	0.05	2.71	1.26	15.04

FUNDING AGENCIES
Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and the University of California Pierce's Disease Grant Program.

TREATMENT THRESHOLDS FOR THE GLASSY-WINGED SHARPSHOOTER BASED ON THE LOCAL EPIDEMIOLOGY OF PIERCE'S DISEASE SPREAD

Project Leader:
Thomas M. Perring
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:
Charles A. Farrar, Rayda K. Krell, and Yong-Lak Park
Department of Entomology
University of California
Riverside, CA 92521

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

ABSTRACT
The current treatment threshold for the glassy-winged sharpshooter (GWSS) as a vector of Pierce's disease (PD) is one insect per tree in citrus. We developed a model to evaluate how the threshold might change in relation to various biological and ecological factors. The model 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. As these factors varied in the model, the GWSS treatment threshold changed. A major limitation of implementing treatment thresholds calculated from the model is that key data for some parameters are lacking. The model is a useful tool for identifying research areas that are needed to further refine a GWSS treatment threshold.

INTRODUCTION
The current treatment threshold recommended to prevent glassy-winged sharpshooter (GWSS) transmission of Pierce's disease (PD) is one GWSS per tree in citrus. This threshold was implemented in Kern County, and was reported to successfully reduce GWSS densities (Wendel et al. 2002). In winter 2003, this threshold directed treatment of almost all citrus acreage in the Temecula and Coachella Valleys. The initial threshold has provided a good start towards refining management criteria for GWSS. However, in the current California budget crisis, it is doubtful that funds will be available to sustain the level of areawide insecticide applications required to respond to this low action threshold. We believe that field-specific thresholds can be developed and we have begun work on a model for this purpose.

In refining the GWSS treatment threshold, the primary question becomes, what is the relationship between insect density and disease incidence? If we can develop this relationship, then we have a much better reference for developing a treatment threshold that will prevent disease transmission. The challenge is that the number of insects required to cause disease will change under different circumstances, therefore, a model is a helpful tool for estimating a threshold number based on key parameters.

In our approach to establishing treatment thresholds we are developing a model that incorporates biological and ecological data. It is common in entomology to calculate a treatment threshold based on the economic injury level (EIL). In a traditional EIL, injury is defined as the amount of injury per pest and is typically expressed in terms of amount of plant tissue consumed per insect (Higley and Pedigo 1996). In a system where an insect transmits a pathogen, injury can be interpreted as the number of plants infected per insect vector.
Currently, there is no tolerance for GWSS-transmitted PD. Therefore, we must assume that the treatment threshold is the number of GWSS required to cause a single PD infection. However, as more information is gained about the GWSS/PD relationship in California, we are learning that the likelihood of GWSS successful transmission of PD will vary under different conditions and therefore the threshold should vary as well.

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
Model Components:
The first challenge in developing the model was to identify the primary parameters affecting the number of insects required to cause a single PD infection. We identified a list of these primary factors and they are listed in Table 1.
The assumption

Table 1. Components of the model for a treatment threshold of an insect vector of plant disease.

Symbol	Description	Parameter Value Range	Reference
T_v	number of insects required to cause a single infection	calculated from model	none
C	proportion insects carrying pathogen	0.00–1.00	none
R	insect transmission efficiency of pathogen	0.01–0.35	Almeida and Purcell 2003
M	proportion insects that will move from sample area to feed on host plant of interest	0.00–1.00	none
P	number of plants a single insect will visit	5–15	none, values estimated
S	plant varietal susceptibility to disease expressed as a proportion	0.04–1.00	Raju and Goheen 1981
E	probability of infection event resulting in disease	0.00–1.00	none

Model Assumptions:

The following assumptions were made for the model:

- GWSS will be monitored by sticky traps in citrus.
- Treatment of GWSS will be applied to citrus.
- The relationship between sticky-trap catches and an absolute population estimate of GWSS is known.

Model Design:

A discrete, dynamic, deterministic model was developed using the model variables described in Table 1. The mathematical relationship between the primary model variables was arranged to yield a threshold result of the number of vectors required to cause a single PD infection:

$$T_v = \frac{1}{C \times R \times M \times P \times S \times E}$$

Model Simulation:

In a model simulation run, we varied two parameters across a range of values from 0.00–1.00 to examine the effect on the GWSS treatment threshold (Figure 1). In this simulation, as the proportions of GWSS carrying PD and moving to grape increased, the threshold decreased. As expected, the threshold was greater than one when these proportions were lower. For example, when the proportions GWSS carrying PD and moving to grape were approximately 0.25 and each GWSS visited five vines (Figure 1A); the treatment threshold was at least 10. When the proportions were very low (<0.05), the threshold increased greatly to over 3,000 GWSS (Figure 1A). The thresholds remained lower over a greater range of proportions when the number of grapevines visited per GWSS was increased to 15 (Figure 1B).

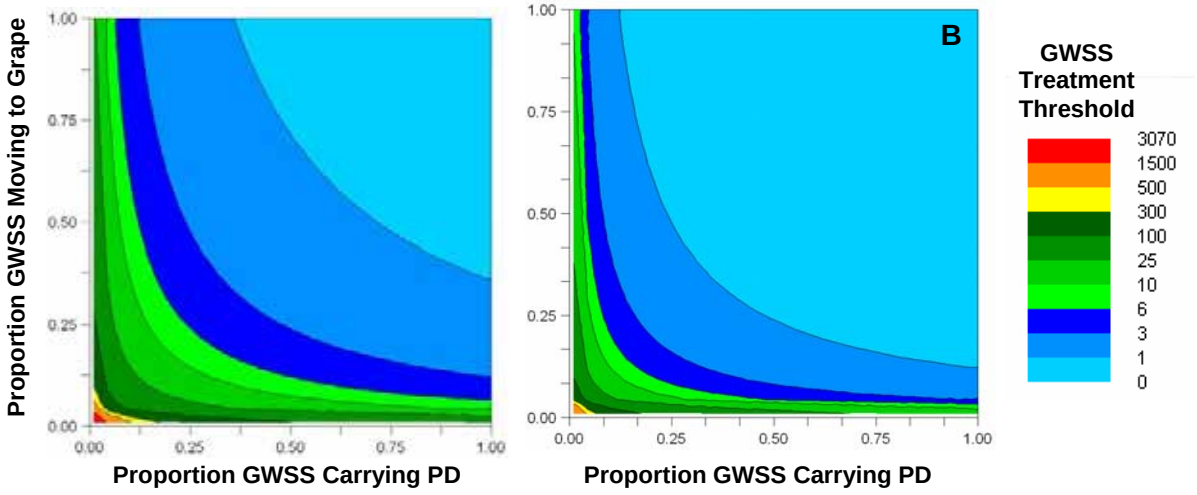

Figure 1. Glassy-winged sharpshooter treatment threshold at varying proportions of GWSS moving to grape and GWSS carrying PD at two different levels of grapevines visited per GWSS: A) 5, and B) 15. Other model parameters were constant: transmission efficiency = 0.35, varietal susceptibility = 0.80, and probability of infection event resulting in disease = 1.00.

The initial threshold model is a useful tool for exploring the effect of various epidemiological parameters on the relationship between GWSS density and PD incidence. However, the primary limitation to implementing treatment thresholds based on the model is that key data are lacking. The model has helped to identify several areas where more research is needed including:

- The relationship between sticky-trap catches and an absolute population estimate of GWSS.

- Methods for determining the percentage of GWSS carrying PD in the field.
- Preference of GWSS to move from citrus to grape.
- Grape varietal susceptibility to PD for common cultivars, especially table grapes.
- Various data on phenological and environmental circumstances under which a *Xylella fastidiosa* infection event leads to PD development.

We will continue to refine the model as additional simulation runs are performed. Additional parameters may be added to the model and others removed if their importance is found to be negligible. Some mathematical relationships may change as more data become available. Realistic model parameters will be evaluated in the model as they become available through current research.

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FUNDING AGENCIES

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EPIDEMIOLOGY OF PIERCE'S DISEASE IN THE COACHELLA VALLEY

Project Leader:
Thomas M. Perring
Department of Entomology
University of California
Riverside, CA 92521

Carmen Gispert
Viticulture Farm Advisor
UC Cooperative Extension Riverside County
Indio, CA

Cooperators:
Charles A. Farrar and Rayda Krell
Department of Entomology
University of California
Riverside, CA

Reporting Period: The results reported here are from work conducted from May 1, 2001 through September 30, 2003.

ABSTRACT
In 2001, no Pierce's disease (PD) was detected in the Coachella Valley. However in June 2002, one diseased grapevine was found in each of two adjacent vineyards. A subsequent census of those vineyards revealed the presence of 13 infected vines. Each of the infections was confirmed with ELISA and bacterial culturing, and PCR was used to confirm the presence of the grape strain of *Xylella fastidiosa* (i.e. Pierce's disease). Surveys in June 2003 identified two additional fields with infected vines, bringing the total number of locations in the valley to three.

Yellow sticky traps have been used to show the seasonal trend of glassy-winged sharpshooter (GWSS) adults. Average counts in the summer of 2002 were higher than in the summer of 2001. Similarly, winter traps in 2003 showed higher densities than winter traps in 2002, suggesting an increasing GWSS population density throughout the valley. However, spring and summer 2003 traps were nearly zero at all locations, indicating successful control of GWSS by the CDFA sponsored vector control program implemented by the Riverside County Agricultural Commissioner's office. Through this study, traps adjacent to citrus groves caught more GWSS than traps not near citrus, however fewer than 35 percent of the traps near citrus caught GWSS on any given week. GWSS count data were managed in a GIS format and this enabled us to place relative importance to citrus groves based on various densities of GWSS caught near the groves. This information could be used to target sites for vector reduction treatments.

INTRODUCTION
Pierce's disease (PD) has been in California for over 100 years (Purcell 1981), but the recent introduction of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, into the state (Sorenson and Gill 1996) has radically changed the epidemiology of this devastating disease. Since 1994, at least 1,500 vineyard acres have been lost to the disease in California, and in the Temecula Valley alone, losses have been estimated at $13 million (Wine Institute 2001). The California grape industry is estimated to contribute $33 billion to the state economy (Wine Institute 2001), and GWSS transmission of PD threatens to destroy one of the state's most valuable commodities.

The rapid losses caused by GWSS-transmitted PD in Temecula suggest that areas where GWSS becomes established experience rapid PD spread and vine decline. We conducted a PD survey of eight vineyards in Temecula and found plant decline or death from PD ranging from 51–87% (Perring et al. 2001). The most plausible explanation for the swiftness and severity of the PD epidemic in Temecula is the unique epidemiology created when GWSS is introduced into an area with endemic PD sources (Purcell and Saunders 1999). In Temecula, the epidemic has mimicked that of grape growing regions in the U.S. where GWSS is endemic. In the southeastern U.S., GWSS-transmitted PD is the major factor limiting grape production (Purcell 1981).

The table grape industry in the Coachella Valley is represented by 10,465 acres of producing vines, which generated grapes valued at $108.5 million in 2001 (Riverside County Agricultural Commissioner, 2001). Pierce's disease (PD) was identified in the Valley in 1983 (Goheen 1984), but until the arrival of the glassy-winged sharpshooter in the early 1990's (Blua et al. 1999), PD was of little concern. In May 2001, we began a study in this desert valley, with the goal of discovering characteristics unique to GWSS-vectored PD epidemics.

OBJECTIVES
The goal of our epidemiological studies in the Coachella Valley is to describe the epidemiology of PD when the primary vector is GWSS, and to use this information to design management strategies to reduce disease spread.
Three objectives are pertinent to this report:
1. Determine the incidence and distribution of PD in the Coachella Valley.
2. Describe the spatial and temporal abundance of GWSS in the Coachella Valley.
3. Determine the relationship of citrus to the abundance of GWSS in vineyards.

RESULTS AND CONCLUSIONS

Results

***PD incidence and distribution*:** For the past three grape growing seasons, we have surveyed the Coachella Valley in search for PD. In 2001, we visually inspected 300 plants in each of 25 vineyards and all vines in a 60-acre vineyard proximal to an area that had PD in 1983. We collected 233 suspected samples and analyzed them with ELISA. None of these plants were positive for *X. fastidiosa*. In 2002, we visually sampled 300 plants in each of 25 vineyards, and visually inspected 35,000 vines randomly distributed throughout the Valley. We analyzed (by ELISA) 268 plants from this survey and found 13 vines with *X. fastidiosa*. Bacteria were confirmed in these plants with selective-media plating and PCR, amplifying for PD-specific DNA. This was the first post-GWSS PD finds in the Valley. This past summer (2003) we visually inspected an estimated 616,400 vines and 478 vines with suspected PD were subjected to ELISA. Five of these 478 vines were positive for PD. Four of these vines were at one field site and the fifth vine was at another site. This brings the number of fields at which PD has been detected in the Coachella Valley to three (Figure 1). We are in the process of characterizing these three fields to determine the extent and patterns of infection in them.

***Spatial and temporal abundance of GWSS*:** We used yellow sticky traps distributed uniformly at one-mile intervals throughout the Coachella Valley to monitor the seasonal cycle of adult sharpshooter activity. GWSS catches rose into the summer of 2001, declined in late July, peaked again in mid-August, and then declined into the fall and winter (Figure 2). Numbers were extremely low until a period of increased activity, presumably by overwintering adults, in January and February 2002, after which counts declined again until May 2002. Average counts in the summer of 2002 were higher than in 2001, suggesting an increasing trend of GWSS numbers in the Coachella Valley. GWSS adult catches in the fall of 2002 were very low (similar to fall 2001) until January 2003. At this time there was a peak in adult activity that was greater than trap catch densities at the same time of the year in 2002. In February 2003, the Riverside County Agricultural Commissioner's Office, with support from CDFA, initiated a GWSS reduction program by treating citrus in the Coachella Valley. From our trapping data, this action appears to have reduced GWSS adults to a very low level that has been maintained this year (Figure 2).

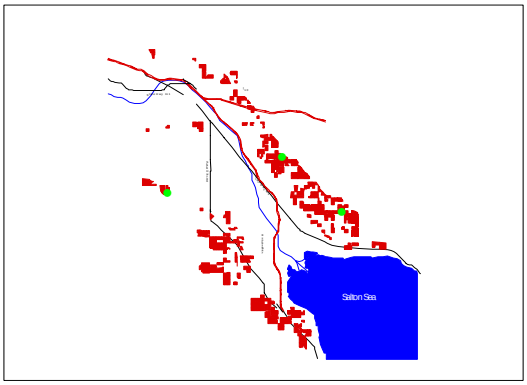


Figure 1. Vineyards (red) and sites (green) inCoachella at which PD has been found.

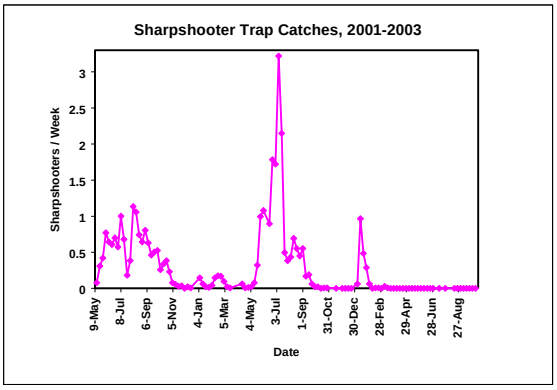
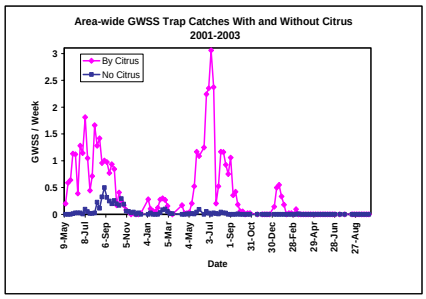
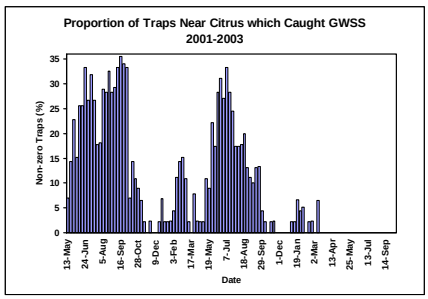


Figure 2. Sharpshooter trap catches, 2001-2003.

***Relationship of citrus to the abundance of GWSS in vineyards*:** Numerous studies have shown citrus to be a key host for GWSS and we studied the contribution of citrus proximity to GWSS densities in grapes near citrus. Prior to the vector reduction program in the February 2003, we found that traps adjacent to citrus caught more GWSS than those not adjacent to citrus (Figure 3). However, the presence of citrus did not always result in elevated GWSS catches; fewer than 35 percent of the traps adjacent to citrus caught GWSS on any given week (Figure 4). This indicates that vector control strategies should be targeted at citrus, but all citrus groves in the Coachella Valley do not need treatment at this time. We also conducted extensive studies at 25 citrus/grape interface study sites. At each site, traps were placed in 4 plots: along the citrus border, within the vineyard adjacent to the citrus (designated "Grapes-Near", Figure 5), 500 ft from the citrus (Grapes-Medium), and 1000 ft from the citrus (Grapes-Far). When GWSS were caught (on 31 of 90 weeks, no GWSS were trapped in the Valley), traps near citrus consistently caught the most GWSS than traps within the vineyards, statistically different on 20 weeks ($P<0.05$, Tukey-Kramer). The effect on PD epidemiology of these decreases in GWSS with distance from citrus are not clear, but Perring et al. (2001) showed PD incidence was higher, on average, in vines next to citrus compared to vines distant from citrus. Our Coachella Valley data support the practice of focusing vector control on citrus and grapes immediately adjacent to the citrus. At the same time, the data indicate that area-wide insecticide applications in vineyards that are not close to citrus are unwarranted in the Coachella Valley.





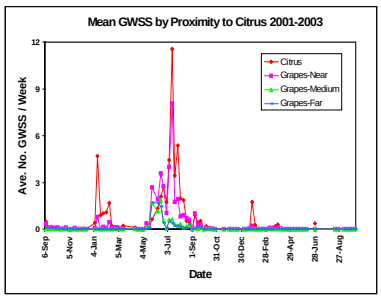


Figure 3 **Figure 4** **Figure 5**

CONCLUSIONS

This year we identified two additional fields in the Coachella Valley with PD, bringing the total to three fields. In each of these fields, many of the vines show severe disease, suggesting they have been infected for some time. Some of the vines show less-severe symptoms that may indicate more recent infections. While we currently have no basis for linking symptom severity to length of infection in mature vines, we are interested in this relationship because it may provide information on primary and secondary spread of PD by GWSS.

Early in our research program, we established a geographic information system (GIS) in which we have managed 90 weeks of data from 156 traps. This provides a powerful tool for data manipulation and allows us to link GWSS densities to spatial information, and use these relationships to identify sites that should be targeted for vector reduction. For example, we can display the traps that have caught more than 0 GWSS per week (or conversely identify traps that have never caught a GWSS) through the course of the study (Figure 6A). Similarly, the GIS can be used to display traps with varying densities of GWSS, (>5, Figure 6B or >20, Figure 6C).

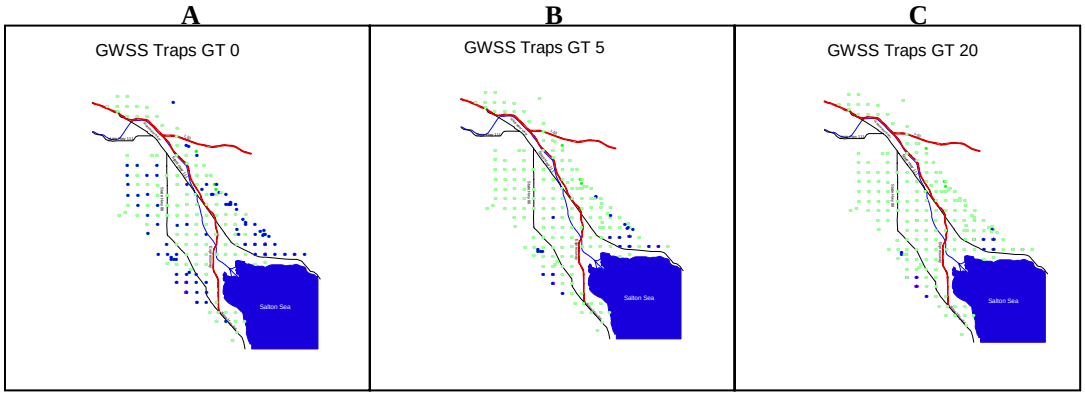


Figure 6. Trap locations (green) and those traps (in blue) that caught greater than (GT) 0 GWSS, GT 5 GWSS, or GT 20 GWSS per week through the course of this study (May 2001 - October 2003).

If we specify a certain distance around each of the traps (we used 500 ft. for this analysis), we can identify citrus blocks near traps with various GWSS densities. From this exercise, we identified the groves within 500 ft. of traps that have caught five or more GWSS/week (Figure 7A) and groves near traps that have caught one or more GWSS/week (Figure 7B) at anytime from May 2001 - October 2003. Using five GWSS/week as a threshold, treatment would be necessary on 4822 acres, while a threshold of one GWSS would result in 8192 acres needing treatment. Depending on resources available for vector reduction, these types of analyses can be extremely useful to prioritize citrus groves for application.

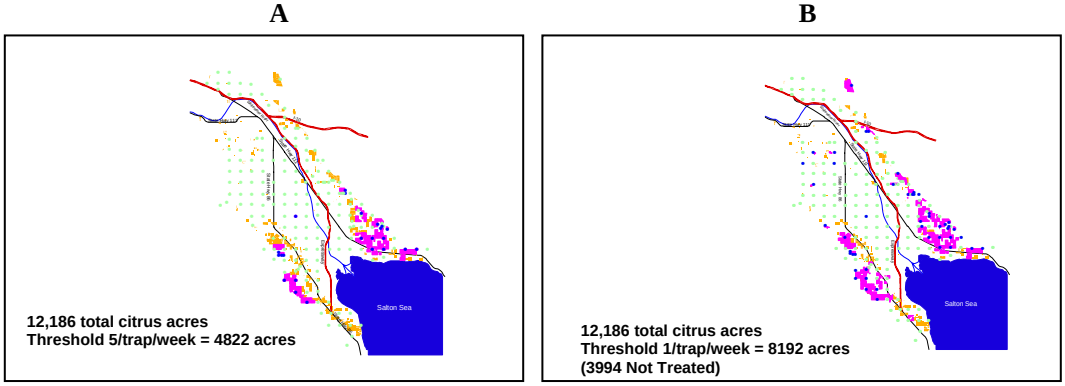


Figure 7. Trap locations (green), citrus groves (orange) and groves within 500 ft. of sticky trap (purple) that caught at least 5 GWSS/week (A) or at least 1 GWSS/week (B) during any week between May 2001 and October 2003.

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FATE OF *XYLELLA FASTIDIOSA* IN ALTERNATE HOSTS

Project Leader:
Alexander H. Purcell
Division of Insect Biology
University of California
Berkeley, CA 94720

Research Associate:
Christina Wistrom
Division of Insect Biology
University of California
Berkeley, CA 94720

Cooperators:
Jennifer Hashim
UC Cooperative Extension Kern County
Bakersfield, CA

Reporting Period: The results reported here are from work conducted from November 2002 to October 2003.

ABSTRACT
Investigations of the fate of the Pierce's disease bacterium *Xylella fastidiosa* (*Xf*) in alternate hosts from which sharpshooters might acquire *Xf* identified bacterial hosts among vineyard weeds, cover crops, field crops and adjacent vegetation common to vineyards in California's San Joaquin Valley. Work for the past year focused on completing additional replications of 15 high-priority weeds to compare mechanical and insect vector inoculation efficiencies, and to determine the fate of bacterial populations over a nine-week period after inoculation with *Xf*. Four things are required for a plant to be a good source of *Xylella* for vector acquisition: it must be an attractive food host to sharpshooters, develop *Xylella* infections frequently when fed on by infective insects, allow systemic growth of the bacteria beyond the inoculation site, and support population growth above 1,000,000 cfu of *Xf* per gram of plant tissue. Although insect survival varied considerably between replications, it averaged 77% on all plants for two days. Ten of the 35 weed species examined were colonized by *Xf* more than 50% of the time in greenhouse studies when plants were maintained in ideal conditions for bacterial growth. Sixteen species supported *Xylella* populations above 1,000,000 cfu/g, thirteen had populations between 1,000,000 and 10,000 cfu/g, and three had populations below 10,000 cfu/g. Finally, 13 species had *Xf* recovery immediately distal to the inoculation site in greater than half of successfully inoculated plants. The plants that had greater than 60% insect survival during inoculation, more than 50% of sites inoculation sites infected in greenhouse tests, that supported bacterial populations of 1,000,000 cfu/g or larger, and had systemic *Xf* movement beyond the inoculation site in more than half the infected inoculation sites were: black nightshade, common sunflower, annual bur-sage, morning glory, poison hemlock and fava bean.

INTRODUCTION
Understanding the fate of *Xylella fastidiosa* in its various hosts is important because of the wide range of plants that the insect vectors feed on (Adlerz, 1980; Purcell, 1976 and unpublished data). Additionally, the bacteria itself survives and multiplies in an unusually large number of plants (Freitag, 1951; Raju *et al.* 1983; Hopkins, 1988). Studies of the fate of *Xf* in four plant species highly preferred by various insect vectors revealed that blackberry, mugwort, and watergrass were propagative hosts but only blackberry allowed systemic movement (within-plant spread) of *Xf* (Hill and Purcell, 1995). In grape and two other hosts, vector transmission occurred only from plants with population densities exceeding 10,000 cells per gram, with efficient transmission occurring only near 10,000,000 (log 7) *Xf* cells per gram. Further studies of the fate of *Xf* in 33 species of riparian plants commonly found in Napa valley revealed that most species were propagative but non-systemic hosts (infected only the inoculated plant cells) of the bacteria and suggested that *Xylella* eventually disappeared from non-systemic hosts (Purcell and Saunders, 1999). Previously reported (Wistrom and Purcell, 2002) data in this study identified 9 alternate hosts that developed *Xf* infections greater then 50% of the time. Seven species supported *Xf* populations over 1,000,000 colony-forming units [cfu] per gram of plant tissue. Bacterial survival in field conditions was tested with 5 previously identified alternate hosts in summer and winter field studies. *Xylella* was recovered less frequently (at 26% of inoculation sites compared to 46% in winter, and 35% of inoculation sites compared to 21% in the summer) and at approximately 10-fold lower populations from field-grown plants from Bakersfield, CA, as compared to greenhouse-grown plants, in ideal conditions for *Xf* growth (Feil and Purcell, 2001) during the first three weeks after inoculation. In general, most non-systemic hosts of *Xf* developed highest populations within 1 to 3 weeks that thereafter decreased, while *Xf* populations in systemic hosts continued to increase or remain at the highest densities attainable in a particular host.

OBJECTIVES
Evaluate the fate of *Xylella fastidiosa* in Central Valley weeds and other crops of interest.

RESULTS
Experiments in the past year focused on repeating inoculations of 15 high-priority weeds to obtain larger sample sizes and comparisons of inoculation efficiency between glassy-winged sharpshooters, blue-green sharpshooters, and mechanical inoculation. Three new host species were tested; curly dock, lambsquarters, 'Moapa' alfalfa. To date, 35 species of common vineyard weeds, crops and other plants have been evaluated. All plants were inoculated with STL, a Pierce's disease strain of

Xf originally isolated from Napa valley. Plants were inoculated with *Xf*-carrying sharpshooters by confining groups of 2 to 4 infective sharpshooters to a 3-cm length of stem, petiole or leaf (depending on plant morphology) in a foam-and-mesh cage for 2 days. Only results from sharpshooter groups capable of successfully transmitting *Xf* were used. Plants were mechanically inoculated by placing 5 µl of turbid (8 to 9 $\log_{10}$ cfu/ml) *Xf* cell suspension in SCP buffer on the stem, leaf or petiole of the plant, which was probed with a #2 insect pin until drawn into the plant. Inoculations were performed on sunny days to maximize uptake of the *Xf* suspension via transpiration. Plants were assayed for presence and population of *Xf* by culture on semi-selective PWG media using the techniques of Davis (1983) and Hill (1995).

Host range experiments with plants inoculated in the greenhouse identified 10 species of weeds that were infected greater than 50% of the time with *Xf*. These were black nightshade, common sunflower, common cocklebur, annual bur-sage, morning glory, marestail, silverleaf nightshade, sacred datura, poison hemlock and fava bean. Another 18 species become infected 20 to 50% of the time when inoculated with *Xf*; johnsongrass, cheeseweed, field bindweed, yellow nutsedge, purple nutsedge, prickly lettuce, southwestern cupgrass, whitestem filaree, curly dock, common purslane, California burclover, black mustard, quinoa, tree tobacco, red gum, 'Ace' tomato, 'Violeta Lunga' eggplant, and 'Moapa' alfalfa. Plants that became infected less than 20% of the time in greenhouse tests were: jojoba, annual sowthistle, prostrate pigweed, watergrass, and blue gum. Only white clover (0 of 3 sites) and red clover (0 of 5) had no *Xf* recovery from any inoculation sites tested. A total of 54 and 74 sites were tested respectively, but either were contaminated with bacteria other than *Xf* or inoculated with non-infective sharpshooters. California burclover, black mustard and alfalfa did not have infections move beyond inoculation sites, however the three species had small sample sizes due to contamination and poor transmission of the sharpshooters. Johnsongrass had *Xf* populations of $\log_{10}5$ cfu/g at the inoculation site, but only developed systemic infections at 2 of 14 infected sites. Other species with fewer than 20% of infections moving beyond the inoculation site were annual sowthistle and yellow nutsedge.

Populations of *Xf* in alternate hosts followed three main patterns, either increasing over nine weeks, remaining steady over the sampling period, and decreasing. *Xf* populations increased in 6 plants, including fava bean, field bindweed, and annual morning glory. Seven plant species had steady populations of *Xf* at 3 and 9 weeks after inoculation. *Xf* populations decreased in 3 species, johnsongrass, sowthistle, and eggplant population from three weeks after inoculation. Species with populations less than 10,000 cfu/ gram of plant material were cheeseweed, jojoba, annual sowthistle, and whitestem filaree. Sharpshooters cannot acquire *Xf* from plants with populations lower than 10,000 cfu/g (Hill and Purcell, 1997).

Some plants other than white clover had a large proportion of contaminated inoculation sites. This appeared to be a function of the plant rather than deficiencies in technique, as contamination problems were consistent across replications and other species tested on the same day were not contaminated. Black nightshade was tested three times, but all samples were contaminated in two replications. Prostrate pigweed had 10 of 19 sites contaminated, and red clover had 10 of 11 sites contaminated. Other complications were low infectivity (poor acquisition of *Xf*) of blue-green sharpshooters used in experiments from October 2002 to February 2003. Only 30% (46 of 152 groups) were infective compared to 94% (119 of 126 groups) from April to July 2003. This was not the case the previous winter (November 2001 to January 2002), where 122 of 128 (95%) of sharpshooter groups transmitted *Xf*. Probable causes for the drop were the condition of the Pierce's disease-infected plants from which the sharpshooters acquired *Xf*, or reduced feeding by sharpshooters.

Five of the 6 plant species that had been inoculated both with blue-green sharpshooters and mechanically had a greater percentage of sites became infected with *Xf* when the plants were insect-inoculated. Poison hemlock was the sole exception (Table 1). Most notably, annual morning glory had 93% (28 of 30 stems) become infected with *Xf* when fed on by infective insects, but 0 of 20 when mechanically inoculated. It is unclear why this is the case; however, it is not due to the disruption of *Xf* infection by excretion of protective latex as results for prickly lettuce show that *Xf* can be mechanically introduced into plants that produce latex (12 of 53 sites infected).

Four other plants had varying results when mechanically inoculated as compared to infected with glassy-winged sharpshooters (GWSS). Cheeseweed developed infections in 29 of 57 (51%) needle-inoculated sites but only in 1 of 12 (8%) insect-inoculations. Similar results were seen for sacred datura (needle inoculation: 43 of 56, compared with 1 of 7 for GW), and red gum (needle inoculation: 12 of 33; 0 of 5 for GW). In previous tests, 30% of GWSS groups transmitted bacteria to seedling grapes, compared to 96% of blue-green sharpshooter groups. It appeared that GWSS were even less efficient at transmitting *Xf* to alternate hosts than they were to grapes.

Table 1. Comparison of insect (BG: blue-green sharpshooter) and mechanical (NI: needle) inoculation.

Plant	Inoc.	**Sites Infected**	% Sites w/ *Xf*	Median [*Xf*]	Systemic Infections	% Sys w/ *Xf*	Systemic [*Xf*]
jojoba	BG	5/41	12	log 4	1/5	20	log 4
jojoba	NI	3/74	4	log 6	2/3	67	log 6
prickly lettuce	BG	35/56	63	log 6	8/33	24	log 3
prickly lettuce	NI	13/54	24	log 6	7/13	54	log 6
cocklebur	BG	54/71	76	log 5	33/54	61	log 4
cocklebur	NI	11/32	34	log 4	7/11	64	log 6
ann. bur-sage	BG	21/27	78	log 6	17/21	81	log 6
ann. bur-sage	NI	10/19	53	log 5	9/10	90	log 4
m. glory	BG	36/43	84	log 5	19/36	84	log 4
m. glory	NI	12/51	24	log 5	7/17	42	log 4
hemlock	BG	14/22	64	log 6	11/14	79	log 6
hemlock	NI	10/15	67	log 4	5/10	50	log 5
quinoa	BG	12/24	50	log 5	2/12	17	log 4
quinoa	NI	13/49	27	log 3	6/13	46	log 4

Sites infected are the *Xylella fastidiosa (Xf)*-infected sites over total number inoculated. Median *Xf* population is in $\log_{10}$ colony-forming-units per gram of plant material (cfu/g) as determined by culture. Systemic infections are *Xf* infections distal to the inoculation site, over number of sites infected, showing the tendency for *Xf* to colonize the plant. Systemic [*Xf*] is the population of *Xf* at distal to the inoculation site in $\log_{10}$ cfu/g.

CONCLUSIONS

For a plant to be a good source from which sharpshooters can acquire *Xf*, the plant must be an attractive food host to sharpshooters, capable of being inoculated with *Xf*, allow the spread of the bacteria beyond the inoculation site, and support population growth above well 10,000 cfu of *Xf* per gram of plant tissue, the threshold for bacterial acquisition by sharpshooters when feeding on a host plant. While most of the plants examined supported some degree of bacterial growth, few have all the traits that are needed to play a role in the propagation of Pierce's disease by serving as inoculum sources for large numbers of sharpshooters.

Although insect survival varied considerably between replications, it averaged 77% on all plants for two days, confined to a small foam-and-mesh cage. All plants studied had insect survival above 60% for all insect-inoculated replications except for southwestern cupgrass, marestail, purslane, sacred datura, tree tobacco and blue gum. Even in this artificial situation, sharpshooters fed on a wide range of plants and potentially exposed them to *Xylella*.

Ten of the 35 weed species examined were inoculated more than 50% of the time in greenhouse studies. Plants were maintained in ideal conditions for bacterial growth in the greenhouse following the two-day inoculation access period with 2 to 4 insects per inoculation site. When similarly inoculated plants were placed outside for the nine-week evaluation period in the previous years' field study, many fewer *Xf* infections survived. As noted in the introduction, 20% fewer infections developed field-grown plants in winter, and 14% less in the summer. Thus it is unlikely that the plant species that had low inoculation efficiencies in the greenhouse will develop infections in the field.

Study results will assist researchers by identifying the biology of *Xf* in various hosts, while grape growers may use this information to determine which types of vegetation are potential inoculum sources, and when to eliminate them. Sixteen species supported *Xylella* populations above 1,000,000 cfu/g, thirteen had populations between 1,000,000 and 10,000 cfu/g, and three had populations below 10,000 cfu/g. Since acquisition efficiency by sharpshooters increases with the bacterial population in the host plant (Hill 1997), plants with bacterial populations below 1,000,000 cfu/g are unlikely to be major inoculum sources. Finally, the bacteria must move beyond the inoculation site throughout the plant to colonize it. The plants that had all the traits; greater than 60% insect survival during inoculation, were infected greater than 50% of the time in greenhouse tests, supported bacterial populations of 1,000,000 cfu/g or larger, and had systemic *Xf* movement beyond the inoculation site at more than 50% of plants where *Xylella* was recovered at the inoculation site, were black nightshade, common sunflower, annual bur-sage, morning glory, poison hemlock and fava bean.

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CHARACTERIZATION AND STUDIES ON THE FUNDAMENTAL MECHANISMS OF *XYLELLA FASTIDIOSA* TRANSMISSION TO GRAPEVINES BY THE GLASSY-WINGED SHARPSHOOTER

Project Leader:
Alexander H. Purcell
Division of Insect Biology
University of California
Berkeley, CA 94720-3112

Researcher:
Rodrigo P.P. Almeida
Department of Plant and Environmental Protection Sciences
University of Hawaii at Manoa
Honolulu, HI 96822

Cooperators:
Elaine Backus
USDA-ARS
San Joaquin Valley Agric. Sci. Ctr.
Parlier, CA 93648

Jennifer Hashim
UC Cooperative Extension
Bakersfield, CA 93307

Barry Hill
CDFA, Plant Pest Diagnostics Branch
Sacramento, CA 95832-1448

Reporting Period: The results reported here are from work conducted from October 2002 to October 2003.

ABSTRACT
The transmission of *Xf* to dormant grape by the glassy-winged sharpshooter (GWSS) was confirmed for a second year in outdoor cage trials in Bakersfield in January 2003, but at lower rates than in February 2002. Attempts to transmit *Xf* with GWSS from dormant grape and almond did not result in transmission. Sharpshooters fed on suspensions of *Xf* in xylem sap acquired *Xf* as determined by culture assays, but all failed to transmit *Xf* from suspensions to grape, suggesting that vector transmission requires a plant factor or environmental conditions not present in cultured cells.

INTRODUCTION
In attempts to reduce the economic impact of the glassy-winged sharpshooter (GWSS, *Homalodisca coagulata*), it is essential to better understand and evaluate GWSS's transmission of the pathogen *Xylella fastidiosa* (*Xf*). *Xf* has been causing diseases in California for a long time, but GWSS is apparently a more effective vector than other sharpshooters previously present in California in the field spread of Pierce's disease. We previously documented that GWSS transmits *Xf* with similar characteristics as other vectors (Purcell 2002). There is no or a very short (minutes) latent period; insects acquire and inoculate *Xf* with only 1 h of plant access period, although efficiency increases with larger access periods. Nymphs were as or more efficient vectors than adults, both for pathogen acquisition and inoculation, but lost infectivity after molting (Almeida and Purcell 2003). Overall, GWSS was at least 50% to 75% less efficient as a vector of *Xf* compared to the blue-green sharpshooter (BGSS) (Purcell and Finlay 1979). One difference in the feeding behavior or GWSS compared to traditional California vectors such as BGSS was that adults transmitted *Xf* with similar efficiency to green tissues as to 2-year old wood of grapevines. Similar to previous results for BGSS (Hill and Purcell 1995), we found no correlation between the amount of the pathogen detection in the head of GWSS by culture and its transmission to plants. Our objectives for the past year were to further characterize *Xf* transmission by GWSS to grapevines and to develop methods by which GWSS could transmit *Xf* from artificial diets so as to be able to better control and experimentally manipulate bacterial acquisition. The final (#3) objective of the project was addressed without success the previous year; no bacterial isolates from GWSS proved to be taken up by GWSS from surface sprays of the bacterial isolates.

OBJECTIVES
1. Characterize the transmission of *Xf* to grapes by GWSS.
2. Develop *in vitro* assays to assess vector transmission of *Xf*.
3. Test the possibility of biological control of *Xf* transmission through competition for attachment site in vector's foregut.

RESULTS
GWSS transmission of *Xf* to dormant grapevines
In a repeat of experiments conducted in an outdoor cage in Bakersfield in 2002, we confirmed that GWSS can transmit *Xf* to dormant grapevines, although rates were much lower than in 2002. We first confined GWSS on PD-grape source plants (strain STL) for 4 days in the greenhouse, then we caged groups of 4 adult GWSS per plant on grape seedlings. We transported 15 of these groups (60 insects total) on grape test plants and transferred one group per vine to a small mesh sleeve cage on field-grown Pinot Noir vines planted in an outdoor cage in Bakersfield, CA. We inoculated 15 field vines each on January 23 and 30, 2003 and removed the insects after one week. We diagnosed all plants after more than 3 months for PD symptoms and for *Xf* by culture assays (Hill and Purcell 1995) and/or by ELISA (Minsavage et al. 1994) in the CDFA Diagnostics Lab in Sacramento, using leaf petioles for diagnoses. Transmission rates in the greenhouse trials were 3 of 15 for January 19-23 inoculations and 4 of 15 for Jan. 26-30 inoculations in the lab on green vines. Only one of the 30 total groups of 4 GWSS transmitted to dormant grape in the field; this same group had also transmitted to a green grape test plant on which the insects were caged before exposure to field test plants. This rate of transmission (3%) was much lower than the 20-30% transmission achieved in identical experiments in February 2002. As we noted in our lab experiments over the previous three years, GWSS transmission to grape is quite variable among experiments (Almeida and Purcell 2003).

Electronic monitoring of sharpshooter probing behavior
This work was conducted together with Dr. Elaine Backus (USDA-ARS, Parlier CA). We used the blue-green sharpshooter (BGSS, *Graphocephala atropunctata*) as our model insect because it is more efficient than the GWSS in transmitting *Xf*. Our results were presented last year (Purcell 2002) and have been submitted for publication. Information from this work establishes benchmarks for future research addressing the mechanisms of *Xf* transmission and sharpshooter ecology.

Develop *in vitro* assays to assess vector transmission of *Xf*
Results have been already discussed in a previous report. In summary, we have demonstrated that sharpshooters can acquire planktonic *Xf* suspended in filter-sterilized grape xylem sap kept in sachets of thin flexible membranes (Parafilm M^R). But these insects did not transmit the pathogen to plants afterwards. During Spring 2003, we used BGSS that had been lab-reared on basil with frequent changes to eliminate any *Xf* attached to the foreguts of the insects. These BGSS were screened on pre-test grape plants to test for infective BGSS before using them in acquisition-feeding experiments. We suspended $\log_{10}6$ cells/ml in filter-sterilized grape sap and fed about 20 µl of suspension through a thinly stretched Parafilm^R membrane to individual BGSS adults. After a 6-8 hour access to feeding on *Xf* suspensions between membranes, we transferred the insects to grape test plants for 4 days, after which we cultured from the heads and bodies of the surviving insects. Unfortunately, half of the pre-test plants became infective, indicating that probably about 20% of the BGSS used were infective before the experiment. Of the BGSS that did not transmit to a pre-test plant, only one of over 30 BGSS transmitted after feeding on sachets of bacterial suspensions, indicating that either the insect(s) picked up *Xf* from the cell suspension or (more probably) that it was already infective but did not transmit to the pre-test plant. We recovered *Xf* from all of the BGSS we cultured except for those where contaminating bacteria made detection of *Xf* impossible. The results suggested that cultured *Xf* cells are not genetically activated in sterile xylem sap to attach to vector foreguts from which they can later be transmitted, or that plant factors not present in cultured cells are required for attachment to the vector.

Biological control of *Xf* transmission through competition for attachment site in vector's foregut
Our results on these experiments have been previously reported (Purcell 2002).

Microscopy of sharpshooter foreguts
We examined BGSS adults foreguts for *Xf* after caging them on PD-vines for 4 days, followed by at least 14 days on grape test plants. Of 19 such BGSS that were adequately dissected for SEM, we found "carpet-like" mats of *Xf* attached to all 10 BGSS examined in scanning electron microscopy that transmitted *Xf* to grape (Figure 1). We did not observe attached *Xf* in any of the 9 insects that did not transmit to grape. Not many cells of *Xf* are required for efficient transmission of *Xf*, but evidently heavy biofilms of *Xf* in the foregut also permit efficient transmission. Our previous reports (Purcell 2002) described similar findings of *Xf* biofilms in the foreguts (precibarium) of GWSS (Brlansky et al. 1983). The general distribution of *Xf* in the foregut of BGSS (Figure 1) in this transmission experiment was similar to that we described earlier (Purcell 2002).

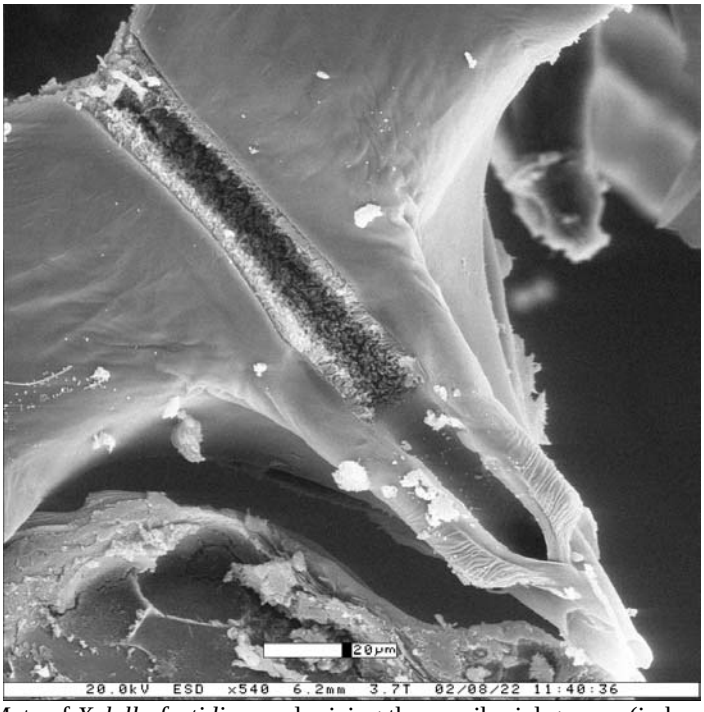

Figure 1. Mats of *Xylella fastidiosa* colonizing the precibarial groove (in hypopharynx) of blue-green sharpshooter 14 days after start of acquisition period on grape with Pierce's disease. Sucking pump chamber is at upper left.

CONCLUSIONS

We confirmed for a second year that GWSS can transmit *Xf* to dormant grapevines in the field. This implies that it is important to minimize GWSS populations feeding on vineyards in winter months as well as the growing season. Our efforts to develop a system to deliver planktonic *Xf* cells to insects were successful, but the ingested cells were not transmitted to plants afterwards, suggesting that either *Xf* responds to environmental signals to activate genes necessary for attachment or that the bacterium requires plant-derived factors for transmission.

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GLASSY-WINGED SHARPSHOOTER TRANSMISSION OF *XYLELLA FASTIDIOSA* TO ALMOND

Project Leader:
Alexander H. Purcell
Division of Insect Biology
University of California
Berkeley, CA 94720-3112

Researcher:
Rodrigo P.P. Almeida
Department of Plant and Environmental Protection Sciences
University of Hawaii at Manoa
Honolulu, HI 96822

Reporting Period: The results reported here are from work conducted from October 2002 to October 2003.

ABSTRACT
The acquisition of *Xylella fastidiosa* (*Xf*) from dormant almond by the glassy-winged sharpshooter (GWSS) was attempted in January 2003 near Bakersfield with 16 groups of 4 adult GWSS per group caged on branches of almond trees with ALS symptoms and confirmed by culturing as having *Xf* infections. None of the surviving GWSS transmitted *Xf* to 16 Non-pareil almond test plants. Culture assays for *Xf* in naturally-infected field almonds and greenhouse plants documented densities of about 1,000,000 to 10, 000,000 live *Xf* per gram of leaf vein. These are 10 to 100 times lower than in grapes with PD symptoms and may explain in part why vector acquisition of *Xf* from almond is lower than from grape. In other experiments, we found that previously reported, genetically-determined groupings of *Xf* strains from almond (Groups I and II) and grape or almond (typed as "Grape" strains) had distinctive biological characteristics that corresponded to the genetic groupings. All strains mechanically inoculated into grape and almond caused almond leaf scorch (ALS), but only grape strains caused Pierce's disease. Almond Group I strains did not grow on PD3 medium, but Almond Group II strains and Grape strains did. Almond strains of *Xf* survived winter dormancy in almond at Berkeley better than did grape strains, but the reverse was true in grape.

INTRODUCTION
One of the unknown but feared impacts of the glassy-winged sharpshooter (GWSS, *Homalodisca coagulata*) in the San Joaquin Valley of California is its role in the spread of almond leaf scorch disease (ALS). GWSS includes numerous species of trees among its favored plant hosts (Turner and Pollard 1959a) and is considered to be one of the most important vectors for the phony peach disease (Turner and Pollard 1959b), so it should be expected to feed on almond. We previously reported that GWSS transmits *Xf* to almond less efficiently than to grape, with insect numbers per plant and days of exposure approximately equal in influence on transmission probability (Purcell 2002; Purcell and Almeida 2002). We also found that GWSS transmitted *Xf* to dormant almond trees in greenhouse experiments. Our objectives for the past year were to determine if GWSS could acquire *Xf* from dormant almond in the field, what populations of *Xf* occurred in ALS-symptomatic plants in the field, and to determine if both grape and almond strains colonized and caused disease in both almond and grape. Some of the results re-summarized here were recently published (Almeida and Purcell 2003b).

OBJECTIVES
1. Determine the efficiencies of acquisition and inoculation of *Xf* by the GWSS to almonds.
2. Quantify populations of *Xf* in infected almonds in the field throughout a season.
3. Determine the ability of the GWSS to inoculate *Xf* to mature (> 1 year) and dormant woody tissues of almond.

RESULTS
GWSS transmission of Xf to almond
Objectives 1 and 3 were completed and the results reported last year (Purcell 2002) and in a publication (Almeida and Purcell 2003b). To briefly summarize: GWSS acquired and inoculated *Xf* from/to almonds with an acquisition efficiency of ~7% per insect per day, and inoculation of 4% per insect per day. This is less than half of GWSS transmission rates to grape (Almeida and Purcell 2003a). GWSS transmitted *Xf* to 1-year old woody tissue of almonds with equal efficiency as to green tissue, and survived about the same on both types of plant tissues. Because of concerns regarding transmission of *Xf* from grape to almonds, we did a transmission experiment using two grape strains (one from Napa Valley, another from Bakersfield), and found that both were transmitted to both grape and almonds. Napa Valley strains were slightly more virulent (more severe symptoms) in grape than were Bakersfield strains.

We tested the ability of GWSS to acquire *Xf* from dormant almonds in the field. In experiments in a commercial 'Non-pareil' almond orchard north of Bakersfield, we caged lab-reared, non-infective GWSS adults. The insects first were tested for four days on grape seedlings to insure that they were *Xf*-free and then transferred in the field to almond branches that had previously tested as positive for ALS symptoms and *Xf* populations by culturing. After one week on dormant almonds we transferred the 16 surviving GWSS (of the initial 64 used) to almond test plants (Non-pareil on Lovell rootstock) in the greenhouse. Almost all of these GWSS survived in the lab, but there was no transmission of *Xf* to almond, as determined by culturing assays (Hill and Purcell 1995).

Populations of Xf in infected almonds in the field and greenhouse
Xf bacterial populations within plants have been correlated with vector transmission efficiency (Hill and Purcell 1997). Following that assumption, we quantified *Xf* from infected almonds in the field growing in 3 different areas of California and

in the laboratory. Details about our experiments have been published (Almeida and Purcell 2003b). We found that bacterial populations were 10- to 100-fold lower in almond than in grapes. We also surveyed bacterial populations in an infected orchard at UC Davis over 4 years (1997-99, and 2001-02). Bacterial populations were low in April, reached 10^6 CFU/g of tissue in June and 10^7 CFU/g in September (Almeida and Purcell 2003b, Almeida and Purcell in press). These low populations suggest that GWSS transmission of *Xf* to almonds during spring and early summer is most likely to occur with infective insects migrating from grape or other plants. During late summer, when bacterial populations increase, transmission from infected to healthy almonds might become important within orchards, but the overwinter survival of *Xf* infections established in August is unknown.

A significant finding from our field inoculations of almond at Davis and Parlier that is important for growers is that first year infections (needle inoculation) are almost certain not to be noticed in visual surveys for ALS symptoms. Further, about half of infections established in early spring die out during the subsequent winter, with even further substantial die-out of infections during the second winter (Almeida and Purcell 2003 in press). Moreover, ALS symptoms from two-year-old infections were not pronounced and would likely be overlooked the second year as well. This means that growers cannot reliably assess the effects of vector control for ALS control on the same or following year's ALS incidence based on ALS symptoms. Extensive ALS symptoms that are noticed for the first time probably result from infections established at least 3 or more years before. In general, ALS symptoms (and presumably *Xf*) spread within trees more rapidly in the more susceptible varieties (e.g., Peerless, Non-pareil) than in less susceptible varieties (e. g., Mission, Carmel), as well as occurring at a higher incidence in the more susceptible varieties (unpublished data).

Biological characteristics of grape and almond Xf strains

PD and ALS have long been considered to be caused by the same strain of *Xf*, but recent genetic studies revealed differences among *Xf* isolated from these host plants (Hendson et al. 2001). We tested the hypothesis that ALS is caused by PD and ALS strains in the field, and found that both groups of *Xf* caused ALS and over wintered within almonds after mechanical inoculation. Under greenhouse conditions, all isolates caused ALS, and all grape isolates caused PD. However, isolates belonging to almond genetic groupings did not cause PD in inoculated grape but systemically infected grape with lower frequency and much lower populations than grape strains. Isolates able to cause both PD and ALS developed 10-fold higher concentrations of *Xf* in grape than in almond. In the laboratory, grape isolates over wintered with higher efficiency in grapes than in almonds; almond isolates over wintered significantly better in almonds than in grapes (results reported in Almeida and Purcell in press).

Based on our *Xf* collection, we never recovered Almond strain isolates from PD grapevines, but have recovered Grape strain isolates from infected almonds. We assigned almond strains into groups I and II based on their genetic characteristics, growth on PD3 solid medium (Davis et al. 1981), and bacterial populations within inoculated grapevines. Our results show that genetically distinct grape and almond strains differ in population behavior and pathogenicity in grape and ability to grow on two different media. Details about the genetic grouping of the various *Xf* strains in California are reported in Hendson et al. (2001).

CONCLUSIONS

In summary, we found that GWSS is a less efficient vector of *Xf* to or from almond in field and lab tests than vectors such as the blue-green sharpshooter and less efficient than in transmitting to grape. GWSS did not acquire *Xf* from or survive well on dormant almond in the field, but transmitted *Xf* to dormant almond in lab tests. This does not mean that GWSS is not a threat for spreading ALS where populations of *Xf*-infective GWSS enter almond orchards. Other factors such as GWSS population levels, infectivity rates, and residence times in orchards, and plant-to-plant movement rates can be as important as transmission efficiency.

The populations of *Xf* in almond were generally 10 to 100-fold less than in comparable weights of leaf veins of PD-grape. This may explain in part why vector transmission of *Xf* from almond is lower than from grape. Growers should be aware that ALS symptoms progress slowly from a single point of infection the same year of infection and most are not noticeable even the second year of infection. Thus most ALS foliar symptoms will not be noticed until the third year or later after infection.

Our finding that grape and almond strain groupings based on genetic characteristics are biologically distinct explains at least in part why PD does not occur near hotspots of ALS. However, our findings cannot explain the reverse situation: "Why doesn't ALS occur near PD hot spots?" The lower rates of overwinter survival of grape strains in almond can only partly explain this anomaly.

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

Project Leader:

Frank Wong
Department of Plant Pathology
University of California
Riverside, CA 92521

Donald A. Cooksey
Department of Plant Pathology
University of California
Riverside, CA 92521

Heather S. Costa
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:

James Downer
UC Cooperative Extension
Ventura County

Mike Henry
UC Cooperative Extension
Riverside County

John Kabashima
UC Cooperative Extension
Orange County

John Karlik
UC Cooperative Extension
Kern County

Michelle LeStrange
UC Cooperative Extension
Tulare County

David Shaw
UC Cooperative Extension
San Diego County

Reporting Period: The progress documented by this report reflects all work initiated July 1, 2003 and completed by October 15, 2003.

ABSTRACT

The xylem-limited bacterial pathogen *Xylella fastidiosa* causes a number of diseases in a wide range of hosts including Pierce's disease of grapevine and leaf scorch of oleander. Recently, the presence of the bacteria has been documented in a number of landscape ornamentals in southern California have showing symptoms typical of those caused by the pathogen. Plants such as sweet gum (liquidambar), olive and ornamental plum have been identified as susceptible hosts of the pathogen. During surveys conducted in the summer of 2003, over 500 samples from plants showing symptoms typical of *X. fastidiosa* infection from five cities in southern California were tested for the presence of the pathogen. Seventy-eight host species were represented in the samplings. Plants from 26 of the species represented tested positive for the presence of the pathogen by ELISA. Current work is focusing on the confirmation of the presence of the pathogen by PCR amplification and the collection of bacterial isolates from these suspected hosts for both genetic and host range characterization.

INTRODUCTION

Xylella fastidiosa is a bacterial plant pathogen that causes a variety of diseases in a broad range of plant hosts including Pierce's disease of grapevines, almond leaf scorch, alfalfa dwarf, citrus variegated chlorosis, leaf scorch of live oak, pear leaf scorch, and oleander leaf scorch (Hopkins 1989; Hartung *et al*. 1994; Purcell and Hopkins, 1996; Purcell *et al*., 1999). Multiple strains of *X. fastidiosa* with different host ranges have been identified (Chen et al 1992, da Costa *et al*. 2000, Henderson *et al*. 2001), but little is known about the diversity of these populations in the urban landscape and their ability to cause loss in both plants of horticultural and agronomic importance.

Until recently, losses of ornamental plants resulting from *X. fastidiosa*-induced diseases were primarily limited to oleander. Since the symptoms of *X. fastidiosa* often mimic those caused by other pathogens or abiotic stresses, problems caused by this bacterium may have been frequently misdiagnosed in the urban environment. However, during recent surveys of potential alternate host plants of PD in southern California; liquidambar, olive, and ornamental plum were found to have symptoms of leaf scorch and tested positive for *X. fastidiosa* using ELISA and PCR analysis of plant tissue. None of these species have been reported as hosts of this pathogen before. In some areas, scorch symptoms were very common on these plants, i.e. in olive plantings in Riverside County, up to 33% of the trees exhibited symptoms. *X. fastidiosa* was isolated from each of these species on specialized media for genetic characterization. Preliminary studies of *X. fastidiosa* isolates from these hosts indicate that the strain(s) present differ from the PD and OLS strains found in grape and oleander respectively (D. Cooksey, *unpublished data*), however, their exact relationship to other previously identified strains, and the host range of these isolates, remains unknown. In order to develop strategies to manage this pathogen, it is essential to determine the potential of these strains to infect other host plants, particularly agricultural crops such as grape, almond, olive and citrus.

The broad host range of this pathogen, and its potential threat to California landscapes, agricultural and forestry crops makes it critical to document and characterize the strains of the pathogen that are present throughout California, and determine the plant host range of each of these strains. The result of this project will provide knowledge base of crop hosts that may be at risk of infection with new strains and will provide a database of ornamental species that can serve as inoculum sources of *X. fastidiosa.* This information is necessary to develop management practices that target the removal of infected plants and sources of inoculum near susceptible crops.

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 AND CONCLUSIONS

Objective 1

Over 500 samples from landscape plants showing symptoms of wilt, dieback or scorch were taken from five cities in distinct locations in southern California. Sampling locations consisted of areas several city blocks in size, from which both suspected host species and plants symptomatic of disease were taken (Table 1).

Originally, only 24 species of possible host plants were to be examined, but it was decided after an initial survey of plants showing symptoms typical of *X. fastidiosa* infection that all plants showing typical symptoms would be sampled. Thus, over 78 distinct landscape ornamental species were sampled in the survey.

A large number of plant samples indicated the presence of *X. fastidiosa* based upon ELISA results only (Table 2). Plants from 26 of the 78 species sampled tested positive. Within a tested species, the number of individual samples testing positive was variable, which was not unexpected. For example, 16 of 23 samples from *Agapanthus africanus* tested positive, while only 3 of 10 samples of *Jacaranda mimosifolia* tested positive. At this time, PCR confirmation using the RST31/RST33 primer pair (Minsavage *et al*. 1994, Pooler *et al*. 1997) for general amplification of *X. fastidiosa* has only been successfully completed for a small subset of these samples. Likewise, in only a few cases has the bacteria been successfully cultured on PD3 or PW agar.

Table 1. ***Origin of samples collected as of October 15, 2003.***

City	County	Samples	Species Represented[a]
Filmore	Ventura	105	45
San Diego	San Diego	102	32
Redlands	San Bernardino	117	37
Riverside	Riverside	97	40
Tustin	Orange	118	29
Total		*539*	*78*

[a] Actual number of plant species sampled per location. Some species sampled in multiple locations, depending of the presence of symptomatic specimens in the sampling area.

Table 2. Partial listing of plant species confirmed or likely to be hosts of *X. fastidiosa* in southern California urban landscapes.

Species	Common Name	ELISA[a]	PCR[b]	Culture[c]
Agapanthus africanus	Lily of the Nile	+	---	---
Alibiza julibrissin	Silk Tree	+	---	---
Cinnamonum camphora	Camphor Tree	+	---	---
Gingko biloba	Gingko	+	---	+
Impatiens spp.	Impatiens	+	---	---
Jacaranda mimosifolia	Jacaranda	+	---	---
Justica spicigera	Mexican Honeysuckle	+	---	---
Liquidambar styraciflua	Sweet Gum	+	+	---
Morus spp.	Mulberry	+	+	---
Nerium oleander	Oleander	+	+	+
Olea europa	Olive	+	+	---
Photina fraseri	Frasier Photina	+	+	---
Prunus dulcis	Almond	+	+	+

(+) indicates a positive test result, while (---) indicates a negative or inconclusive result.

[a] Positive reactions using a commercially available ELISA kit (Agdia)

[b] Amplification of product using RST31/RST33 primer set

[c] Isolation of *X. fastidiosa* from plant extracts on PD3 or PW media

Objective 2

So far, isolates available for characterization include those from Gingko, Oleander, and Sweet Plum. No additional work will be performed until a larger number of isolates becomes available.

The current focus of the work is to continue to collect and identify hosts of *X. fastidiosa* in landscape hosts, confirm the presence of the pathogen using the RTS31/RST33 PCR primers and obtain isolates of the bacterium from these hosts.

Objectives 3, 4 and 5
To be completed upon the successful collection of additional *X. fastidiosa* isolates.

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FUNDING AGENCIES

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Section 4A: Bacteria-Insect and Bacteria-Plant Interactions

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

Project Leader:
Elaine Backus
USDA-ARS-PWA
Exotic & Invasive Dis. & Pests
Parlier, CA 93648

Personnel:
Fengming Yan
College of Life Sciences
Peking University
Beijing, China

Javad Habibi
Department of Entomology
University of Missouri
Columbia, MO 65211

William Bennett
Private consultant
744 Elktown Rd.
Otterville,MO 65348

Cooperators:
Matthew Blua
Department of Entomology
University of California
Riverside, CA 92521

Alexander Purcell
Division of Insect Biology
University of California
Berkeley, CA 92521

Edwin Civerolo
USDA-ARS-PWA
Exotic & Invasive Diseases & Pests
Parlier, CA 93648

Reporting Period: The results reported here are from work conducted from November 1, 2002 to September 30, 2003.

ABSTRACT

Backus is studying the stylet penetration (probing) behaviors of the glassy-winged sharpshooter (GWSS), and how they interact with populations of *Xylella fastidiosa* (*Xf*) to facilitate transmission to grape. Electropenetration graph (EPG) monitoring is combined with videotaping of stylet movements in artificial diet and histology of fed-upon plant tissues. For Objective 1, AC EPG waveforms have been identified, characterized, and correlated with salivary sheath formation, stylet advancement, ingestion, and sheath terminations in various cell types in grape. Results show that most probes are very long (>8 hrs), and sustained ingestion events longer than 2 min generally occur in xylem. For Objective 2, a 2 x 4 factorial test was performed, using 2 probing treatments (short probe [pathway + < 1 min ingestion] or long probe [pathway + 1 hr sustained ingestion]) and 4 bacterial detection methods (PCR, culturing, immunocytochemistry and symptoms). Results show that *Xf* inoculation can be PCR-detected from both short and long probes, and thus it occurs during pathway or the first minute of ingestion. Durations for waveforms B1, C and N were significantly different between PCR-positive and PCR-negative plants, for long probes only. This suggests that inoculation occurs when B1 is coupled with stylet tips in xylem. The bacterial detection methods compared have differential sensitivities; immunocytochemistry is the most sensitive, PCR is intermediate and culturing is the least sensitive. The more sensitive the test, the earlier and more frequently the bacteria were detected. Present results support the following inoculation hypothesis. *Xf* bacteria exit the stylets during brief stylet activities represented by the B1 waveform, probably within seconds of the first puncture of a penetrated cell, either along the path or in the xylem. Proper placement of the bacteria appears to be crucial; placement in xylem leads to growth of the bacteria sufficient for detection by less sensitive methods. Further analysis will test this hypothesis, and also associate this behavior with appearance of bacteria in the head. This research provides crucial baseline data for future development of a Stylet Penetration Index for inoculation behavior.

INTRODUCTION

Almost nothing was known, until this work, about the stylet penetration (probing) behaviors of the glassy-winged sharpshooter (GWSS), and how they interact with populations of *Xylella fastidiosa* (*Xf*) to facilitate transmission to grapevine. This project is combining the three most successful methods of studying leafhopper feeding (i.e. histology of fed-upon plant tissues, videotaping of feeding on transparent diets, and electrical penetration graph [EPG] monitoring) to identify most details of feeding. This research will provide crucial baseline data for the present projects of collaborators, as well as for the future development of a Stylet Penetration Index for PD inoculation behavior to be used to screen differences among grape varieties and other uses.

OBJECTIVES

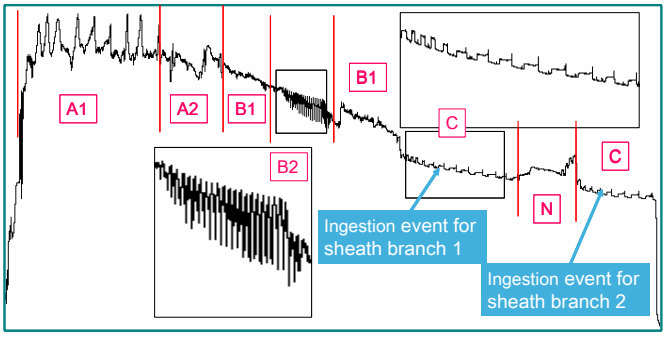
1. Identify and quantify all feeding behaviors of GWSS on grapevine, and correlate them with location of mouthparts (stylets) in the plant and presence/ population size of *Xf* in the foregut.
2. Identify the role of specific stylet activities in *Xf* transmission, including both the mechanisms of acquisition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

RESULTS

The first 5 months of this year were spent moving the entire project from the University of Missouri to Backus's new employer, the USDA-ARS in Parlier, California. Yan spent one month in California completing the lab work for one experiment, then moved to other employment after one year of work on this project. Habibi was able to continue his histology work.

Objective 1 - Waveform Correlations:

BGSS Study: Backus worked with Almeida (former student of Purcell) to complete and submit their publication on waveform correlations of blue-green sharpshooter (BGSS), *Graphocephala atropunctata* (Almeida & Backus, *in revision*). GWSS Experiments 1, 2 and 3: 1) Backus and Bennett completed the development of the new AC/DC monitor and used it to preliminarily record GWSS waveforms; work is continuing. 2) Habibi completed histological preparation and micrography of the 98 correlation probes discussed last year. 3) Yan completed the artificial diet videomicrography. Synthesis of all correlation data is underway, for a detailed manuscript on AC waveforms correlations (*in prep*). Current AC waveform interpretations from these results are shown in Figure 1 and Table 1.



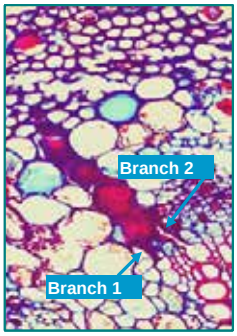


Figure 1. Waveform trace from an interrupted grape probe, with its correlated salivary sheath. The insect's stylets were pulled out after < 1 minute of the second C (ingestion) event. When stylets are removed so abruptly, the salivary sheath is left open. Thus, this event corresponds to branch no. 2 of the sheath, in xylem, and branch no. 1, in parenchyma, is from the first C event.

Objective 2 - Inoculation Behavior

GWSS Experiment 4 – EPG Waveforms. Design and Methods. We used now-standard waveform interrupting and plant techniques to correlate waveforms and salivary sheathes with inoculation of *Xylella* to healthy grapevine petioles. Eight treatments were performed, using a 2 x 4 factorial, randomized complete block design with 10 replicates. The treatment levels were *two types of probes* and *four Xf detection methods*, as follows:

Types of probes:

1) 3 EPG-monitored probes each containing pathway + < 1 minute of ingestion, or

2) 1 EPG-monitored probe containing pathway + 1 hr of ingestion (including any interruptions)

NOTE: Correlation results (e.g. Figure 1) suggest that during long durations (>7-10 min) of ingestion the stylet tips are in xylem; short durations (0.5-2 min) are often performed in other cell types.

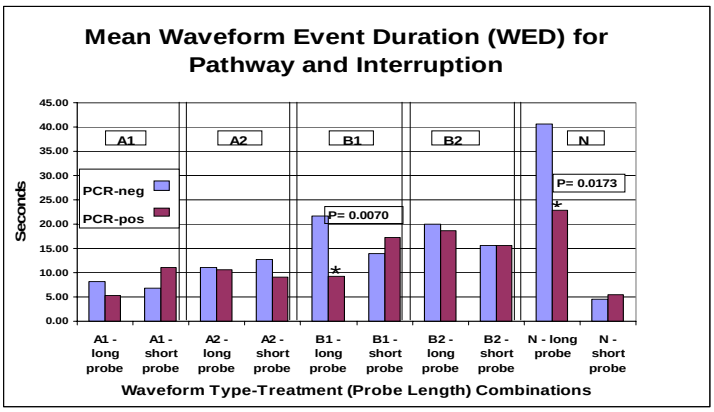
Xf detection methods: Probed plants were held in the greenhouse, then the 2-mm length of petiole immediately around the probe site was excised and tested via: 1) PCR (by Civerolo), after 6 week holding time, or 2) bacterial culturing (by Purcell), after 6 week holding time, or 3) symptoms, after 3 month holding time, or 4) immunocytochemical detection of both salivary sheaths and *Xylella* (by Habibi), after 5 d holding time.

Results to date: PCR and culture tests are complete for both probing treatments (Table 2; Figure 2). Immunocytochemistry is complete for long probes (Figure 3); work is in progress for the short probes. Symptoms proved unreliable.

Table 2: Number of grape samples containing identified GWSS probes that was positive out of the total number tested, using each of the three bacterial detection methods.

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

PCR successfully detected *Xf* amplicons inoculated by both short and long probes (Table 2). There was no significant difference (Proc CATMOD) between the probe treatments (p = 0.9807). Therefore, success of PCR recovery from identified probes was 45%. This far exceeded the recovery via culture (5.6%).



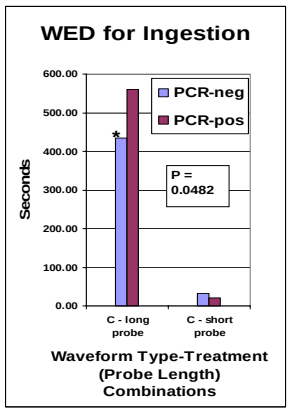
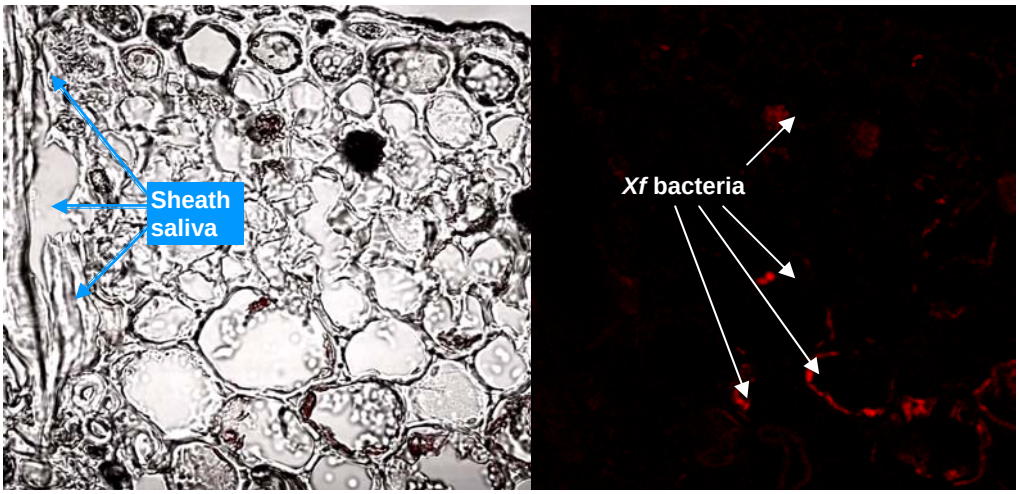


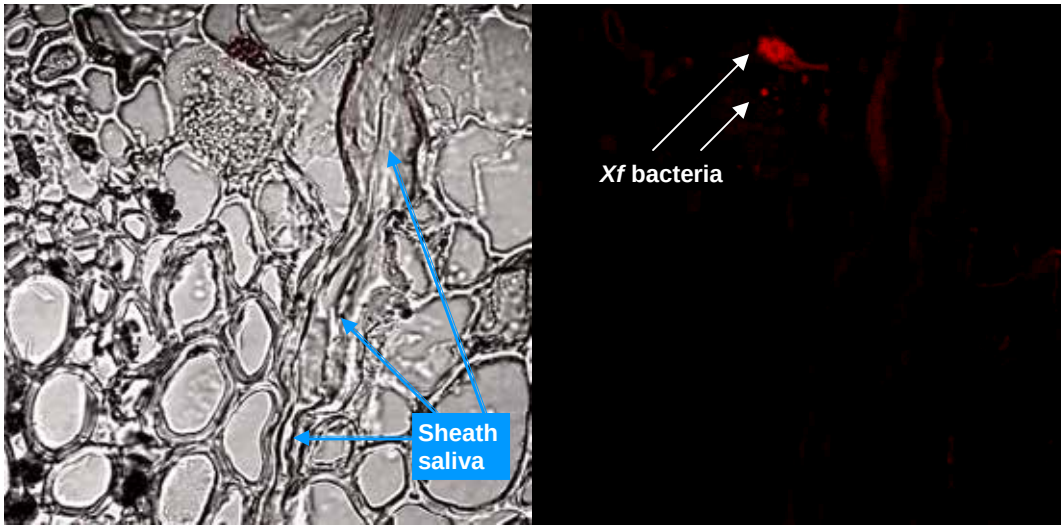
Figure. 2. Mean durations for each uninterrupted occurrence (= waveform event) of a waveform type, compared (ANOVA and LSD) between short and long probe treatments.

There were distinct differences in waveform durations among probe and bacterial detection treatments (Figure. 2). Long probes showed significant differences between PCR-positive and PCR-negative plants. Positive probes had: 1) shorter B1 pathway, 2) shorter N interruption, and 3) longer C ingestion activities. Short probes displayed no significant differences in waveform durations between positives and negatives.



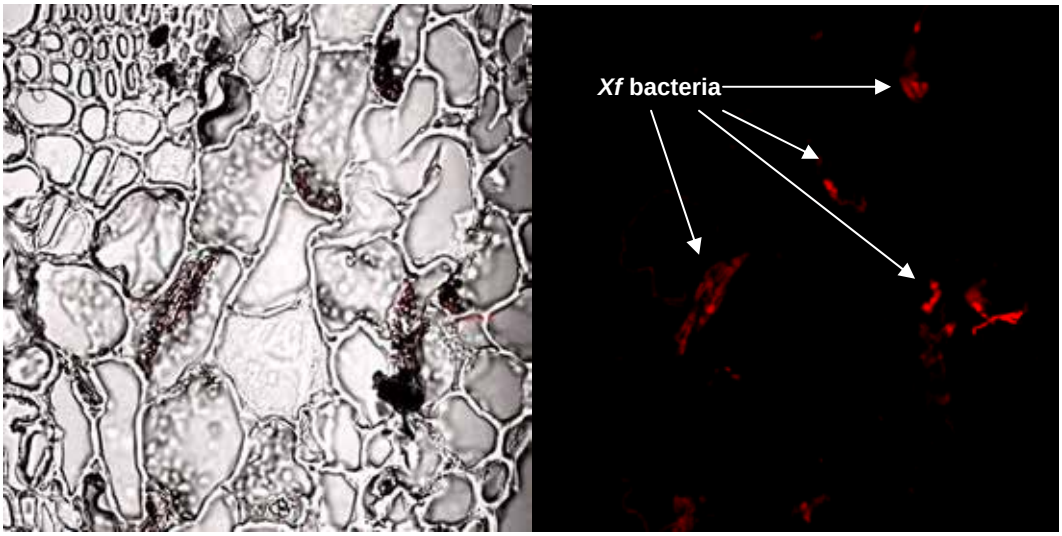
Numerous aggregations of what appear to be bacteria are visible in the transmitted light view (left). But, only a few aggregations clearly fluoresce, indicating binding with the *Xf* antibody. Size of aggregation and degree of fluores-cence is low at 5 days.

Figure 3. Confocal laser scanning micrographs of an unstained salivary sheath in grape tissue from Expt. 4, afte text). Transmitted light view (left) is overlaid with half of the pixels of the laser-excited fluorescent image (right). *Xf* aggregations are very bright (arrows, right); less bright signal is probably autofluroescence of cell walls and sheath saliva.



At 10 days after a probe, the size of the *Xf* aggregation is larger than at 5 days (Figure 3). Bacteria lie relatively close to salivary sheath.

Figure 4. Confocal laser scanning micrographs of the unstained salivary sheath in grape tissue, as in Figure 3. Tissue is from Expt. 6, after 10 days holding time (see text). *Xf* aggregations are very bright (arrows); less bright signal is probably autofluroescence.



At 40 days after the probe, *Xf* aggregations are seen much more frequently, in sections further away from the probe. Size of aggre-gations is also larger.

Figure 5. Confocal laser scanning micrograph of bacteria as in Fig.ure 4, but in a section some distance from the section with the salivary sheath (sheath not shown). Tissue is from Expt. 6 but after 40 days holding time. *Xf* aggregations are very bright (arrows); less bright signal is probably autofluroescence.

We confirm findings of Almeida and Purcell (2002) that adult GWSS can inoculate a healthy grapevine plant in less than 1 hr. However, our results improve upon theirs, which showed only a 19.6% individual inoculation rate; ours was 45% via PCR and 75% via immunocytochemistry. Thus, exact knowledge of the duration and location of a 1-hr probe, via EPG, increases the likelihood of subsequent detection. Inoculation by short probes, however, is not as easily detectable via PCR as inoculation by long probes; 3 times as many short probes had to be made into the same petiole for the same detection efficiency as 1 long probe. Also, the large difference between PCR and culturing results suggests that few insect inoculations inject sufficient quantity or quality of *Xf* for later culturing. This could be due to: 1) very few bacteria injected, 2) bacteria injected into non-xylem cells or 3) killed in the process of inoculation or 4) transported away from the site of injection, or 5) insufficient holding time for bacterial colony growth. Any combination of these is also possible.

Experiment 5 – Single Probes: To test the inoculation efficiency of single, short probes, we repeated Expt. 4 with only 1 short probe instead of 3, and used only PCR for detection. Two of the 8 probes performed (25%) were PCR-positive.

Experiment 6 – Sheath Time Course: We also tested how long a GWSS salivary sheath remains intact in a grape plant, and the location and spread of *Xf* in relation to salivary sheaths of identified probes. We repeated Expt. 4 using only immunocytochemistry to detect bacteria but varying holding times (10, 20, 40 and 80 days). We found that salivary sheaths were completely intact up to 40 days after probing; only at 80 days were the sheaths slightly dissolved and diffuse. At 10 days, fluorescently labeled *Xf* aggregations were detectable in xylem cells adjoining the salivary sheath (Figure 4), while at 20 (data not shown) and 40 days (Figure 5), *Xf* aggregations were visible in xylem cells several sections above and below, and several cell layers lateral to, the sheath. Thus, *Xf* can move laterally in the plant within 20-40 days after insect inoculation.

Interpretation of Overall Results

Xf inoculation can occur during both short and long probes. Thus, bacteria must exit the stylets during pathway or the first few seconds of ingestion (the phases common to both probe lengths). But there is a higher probability of detecting *Xf* inoculation during long probes than during short ones. The detection methods compared have differential sensitivities; immunocytochemistry is the most sensitive, PCR is intermediate and culturing is the least sensitive. Only long probes had waveform durations that were significantly different among PCR-positive and PCR-negative probes. The longer the duration of the C (ingestion) waveform (therefore the shorter the duration of N [interruption]), the greater was the likelihood that inoculated *Xf* could be detected later. We believe this is because the longer the C, the more likely the stylets were in xylem. Thus, the greatest likelihood of PCR detection of inoculation (in the vicinity of the probe) is when certain pathway activities are performed in xylem cells. Inoculating, xylem-ingestion probes have shorter durations of B1 pathway than non-xylem-ingestion probes.

CONCLUSIONS

Results to date, taken together, support the following hypothesis. *Xf* bacteria exit the stylets during brief stylet activities represented by the B1 waveform, probably within seconds of the first puncture of any penetrated cell along the path to xylem. Proper placement of the bacteria appears to be crucial; placement in xylem leads to growth of the bacteria sufficient for detection by the less sensitive methods. The more sensitive the test, the earlier the bacteria can be detected. Further analysis will test this hypothesis, and also associate waveforms with appearance of bacteria in the head.

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

- The first means of empirically tracking in real-time the entire insect inoculation process, to accurately pinpoint the timing and site of bacterial injection. Because the protocol is now standardized and repeatable, it can be used to document timing of transmission and other insect-bacteria-plant interactions, e.g. 1) time course studies to trace the movement of insect-inoculated (as opposed to mechanically inoculated) *Xf* through the plant, and 2) studies to explain Purcell's epidemiological concept of how many "bug visits" to a plant are required to develop a chronic PD infection.
- The first accurate knowledge of GWSS feeding behavior, at the real-time instant it occurs. This knowledge and protocol can be used for numerous future studies of feeding, e.g. testing the acceptability of artificial diet mixtures, or of various host and non-host plants.
- The first findings that EPG waveforms differ among inoculating and non-inoculating probes. Therefore, we now have evidence that we can develop a Stylet Penetration Index that would speed the testing of resistant varieties of grape and other host crops. Completing development of such an Index will be a major goal of future work.
- Crucial EPG waveform correlations and better protocols for the next studies on *Xf* transmission mechanisms.

Table 1. Current definitions of the AC EPG waveform phases, families and types of glassy-winged sharpshooter.

Waveform Phase	Waveform Family	Waveform Type	Waveform Characteristics	Proposed Biological Meaning	
				Plant Tissue/Cell	Insect Activity
Pathway	A	A1	Highest amplitude, hump-like waveform at beginning of probe usually w/ spikes at the top	Parenchyma or mesophyll	Major salivary sheath formation, deep extension/retraction of stylets, some watery salivation
		A2	Medium amplitude, variable slope; irregular high frequency with occasional 'trenches' and/or potential drops	Parenchyma or mesophyll	Lengthening and/or hardening of salivary sheath; some watery salivation
	B	B1	Short, single- or multi-peak 'bursts' w/in irregular, wave-like sections	Xylem, parenchyma or pith	Minor sheath additions, watery salivation, tip fluttering; internal
		B2	Extremely regular, stereotypical pattern (4~5 s), with distinct phrases	Xylem, parenchyma or pith	muscle and/or valve/pump movement?
Ingestion	C	C (to be subdivided)	Regular, low frequency with distinct phrases	Xylem, parenchyma or pith	Ingestion (watery excretory droplets correlated)
Interruption	N	N (to be subdivided)	Irregular, appears A-like, but occurs during C; ave. duration 16 sec	Xylem, parenchyma or pith	Salivary sheath extension or branching

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

Project Leader:
Blake Bextine
Department of Entomology
University of California
Riverside, 92521

Project Director:
Thomas A. Miller
Department of Entomology
University of California
Riverside, 92521

Consultant:
Frank Richards
Yale Medical School
New Haven, CT 06520

Collaborators:
Carol Lauzon
Department of Biological Sciences
California State University, Hayward
Hayward, CA 94542

David Lampe
Department of Biological Sciences
Duquesne University
Pittsburgh, PA 19219

Don Cooksey
Department of Plant Pathology
University of California,
Riverside, 92521

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ABSTRACT

Xylella fastidiosa (*Xf*), which causes Pierce's disease (PD) in grapevines, is transmitted by the glassy-winged sharpshooter (GWSS). Paratransgenesis employs symbiotic bacteria to deliver anti-*Xf* 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, as does *Xf*, and the xylem of plants. In this report, we describe the interaction of *Axd* with plants, GWSS predators, and alternate *Xf*-vectors and report preliminary data on inhibition of transmission by anti-*Xf* factors. *Axd* colonized and traveled within 6 host plants, reaching the highest titers in lemon. *Axd* colonized the foregut region of two alternate *Xf*-vectors, the blue green and smoke tree sharpshooters, and was not identified in predatory arthropods that fed on *Axd*-positive GWSS. 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 principle 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.

Paratransgenic approaches to pathogen control are currently being developed to deliver anti-pathogen strategies to disrupt Triatomid transmission of *Trypanosoma cruzi* (1), to prevent colitis in mammals (2, 7), and to interfere with transmission of HIV(4). 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 (5, 8). *Axd*, genetically marked with DsRed or EGFP protein, colonized the cibarium of GWSS for up to 35 days, the longest period tested (3).

Two categories 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 environmental endpoints of *Axd* in plants, GWSS predators, and alternate insect vectors.
2. Test the ability of anti-pathogens to disrupt *Xf* disease cycle.

RESULTS

Axd movement within and colonization of six host plants.

Plants treated with *Axd* showed no abnormalities (stunting, chlorosis, necrosis, etc.) two weeks after inoculation, despite relatively high titers in several hosts as compared to control plants. The melting temperature of the 199 bp *Axd* EGFP insert sequence is 92.1°C. Comparing PCR product melting temperature to that of the primer dimer (80.6°C), conformations were made by increasing the temperature from 72 to 99°C at a rate of 1°C/45s and measuring fluorescence every 45s, then plotting results on the melting curve. A standard curve and a regression line (R=0.983, R2=0.967, efficiency=0.73, M=0.239, B=10.362) was constructed based the amplification of known standards with plant background. Standards were tested in the presence of each host plant and no significant differences were found, therefore differences in quantification were not attributed to deleterious plant effects on PCR.

Axd was found most consistently (44 of 45) and in the highest concentrations in lemon plants in both replications (Table 1). In both replications, lemon plants were found to have greater than 3 million *Axd* cells per 2 cm of tissue, almost one order of

magnitude higher than in other hosts tested. While all inoculated sweet orange plants (25 of 25) were positive with relatively high titers (943,305 cells/2 cm) of *Axd* in the first replication, only 13 of 20 were positive in replication 2 with a lower mean titer (19,458 cells/2 cm). Grapevine, periwinkle, and crepe myrtle had lower numbers of positive plants and fewer cells per 2 cm cut. Chrysanthemum was only replicated once and 19 of 20 plants were positive with a relatively high titer of *Axd* (151,108 cells/2 cm).

Table 1. Axd populations per 2 cm stem tissue (5 cm from inoculation point) in different host plants determined by QRT-PCR (2 wks post inoculation).

Host Plant	Replication 1		Replication 2	
	Number of cells per positive plant	Positive hosts	Number of cells per positive plant	Positive hosts
Lemon	3,591,427	25/25	3,034,792	19/20
Sweet Orange	943,305	25/25	19,458	13/20
Crepe Myrtle	884,770	8/25	29,235	2/20
Periwinkle	304,820	10/25	284,164	16/20
Grape	18,225	24/25	71,982	6/20
Chrysanthemum	NA	NA	151,108	19/20

*All negative controls were negative.

Acquisition of Axd by GWSS predators

Using a plant-based artificial feeding system (AFS) (3), GWSS adults were fed *Axd* marked with a dsRed protein. After feeding from the AFS, GWSS were maintained on chrysanthemum, and fed to predators over a 2 week period. Samples GWSS from this colony all tested positive for the presence of dsRed-*Axd*. Predators tested negative for the presence of *Axd*; 3 arachnid species (n=5), 1 Mantidae species (n=5), and 1 Reduviidae species (n=5) by fluorescent microscopy.

Acquisition of Axd by alternate Xf-vectors

In replication one, blue green sharpshooters were offered dsRed *Axd* through an artificial membrane (6) and smoke tree sharpshooters were offered dsRed *Axd* through the plant-based AFS (3). The foreguts of all dsRed *Axd*-fed insects were viewed under fluorescent microscopy and were positive at 1d, 7d, and 14d post-exposure, while all control (non-dsRed Axd-fed) insects were negative. In replication two, both blue green and smoke tree sharpshooters were offered dsRed *Axd* through the plant-based AFS and tested positive by fluorescent microscopy on all dates.

Axd in California vineyards

In July of 2003, we received a permit from the EPA to test for the environmental endpoint of *Axd* when inoculated into grapevines by needle inoculation, foliar application, and soil drench. We are currently analyzing samples from these experiments, which were conducted in Riverside, Bakersfield, Temecula, and Napa. In these caged field trials, our goal is to determine if *Axd* colonized different tissues of these grapevines, especially fruit.

Interruption of PD cycle

In a preliminary experiment, 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 acquisition access period (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, respectfully), 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=X14) at 500µg/ml. These experiments are being replicated.

CONCLUSIONS

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 at this time, two other native sharpshooters also transmit the vehicle bacterium, *Axd* and several plants can serve as hosts.

Movement of *Axd* beyond grapevines is an issue that has to be addressed, not just for permitting purposes, but also to gain acceptance from the producers and consumers. Predatory arthropods feed GWSS containing high titers of *Axd* did not acquire the marked bacteria.

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.

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ROLES OF *XYLELLA FASTIDIOSA* PROTEINS IN VIRULENCE

Project Leader:
George Bruening
Department of Plant Pathology
University of California,
Davis 95616

Cooperators:
Edwin Civerolo
San Joaquin Valley Agricultural Sciences Center
Exotic and Invasive Diseases and Pest Research
USDA-ARS-PWA, Parlier, CA

Abhaya M. Dandekar
Department of Pomology
Univ. of California,
Davis, CA

Goutam Gupta
MS M888
Biology Division
Los Alamos Nat. Lab.

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ABSTRACT
This work derives from a preliminary experiment by Civerolo and Bruening in which *Chenopodium quinoa* (Cq) was found to develop a localized chlorosis 24-48 hr after leaves were infiltrated with a suspension of live or heat-killed *Xylella fastidiosa* (*Xf*) cells. Excised electrophoresis gel regions were assayed for chlorosis-inducing activity, which was associated with a protein band with mobility corresponding to an estimated mass of 40K. Mass spectrometry of material the gel band revealed peptides corresponding to about 40% of the peptides predicted for the mopB gene. *Xf* mopB is an ompA protein. Members of the ompA group are located in the outer membrane of Gram-negative bacteria. The mopB translation product, signal peptide, and mature mopB protein were identified. In the present period, we showed that fluorescent anti-mopB IgG binds to intact *Xf* cells grown in liquid culture, confirming the location of mopB on the *Xf* cell surface. Previously we found that *Xf* cells extracted with SDS under specific conditions are depleted in most non-mopB proteins. This observation was extended by solubilizing mopB from the SDS-extracted cells at a slightly elevated pH, at 30°C in the presence of SDS and sodium perchlorate. Only traces of other proteins contaminated the mopB preparation. We postulate that mopB, as the major outer membrane protein of *Xf*, may participate in an interaction between *Xf* and the xylem sites at which colonization of the plant by *Xf* is initiated. Understanding a mopB-xylem interaction could direct strategies for interfering with *Xf* infection of grape and other *Xf* hosts. We demonstrated that thin balsa wood squares are able to absorb mopB from a mixture of mopB with other proteins, in the presence of non-ionic detergents known to be effective in solubilizing integral membrane proteins. Cellulose, in the form of washed filter paper, was similarly selective. These results support, but do not prove, our hypothesis about a role for mopB in *Xf* infection, possibly acting to bind *Xf* to the xylem interior. It may be possible to exploit mopB as a target for controlling Pierce's disease.

INTRODUCTION
The bacterium, *Xylella fastidiosa* (*Xf*), is the causative agent of Pierce's disease of grape. We reported previously the observation that untreated or heat-killed suspensions of 10^6 to 10^8 *Xf* cells/ml, pressure infiltrated into leaves of *Chenopodium quinoa* (Cq), induced a chlorosis in 24-48hr that conformed to the infiltrated area. The chlorosis reaction was the basis semi-quantitative assay for potency of *Xf*-derived preparations. The chlorosis-inducing activity survived treatment with the protein-denaturing detergent sodium dodecyl sulfate (SDS). Nevertheless, proteases inactivated the chlorosis-inducing activity. Extraction of *Xf* suspensions with SDS under mild conditions (30˚C, pH approx. 8.5 buffer) removed many *Xf* proteins (analysis by gel electrophoresis, SDS-PAGE) from the insoluble fraction, but the chlorosis-inducing activity remained insoluble. Guided by stained bands in an SDS-PAGE gel, unstained gel segments were extracted, concentrated and tested by infiltration of Cq leaves. The bulk of the chlorosis-inducing activity was associated with material with mobility corresponding to molecular weight of about 40K. Analysis of tryptic digests by mass spectrometry revealed peptides derived from the *Xf* outer membrane protein mopB. The abundance of *Xf* mopB suggests that it is the major outer membrane protein of the bacterium. Although a minor contaminant of the mopB preparations could be responsible for the Cq chlorosis, most likely the chlorosis inducing agent is mopB itself. We identified the likely start of translation for the mopB gene, MKKKILT…(corresponding to a 40.7K translation product), recognized a candidate 22 amino acid residue signal peptide, and determined that the pyroglutaminyl-terminated peptide pyro-QEFDDR in tryptic digests mapped to the mopB gene sequence (Simpson, Reinach et al. 2000). Results from other experiments suggest that the pyroglutaminyl residue is the natural amino-terminal end of mature *Xf* mopB protein, predicted molecular weight 38.5, and is <u>not</u> created as an artifact of our analysis by cyclization of an amino-terminal glutamine residue.

Xf mopB was further purified by solubilizing the insoluble fraction (from 30˚C SDS extraction of *Xf* cells) with hot SDS solution and chromatographing on 6% agarose beads. Pooled fractions were concentrated and used to raise polyclonal antibody. Attempts at cloning *Xf* mopB in *E. coli*, using constructions that encompassed the entire *Xf* mopB gene, including its putative promoter, were not successful. However, an inducible bacteriophage T7 RNA polymerase and T7 promoter system was adapted to create *E. coli* cultures that, when induced with IPTG, generated, at low levels, a protein with the mobility and immunological properties of mopB (work of Paul Feldstein). *Xf* mopB accumulation may sicken *E. coli*, accounting for the low level accumulation and requiring another approach for mopB production in *E. coli*.

OBJECTIVES

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

RESULTS AND CONCLUSIONS

Xf mopB appears to be accessible on the outside of intact Xf cells

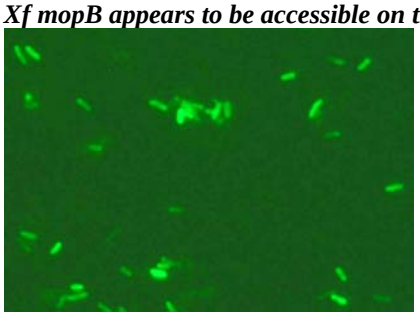


Figure 1. Fluorescent immuno-staining of *Xf* cells IgG from polyclonal antiserum against mopB and IgG prepared from the corresponding pre-immune serum each were labeled with Alexa 488 (fluorescein succinimidyl ester). *Xf* cells grown in liquid culture were exposed to the labeled IgG, diluted with SCP, washed twice with SCP, and examined in an epifluorescence microscope. Labeled *Xf* cells were observed (image at left) for the anti-mopB IgG, whereas the field for cells exposed to pre-immune IgG was dark (not shown). These results are consistent with mopB being accessible on the outside of the *Xf* cells. We have found *Xf* cells grown on agar plates (PD3 medium) to be a more suitable source for purification of mopB than cells grown in liquid PD3 culture. However, our attempts to label even very extensively washed cells from solid medium with fluorescent IgG against mopB were not successful (photomicrographs by Steve A. Wilson). It is possible that *Xf* cells grown on solid medium are encapsulated sufficiently to prevent access of the labeled IgG. We also observed that *Xf* cells from solid medium adhered readily to plastic surfaces, whereas *Xf* cells from liquid culture did not.

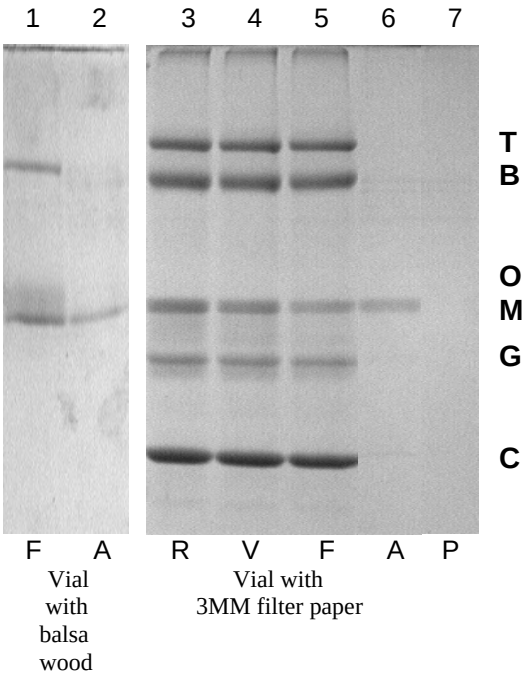
Purification of Xf mopB in soluble form

The methods we previously developed allowed us to purify *Xf* mopB in a soluble form, but only after heating, and presumably denaturing, mopB in SDS solution. Membrane-bound proteins such as mopB are notoriously difficult to coax into a soluble, purified form under mild conditions. Our starting material for trials of solubilization procedures was the insoluble fraction, enriched in mopB, that was obtained as described above by extraction of *Xf* cells with SDS at pH 8.5 and 30°C. We found, by testing a variety of conditions, that increasing the pH to 8.8 and including $NaClO_4$ in the extraction medium efficiently solubilized mopB at 30°C, whereas most of the other remaining *Xf* proteins remained in the insoluble fraction. Centrifugal filtration allowed us to remove excess SDS and $NaClO_4$, creating a very finely divided suspension that appears to dissolve in certain non-ionic detergent solutions. Analysis by SDS-PAGE suggests that the mopB is as pure as mopB recovered after solubilization in hot SDS and chromatography on 6% agaraose beads, but, of course, more likely to be biologically active (work of Jamal Buzayan).

A functional assay for Xf mopB and its implications

The Introduction, above, reports our observation of chlorosis-inducing activity of *Xf* mopB and our characterization of the mopB protein in the context of that activity. Little was revealed about functions of mopB in the context of Pierce's disease. Part of the work conducted in the past year was devoted to identifying possible mopB functions in the initiation of infections. The *Pseudomonas fluorescens* ompA-group, major out membrane protein OprF and the *Xf* mopB protein, unlike most other ompA proteins, have a proline-rich region preceding the carboxyl end region of similarity generally shared by ompA proteins. *P. fluorescens* competes against certain root-pathogenic fungi because of its ability to colonize root surfaces. De Mot and Vanderleyden (1991) purified OprF and demonstrated that OprF binds tightly to roots and probably is responsible for some aspect of the root-adhesion capabilities of *P. fluorescens*. This supposition also is consistent with mutational studies (Deflaun, Marshall et al. 1994). Therefore, we postulated that mopB may contribute to *Xf* virulence by adhering to xylem element interior surfaces. We selected balsa wood as a model for grape xylem because balsa wood is highly porous and readily available. The figure below reveals specific absorption of mopB to balsa wood in the presence of two other proteins, bovine serum albumin and ovalbumin. Absorption was not observed for mopB exposed to SDS at an elevated temperature, validating the new mopB purification method, based on solubilization in SDS and $NaClO_4$, as an improved approach to obtain functional mopB. We believe that absorption to balsa wood, which is composed largely of xylem, constitutes a functional assay for mopB that may have implications for strategies designed to interfere with *Xf* infection.

Figure 2. Binding of *Xf* mopB to balsa wood and to filter paper. Binding of *Xf* mopB to balsa wood and to filter paper. Lanes 1 and 2: Partially purified *Xf* mopB (migration position indicated by "M" on the right) was mixed with bovine serum albumin (BSA, "B") and ovalbumin ("O"). 1 mL of the mixture was exposed to a 2.5 x 2.5cm piece of 1.3mm thick balsa wood for 90min at room temperature with orbital shaking at 100rpm in SCP (succinate, citrate, phosphate) buffer containing 2mg/mL NP-40 non-ionic detergent. The liquid was combined with two 0.5mL rinses of the balsa wood (free fraction "F" lane 1). The balsa wood was eluted at 65°C with pH9 Tris-buffered 8mg/mL SDS, 10mg/mL mercaptoethanol (absorbed fraction "A" lane 2). The F and A fractions were concentrated by centrifugation over a 30K cut-off filter. Results show that part of the mopB protein but little or none of the BSA and ovalbumin (broad band) was absorbed by balsa wood. Lanes 3 through 7: Conditions were as for lanes 1 and 2 except that the reaction volume was 0.25mL, the sample contained transferring (T), BSA (B), glyceraldehydes phosphate dehydrogenase (G) and carboxyanhydrase (C) in addition to mopB (M), and the absorbent was two 8mm disks of Whatman 3MM paper. Lanes 5 and 6 analyze the F and A fractions and show selective absorption of mopB from the protein mixture. Lane 3 received the sample only; lane 4 corresponds to lane 5, except no filter paper was placed in the vial. Lane 7 corresponds to lane 6 except that the filter paper received no sample.



Cellulose is a principal component of xylem. The figure, just above, reveals that cellulose, in the form of washed Whatman 3MM filter paper, also selectively binds mopB in the presence of other proteins. The mopB band is diminished in intensity in lane 5 compared to lane 4, and the "missing" mopB was recovered in lane 6 as material eluted from the paper with hot, alkaline SDS solution.

Subsequently, we have shown that *Xf* cells bind tightly to 3MM filter paper, and that the proteins eluted from the paper by hot SDS include a protein with the mobility of mopB. Although additional experiments will be needed to demonstrate that the binding by cellulose of *Xf* cells is the result of mopB binding to cellulose, our results suggest that mopB may be an essential factor in the attachment of *Xf* cells to cellulose in the interior of xylem elements.

The proposed role of mopB in binding of *Xf* cells to the interior of xylem elements suggests potential transgenic and chemical strategies for interfering with *Xf* infection of grape. Transgenic approaches would involve a grape transgene, encoding a mopB-binding protein, a mopB-inactivating protein, or a hybrid protein capable to binding to mopB and inactivating *Xf* bacterial cells (this last approach being taken by Dandekar and Gupta) expressed in rootstock or scion and targeted to xylem. The expressed transgene protein presumably could overcome the small dose of GWSS-injected *Xf* inoculum and prevent or reduce the incidence of Pierce's disease development. Similarly, a chemical that is capable of being taken up by the grapevine and that interfered with mopB binding to cellulose could be applied as a prophylactic when survey information indicated invasion into the vineyard area of *Xf*-bearing GWSS.

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CONTROL OF PIERCE'S DISEASE THROUGH DEGRADATION OF XANTHAN GUM

Project Leader:
Donald A. Cooksey
Department of Plant Pathology
University of California
Riverside 92521

Cooperator:
Neal L. Schiller
Division of Biomedical Sciences
University of California
Riverside, CA 92521

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ABSTRACT
Endophytic xanthan gum-degrading bacteria isolated by enrichment culture were characterized for their mode and dynamics of xanthan degradation, colonization of plants, and interactions with *Xylella fastidiosa*. In vitro, growth and biofilm production by the endophytic xanthan degrader from oleander, *Acinetobacter johnsonii* GX123, was enhanced by xanthan gum as a substrate and by cells of *X. fastidiosa* added to a minimal medium. Xanthan gum was degraded rapidly during log-phase growth the this endophyte, and viscosity was reduced almost to non-detectable levels. GX123 colonized stems and leaves of oleander systemically, and systemic colonization was enhanced by co-inoculation with *X. fastidiosa*. Its effects on symptom expression are still under investigation.

INTRODUCTION
Pierce's disease of grapevine and other leaf scorch diseases caused by *Xylella fastidiosa* are associated with aggregation of the bacteria in xylem vessels, formation of a gummy matrix, and subsequent blockage of water uptake. In the closely-related pathogen, *Xanthomonas campestris*, 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 *X. fastidiosa* (Simpson et al., 2000; Bhattacharyya, A., et al. 2002; Van Sluys et al., 2003) revealed that this pathogen also has genes for xanthan gum production. In Pierce's disease, xanthan 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 has been studied extensively (Becker et al., 1998). Bacteria that produce xanthan-degrading enzymes have been isolated from soils by several researchers, using enrichment techniques with xanthan gum as the sole carbon source (Sutherland, 1987; Ruijssenaars et al., 2000).

This project is to identify bacteria that produce xanthan-degrading enzymes to target this specific virulence factor of *X. fastidiosa*. This approach has the potential to significantly reduce damage caused by Pierce's disease in grapes and potentially in other hosts of *X. fastidiosa*, such as almonds and oleander. If xanthan gum is important in the aggregation of the pathogen in the insect vector, then our approach may also reduce the efficiency of transmission of Pierce's disease. 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 xanthases and xanthan lyases, we will facilitate possible efforts to transform grapevines to produce these enzymes.

Previously, we used modified xanthan gum that mimicks *Xylella* xanthan from a *Xanthomonas* mutant as the sole carbon source for enrichment culture from Pierce's disease infected grapevines. The xanthan gum biosynthetic operon in the *Xylella* genome is different than the bacterium from which commercial xanthan gum is prepared, *Xanthomonas campestris*. However, it is not feasible to produce enough xanthan gum for our studies from the slow-growing *Xylella fastidiosa*, so we genetically modified a strain of the fast-growing *Xanthomonas campestris* to produce a modified xanthan gum that is predicted to have the same chemical structure as that from *Xylella*. This was accomplished by deleting the *gumI* gene from the biosynthetic operon. We reported last year that over 100 bacterial strains were initially recovered from enrichment experiments, and 11 were subsequently confirmed to effectively degrade xanthan gum. These strains were then tested for cellulase activity. Degradation of the cellulosic backbone of the xanthan polymer would be desirable, but we do not want enzymes that recognize and degrade plant cellulose. Six of the strains had low or non-detectable cellulase activity and were further characterized.

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

Characterization of endophytic bacteria that degrade xanthan gum

The xanthan-degrading bacteria included both gram-negative and gram-positive bacteria, as identified by fatty acid methyl ester analysis and 16S rDNA sequencing. One particular strain (GX123) with high xanthan-degrading activity but no cellulase activity that was isolated from oleander was identified as *Acinetobacter johnsonii* and was characterized in more detail. In batch culture, addition of 0.02% xanthan gum to a minimal medium allowed growth of GX123 in vitro and greatly enhanced biofilm formation by this strain. The measured relative viscosity of xanthan gum rapidly decreased during log-phase growth of GX123 from a value of 7.6 to 2.3 over 24 hours and to 0.2 by 56 hours. Addition of cells of *Xylella fastidiosa* instead of xanthan gum also greatly enhanced both growth and biofilm formation by strain GX123.

Colonization of oleander plants by *Acinetobacter johnsonii* GX123 was examined with and without coinoculations with *Xylella fastidiosa*. In single inoculations, the endophyte spread several centimeters upward from stem inoculation points to achieve populations of 3×10^3 CFU/g after 7 days and 4×10^5 CFU/g after 21 days. It also spread to the first leaf above the inoculation point in each inoculated plant by 7 days (3.2×10^3 CFU/g) and was up to 2×10^5 CFU/g by 21 days in the first leaf. GX123 was not detectable 4 cm below the inoculation point 7 days after inoculation, but after 21 days, it was present at 3.2×10^4 CFU/g at this distance below the inoculation point. However, it was never detected as far as 8 cm below the inoculation point in the single inoculations, even after following populations for 105 days. In co-inoculations with *X. fastidiosa*, the endophyte generally reached higher populations levels in stems and leaves above the inoculation points and spread downward further and more quickly. Unfortunately, Pierce's disease symptoms have not yet developed in these plants, so that the effect of the endophyte on disease expression has not yet been determined.

CONCLUSIONS

The xanthan gum-degrading endophyte *Acinetobacter johnsonii* GX123 is a good candidate as a possible biocontrol agent for Pierce's disease, since it effectively degrades xanthan gum and successfully colonized plants above and below inoculation points, including systemic leaves. The enhancement of growth of this endophyte in vitro, as well as in plants, when *X. fastidiosa* was co-inoculated suggests that it benefits from the presence of this bacterium, possibly growing on the xanthan gum present on and released from *X. fastidiosa* cells. Its effects on symptom expression are still under investigation.

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FUNDING AGENCIES

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

Project Leaders:
Donald A. Cooksey and James Borneman
Department of Plant Pathology
University of California
Riverside 92521

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

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

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

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

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

OBJECTIVES

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

RESULTS

During the first three months of this project, we have extracted DNA from replicated samples of healthy and *Xylella*-infected grapevines, amplified rRNA genes from these extracts, and are in the process of cloning and constructing arrays of these genes from each sample. Preliminary experiments will determine how many random clones we should expect to include for a library representative of the majority of microorganisms present in a grapevine sample.

CONCLUSIONS

This work will contribute significantly to fundamental information on microbial communities in the grapevine xylem, in which *Xylella fastidiosa* multiplies and causes vascular occlusions. Researchers working on biological control of the pathogen, as well as disease resistance in grapevine cultivars, will benefit from the information gained in this work. The work should enhance discovery of potential biological control agents for Pierce's disease and the implementation of biological control efforts underway.

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INSECT-SYMBIOTIC BACTERIA INHIBITORY TO *XYLELLA FASTIDIOSA* IN SHARPSHOOTERS: TOXIC PEPTIDES AGAINST *XYLELLA*

Project Leader:
Donald A. Cooksey
Department of Plant Pathology
University of California
Riverside, CA 92521

Project Director:
Thomas Miller
Department of Entomology
University of California
Riverside, CA 92521

Collaborators:
David Lampe
Biology Department
Duquesne University
Pittsburgh, PA 19219

Carol Lauzon
Dept. of Biological Sciences
California state University
Hayward, CA 94542

Blake Bextine
Dept. of Entomology
University of California
Riverside, 92521

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ABSTRACT
Eleven strains of the pathogen, *Xylella fastidiosa*, and the glassy-winged sharpshooter (GWSS) gut bacterium, *Alcaligenes xylosoxidans denitrificans*, were screened for sensitivity to 41antimicrobial peptides (in addition to 18 screened previously), and more detailed studies of effective inhibitory concentrations of these peptides were conducted. Of 28 additional peptides found to have toxicity toward *X. fastidiosa*, 25 were also toxic to *Alcaligenes*, leaving three as additional candidates for engineering of this GWSS gut bacterium in addition to one found last year. Genes encoding these peptides are being designed and constructed with appropriate promoters and signal peptides for expression and secretion by *Alcaligenes*. Another 89 antimicrobial peptides derived from a combinatorial peptide library were also recently obtained and will be tested against *Xylella* and *Alcaligenes xylosoxidans denitrificans*. The results support the idea that this glassy-winged sharpshooter gut bacterium could be engineered to produce a peptide with toxicity toward the Pierce's disease pathogen.

INTRODUCTION
The destructive potential of *Xylella fastidiosa* in grapevine and other hosts has been greatly increased by the appearance and rapid spread of the glassy-winged sharpshooter (GWSS) in California. This insect vector acquires and carries the pathogen in its mouthparts and transmits the disease to other plants during subsequent feeding. In addition to control measures directed toward reducing populations of the insect, a reduction of the ability of the insect to acquire, maintain, and transmit the pathogen could greatly enhance control. The overall goal of this project is to genetically transform glassy-winged sharpshooter endosymbionts to produce toxic substances that would inhibit or kill *Xylella fastidiosa* and reduce disease transmission. In our component of the project, we have been screening antimicrobial substances against *Xylella*, as well as against the endosymbionts that have been selected for transformation.

Antimicrobial peptides represent one of the most widely distributed forms of natural defense against bacteria and fungi and are now being developed for a variety of medical and agricultural applications. Examples of their use against bacterial pathogens of plants include the transformation of a synthetic cecropin gene into tobacco plants to produce the cecropin peptide *in planta* in an attempt to provide resistance to the leaf pathogen *P. syringae* pv. *tabaci* (Hightower et al., 1994). Harakava et al. (1999) infected tobacco plants with a PD strain of *X. fastidiosa* and used this host pathogen system as a model for testing expression of cloned genes that may give resistance to *X. fastidiosa*. Two antibacterial peptides, cecropins A and B effectively killed a PD strain in an *in vitro* assay. A preliminary study also reported sensitivity of *X. fastidiosa* to Magainin 2 (Momol et al., 2000).

OBJECTIVES
1. Identify toxic peptides effective against *Xylella fastidiosa* but non-toxic to selected endosymbiotic bacteria.
2. Design and construct genes encoding antimicrobial peptides to be expressed and secreted by endosymbiotic bacteria.

RESULTS
Last year, we reported that six strains of *X. fastidiosa* from grape and almond had been used in screening 18 antimicrobial peptides, with four peptides found to be toxic to all strains. One of these was non-toxic to *A. xylosoxidans denitrificans*, one of the natural gut bacteria from the glassy-winged sharpshooter that we are targeting for genetic transformation. During this past year, more extensive studies of antimicrobial peptide sensitivity of 11 strains of the pathogen, *X. fastidiosa*, and of the GWSS gut bacterium, *A. xylosoxidans denitrificans* were conducted, including 41 additional antimicrobial peptides and more detailed studies of effective inhibitory concentrations of these peptides. Of 28 additional peptides with toxicity toward *X. fastidiosa*, 25 were also toxic to *Alcaligenes*, leaving three as additional candidates for engineering of this GWSS gut bacterium. Genes encoding these peptides are being designed and constructed with appropriate promoters and signal peptides for expression and secretion by *Alcaligenes*.

Another 89 antimicrobial peptides derived from a combinatorial peptide library were also recently obtained and will be tested against *Xylella* and *A. xylosoxidans denitrificans.*

CONCLUSIONS

Several antimicrobial peptides were found with toxicity toward *X. fastidiosa* but not against the glassy-winged sharpshooter gut bacterium, *A. xylosoxidans* denitrificans, suggesting that this bacterium could be engineered to produce a peptide with toxicity toward the Pierce's disease pathogen.

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IMPACT OF MULTIPLE-STRAIN INFECTIONS OF *XYLELLA FASTIDIOSA* ON ACQUISITION AND TRANSMISSION BY THE GLASSY-WINGED SHARPSHOOTER

Project Leaders:
Heather S. Costa
Department of Entomology
University of California
Riverside, CA 92521

Donald A. Cooksey
Department of Plant Pathology
University of California
Riverside, CA 92521

Carmen Gispert
UC Cooperative Extension
Riverside/San Diego/San Bernadino Counties
Indio, CA

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ABSTRACT
In this project, studies were conducted to 1) develop a method to detect and differentiate multiple strains of *Xylella fastidiosa* in individual glassy-winged sharpshooters (GWSS,) and 2) determine the relative ability of an individual GWSS to simultaneously retain or transmit two strains of *X. fastidiosa*. Strain-specific primers were developed that can detect and differentiate the Pierce's disease strain (PD) and the oleander strain (OLS) present in extracts from individual insects fed on *X. fastidiosa*-infected grape and oleander plants. After feeding on infected grape and oleander source plants for one day each, 76% of surviving adults tested positive for one or both strains of *X. fastidiosa.* The majority of individuals tested positive for only one strain of the pathogen (29% PD, 41% OLS), and only 7% tested positive for both strains; 24 % tested negative. Overall, individual insects transmitted the pathogen 39% of the time (13% PD, 26%OLS). Thus, only about half the insects that tested positive for *X. fastidiosa* actually transmitted the pathogen to a susceptible host. Although each individual used in transmission studies was exposed to both strains of the pathogen and both types of test plants, in all cases an individual insect transmitted only one strain of the pathogen, never both.

OBJECTIVES
Assess the ability of glassy-winged sharpshooter exposed to two strains of *X. fastidiosa* to transmit either strain of the pathogen.

INTRODUCTION
Xylella fastidiosa is a bacterial plant pathogen that causes a variety of diseases in a broad range of plant hosts including Pierce's disease of grapevines, almond leaf scorch, alfalfa dwarf, citrus variegated chlorosis, leaf scorch of live oak, pear leaf scorch, and oleander leaf scorch (Brlansky et al., 1982; Hopkins, 1989; Hartung et al., 1994; Purcell and Hopkins, 1996; Purcell et al., 1999). The genetic diversity of additional strains has been examined (Pooler and Hartung, 1995; Albibi et al., 1998; Chen et al., 1992; da Costa et al. 2000; Hendson et al. 2001).

Two strains of this pathogen that are presently causing severe economic losses in California are the Pierce's disease (PD) strain that infects grape and other hosts, and the oleander leaf scorch (OLS) strain that infects oleander (Blua et al., 1999; Purcell and Saunders, 1999, Purcell et al., 1999). The PD strain does not infect oleander and the OLS strain does not infect grape. The grape strain appears to have a broader host range than the oleander strain, however the complete host range of each strain is not really known.

The pathogen is spread from plant to plant by leafhoppers. Several leafhopper vectors transmit this pathogen, but the dominant vector in Southern California is the glassy-winged sharpshooter, *Homalodisca coagulata* (Say) (Blua et al., 1999; Sorensen and Gill, 1996, Purcell and Saunders 1999). This insect feeds on a very broad range of plant hosts and is capable of transmitting both the grape and oleander strain of the pathogen (Purcell, 1990; Purcell et al., 1999; Costa et al. 2000). The high mobility of this insect, and its utilization of large number of host plants provide this vector with a great opportunity to be exposed to more than one strain of the *Xylella* pathogen in its lifetime. In this project, studies were conducted to determine the relative ability of an individual glassy-winged sharpshooter to simultaneously retain or transmit two different strains of *X. fastidiosa*.

RESULTS
DNA extraction and amplification
Both the commercial DNA extraction kit and immunocapture techniques we used effectively extracted enough bacterial DNA from plant and insect tissue to allow detection by PCR, however, the commercial kit extraction method was preferred because it was faster and less complicated than the immunocapture techniques. The mixing of strain specific primers in a single reaction minimized the number of samples needed and amount of handling required to get a strain specific identification. The use of the mesh-lined sample homogenization bags for extraction also greatly increased the speed of processing samples compared to using liquid nitrogen or grinding in microcentrifuge tubes. In addition, the use of the Ready-To-Go® beads

simplified and standardized the reagents required to perform PCR. Thus the processing of samples could be performed quickly, with relatively little specialized training if personnel had basic laboratory skills and the necessary equipment was available.

Use of strain specific primers on insects

Strain-specific primer sets were developed to detect and differentiate the PD and OLS strains in individual insects. Extractions of individual insects fed on *X. fastidiosa*-infected oleander that were amplified with a mixture of the PD and OLS specific primer pairs produced only a 638 bp band, which is the size of the oleander-specific product. When extracts from individual insects that fed on infected grapevine were amplified with the same mixture of primers, only a 412 bp band was produced, which is the size of the PD specific product. In some cases, when individual insects were allowed to sequentially feed on both infected grapevine and infected oleander, the products of both primers pairs were produced.

Transmission experiments

Mortality rates of insects feeding on infected plants were higher than expected (57%). Few insects managed to survive exposure to both infected source plants and subsequent exposure to test plants, likely because of repeated handling during the course of the experiments. Only insects that were alive at the end of the experiment were analyzed. After feeding on infected grape and oleander source plants for one day each, 76% of surviving adults tested positive for one or both strains of *X. fastidiosa*. The majority of individuals tested positive for only one strain of the pathogen (29% with PD, 41% OLS), 7% tested positive for both strains, and 24 % tested negative. Overall, surviving insects transmitted one the pathogen 39% of the time (PD 13%, OLS 26%). Thus, only about half the insects that tested positive for *X fastidiosa* actually transmitted the pathogen to a susceptible host. No individual insect transmitted both strains of the pathogen.

CONCLUSIONS

The strain-specific detection method we have described is a practical tool that can be used to differentiate strains of *X. fastidiosa* when multiple strains are being used in controlled experiments. In addition, it will also be useful to screen field-collected insects to determine which strains of the pathogen local populations are carrying. Although false negatives may occur, this method can still provide an indication that infected insects are present in an area, and provide an estimate of the relative numbers that are infected with each strain. Additional strain-specific primer pairs could be designed to detect additional strains of this pathogen that may be present in different geographic areas.

In our studies, the detection of the pathogen in an individual insect using PCR did not always indicate the propensity to transmit the pathogen to a test plant; many insects that tested positive did not transmit the pathogen. The reasons for this are not known. Other studies using media culture to isolate bacteria from insects also found that neither the detection of bacteria in insect heads, nor the estimated numbers of bacteria present, predicted the propensity of the insect to transmit *X. fastidiosa* (Hill and Purcell 1995, Almeida and Purcell 2003). One of the more interesting results observed was that although all surviving individuals were exposed to both strains of the pathogen, and both types of susceptible test plants, in no case did a single individual transmit both strains of the pathogen. Thus, there must be other factors that are contributing to an individual's ability to successfully retain and transmit the pathogen after exposure.

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

Project Leaders:

David Gilchrist
Department of Plant Pathology
University of California
Davis, CA 95616

James E. Lincoln
Department of Plant Pathology
University of California
Davis, CA 95616

Cooperators:

Andrew Walker
Department of Viticulture and Enology
University of California
Davis CA 95616

Steven Lindow
Department of Plant and Microbial Biology
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted from October 2002 to October 2003.

ABSTRACT

Our overall objective is to identify novel genes from cDNA libraries of either grape or heterologous plants that, when expressed in grape, will disrupt infection, spread or symptom development of the xylem-limited bacteria, *Xylella fastidiosa* (*Xf*). Furthermore, we are specifically characterizing the effect of the genetic disruption of Pierce's disease (PD) symptoms on the movement and establishment of the bacterium in the xylem of susceptible grape plants. Specific objectives are to: a) create cDNA libraries from several different grape backgrounds, including two with PD resistance; b) develop a functional *A. rhizogenes*-based cDNA screen in grape; c) examine the morphological and cytological features of cell death in symptomatic tissues; and d) investigate the potential of blocking PD symptom expression and disease impact with anti-apoptotic transgenes. To date we have developed a *Agrobacterium. rhizogenes*-based transformation procedure that results in the induction of transformed roots from infected or healthy vegetative tissue sections following co-cultivation with the transforming bacteria. Each emerging root is an independent transformation event. We then used this technique to develop a functional cDNA library screen (each root contains a different cDNA library member) for genes that block either bacterial multiplication, movement, or symptom expression. The only genes that will be identified will be those that directly affect the ability of the pathogen to cause disease and is not dependent on DNA sequence relationships. We have made excellent progress in creating an extensive library of full-length cDNAs from several resistant sources as well as susceptible Chardonnay and conducting initial screens of the libraries.

INTRODUCTION

The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that, when expressed in grape, will disrupt infection, spread or symptom development of the xylem-limited bacteria, *Xylella fastidiosa* (*X.f.*). Recent published information from our laboratory confirms that specific transgenes from homologous or heterologous hosts, as well as chemical inhibitors of apoptotic proteases (3) that block programmed cell death (PCD) (1) during plant disease development (4), can arrest both symptom development and microbial growth *in planta* in a range of plant-microbe interactions (3, 4, 5). PCD is now well established as a key pathway involving many gene products in numerous diseases of animals and plants. Since our previous work (4, 5) established that the p35 gene (a gene encoded by an animal virus to block PCD in infected host cells) could block growth of several bacterial and fungal pathogens in infected tissue of several plant species, we transformed grape tissue with the p35 transgene and observed that the infected tissue remained asymptomatic. We are now assessing the effect of the p35 transgene on *X.f.* movement or growth in transformed grape tissues using the GFP-transformed *X.f.*, provided by Dr. Lindow. A corollary to this observation is that the *.X.f* -triggered death observed in PD occurs in the absence of apparent water stress and can be observed in young tissues before pronounced symptoms develop. This conserved genetically determined PCD process can be studied by biochemical, cytological and genetic techniques and can be transgenically manipulated by techniques developed in our laboratory (3,4). We believe that examination of the molecular basis of cell death in pre-symptomatic and symptomatic tissues, along with the immediate assessment of the effect of expressing anti-apoptotic transgenes in PD infected tissues on the development of death-related symptoms in grape, will be very informative in the short run in terms of PD biology and physiology. In a longer time frame these data will likely yield genetic strategies for protection of grape against infection by *X.f.* in years not decades. The immediate goal is to rapidly identify novel resistance genes in grape that block any one of several required steps in the infection and spread of *Xf* in the xylem.

OBJECTIVES

1. Construct cDNA libraries from several different grape backgrounds from infected and uninfected grape tissue, including libraries from lines with PD resistance;
2. Develop a functional *A. rhizogenes*-based cDNA screen in grape;
3. Examine the morphological and cytological features of cell death in symptomatic tissues using Dr. Lindow's GFP transformed *X.f*;

4. Investigate the potential of blocking PD symptom expression with anti-apoptotic transgenes.

RESULTS

Construction of cDNA libraries

The construction of a grape cDNA library initially proved much more difficult than we had experienced in making libraries from 4 other plant species. Isolation of mRNA was not difficult but the grape tissue contains high levels of phenolic compounds in an oxidative environment that contaminate the RNA, rendering it difficult to reverse transcribe. However, we now have an efficient protocol for generating full-length cDNA libraries from grape using an antioxidant cocktail during homogenization and CsCl gradient purification of RNA. The libraries have an average insert size of 1000bp and sequence analysis of random inserts reveals that the cDNAs appear full-length. The goal is to create libraries each containing a minimum of 500,000 cDNAs from PD susceptible *Vitis vinifera* (Chardonnay), and resistant sources, *Muscadinia rotundifolia* (Cowart), and *Vitis shuttleworthii* (Hanes City). These libraries will be immediately available to all grape researchers in this program and will represent the largest available source of full-length cDNAs. We have to date constructed approximately 150,000 independent grape cDNAs derived from healthy and *Xf* infected Chardonnay and then ligated into a plant transformation binary vector, CB5, which is a derivative of pBIN19 and uses the CaMV 35S promoter for high level, constitutive expression. We have generated approximately 100,000 independent grape cDNAs each from *M. rotundifolia* (Cowart) and *V. shuttleworthii* (Hanes City). The susceptible Chardonnay will be used as a recipient host to screen cDNA libraries of Chardonnay, Cowart and Hanes City that express differing levels or forms of resistance to PD. Cowart and Hanes City are two primary sources of resistance being used in Dr. Walker's genetics program. As potential cloned resistance genes become available they also will be used in attempts to identify homologues from the Chardonnay cDNA library that may provide resistance by simple alteration in expression level within the homologous host.

Screening of cDNA libraries

The cDNA library has been moved into *A. rhizogenes* in preparation of transformation of infected grape explants for the purpose of finding cDNAs that will block the death of infected tissues. The library will be screened in sets of 50,000 cDNAs to improve the efficiency in terms of handling numbers of symptom blocking cDNAs. However, based on previous experience with tomato, we expect that less than 0.01% of the cDNAs will effectively protect against PCD and/or the disease development. This underscores the need for a highly effective functional screen. In order to provide sufficient *X f*–infected tissue for transformation, we developed a micro-propagation technique for producing clones of sterile grape plants in small plastic boxes that can be inoculated with *Xf* under sterile conditions. The micro-propagated plants are much more efficiently transformed than the greenhouse-derived tissue, which tends to be more highly lignified and produces fewer transformed roots. As a means of fast tracking the cDNA screen while perfecting the grape transformation procedure, we have screened approximately 30,000 members of the Chardonnay cDNA library by *A. rhizogenes* transformation of tomato cotyledons. The resulting roots were subject to PCD induction by treatment with the mycotoxin FB1. PCR was used to amplify the Chardonnay cDNA insertion from the surviving tomato roots. The cDNA inserts were then cloned and sequenced. Using this analysis of the Chardonnay cDNA library, we so far have found several grape full-length cDNAs (encoding open reading frames) that protect tomato from PCD. These positive grape genes will be tested individually in Chardonnay. Lastly, this analysis of the Chardonnay cDNA library in tomato demonstrates that the cDNA library is functional in transformation and expression. It is important to emphasize that this screen is not dependent on the presence or role of PCD in PD but will, in fact, detect any gene that affects the integrity of the bacterium in the infected tissue or the ability of the bacterium to elicit symptoms of PD, regardless of whether the step being affected is strictly dependent on the induction of PCD.

Evaluation of the effect of blocking PD symptoms on bacterial growth and movement

We have begun to evaluate the effect of experimental transgenes on grape tissue bearing *X.f* in xylem elements with various cell death markers and GFP-marked bacteria. By using the GFP-tagged *Xf*, this is a direct functional assay for genes that block bacterial movement or accumulation in the xylem of newly differentiated grape tissue (6). Previously we developed a yeast-based surrogate screen for endogenous anti-PCD plant genes from a tomato cDNA library. We obtained 12 genes from tomato from more than 500,000 cDNAs screened in yeast that also block PCD in the *A. rhizogenes*-tomato root functional disease assay. In order to jump-start the functional assay in grape we will immediately test these genes in the *A. rhizogenes*-transformed PD-infected grape system. Homologues of the tomato genes can be cloned from grape to provide authentic grape genes to use in the very near future; the focus being on any grape homologues of tomato genes that block PD in grape. Of particular interest is the possibility that PD blocking signals initiated with transgenes will move systemically through the vascular system from transformed rootstocks to upper regions of grafted cultivated grape tissue affording protection against systemic movement or activity of *Xf* without genetically engineering the cultivated grape.

Plant transformation with putative resistance genes and whole plant regeneration

Two approaches are being taken. In the first approach, transformed test plants will be obtained by standard *A. tumefaciens* methods by the UC Davis Plant Transformation Facility which now performs grape transformations. To this end, we have initiated transformations of Chardonnay with the baculovirus p35 gene as well as a tomato gene that protects tomato roots from PCD induced by the fungal mycotoxin FB1. In the second approach, we also will pursue regeneration of whole plants from transformed grape roots as a means of direct introgression of cloned resistance genes into a susceptible host plant while maintaining the clonal integrity of the recipient plant following transformation. While derived from basic molecular genetic research, our immediate goal is to rapidly identify resistance genes in grape genotypes that block any one of several required steps in the infection and spread of *X.f* in the xylem and get these into the hands of breeders and viticulturists for immediate

evaluation. As indicated earlier, regeneration in this manner will permit the direct establishment of a propagative plant that will maintain the clonal integrity of the untransformed parental material. More than 120 plants have been transformed by *A. rhizogenes*. Regeneration from transformed roots has been reported from 31 with no indication of unusual difficulties. We do not expect that regeneration will be trivial but are optimistic that, with systematic examination of growth conditions and hormonal regulation of development, regeneration will be possible. However, the first approach will ensure that we have adequate transformed test plants to evaluate under controlled glasshouse conditions in the shortest time possible.

CONCLUSIONS:

We are currently adding monthly an additional 50,000 cDNA inserts each of Cowart, Hanes City and Resistant tester line 8909-15 cloned into the binary vector CB5 (for direct transformation into the *A. rhizogenes* functional screen in Chardonnay). The tissue source for these library sets is from field grown plants. The average insert size is 1000 bases. We anticipate the development of libraries of each of the resistant source with 500,000 members within 12 months. Additional library subsets are being constructed from *X. fastidiosa*-infected tissue that we have developed from micro-propagated sterile cultured plants grown in individual plastic boxes and infected under sterile conditions. This to ensure that the only biotic stress these plants will have experienced is from *X.f* and would, therefore, contain *Xf* specifically induced genes, without confounding by other biotic stress induction as would likely occur in the field or greenhouse grown plants. Plants produced under these same conditions also are the source of *Xf* infected stem sections used for transformation in the *A. rhizogenes* functional screen.

The first grape library has been pre-tested by screening the grape cDNAs in tomato to determine if the library contains anti-PCD genes. Additional considerations that are part of the critical requirements for a comprehensive library from grape lines are that these or other genes will block the development of the bacterium or the symptoms associated with the disease in grape. Hence, we screened 30,000 members of a grape cDNA library by *A. rhizogenes* transformation of tomato cotyledons. Toxin-induced PCD resistant tomato roots were isolated; the protecting grape cDNA insert was recovered by PCR, and sequenced. These genes have now been re-cloned and are being tested in grape against PD-infected stem sections. Based on the pre-test in tomato, a limited number of grape genes appear to effectively protect against PCD as was observed earlier with the screen of tomato libraries in tomato for anti-PCD (disease protecting) genes. Two strongly protecting open reading frames (ORFs) share sequence homology to respectively, glutathione-S-transferase (a protein that has been reported to be involved in disease resistance) and an unidentified expressed plant protein. The fact that a small percentage of the cDNAs appear to protect is encouraging in that we expect that the genome of grape will contain only a few genes that can effectively protect plant cells against pathogen-secreted signaling molecules that lead to disease development. We fully expect to have several novel genes identified within the first year of funding and will proceed to study their mode of action as proposed.

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FUNDING AGENCIES

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THE DEVELOPMENT OF PIERCE'S DISEASE IN XYLEM: THE ROLES OF VESSEL CAVITATION, CELL WALL METABOLISM AND VESSEL OCCLUSION

Project Leaders:

John Labavitch
Department of Pomology
University of California
Davis, CA 95616

Mark Matthews
Department of Viticulture and Enology
University of California
Davis, CA 95616

Cooperators:

L. Carl Greve
Department of Pomology
University of California,
Davis, CA 95616

Susan Lurie
The Volcani Institute
Bet-Dagan, Israel

Eleanor Thorne
Department of Viticulture and Enology
University of California
Davis, CA 95616

Alonso Perez
Department of Pomology
University of California
Davis, CA 95616

Tom Rost
Section of Plant Biology
University of California
Davis, CA 95616

Josh Stevenson
Section of Plant Biology
University of California
Davis, CA 95616

Caroline Roper
Department of Plant Pathology
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted from October 1, 2002 to October 1, 2003.

INTRODUCTION

This proposal is directed toward discovering the plant responses to infection that are fundamental to the progression of Pierce's disease (PD) in grapevine. The disease is caused by the growth of the bacterium *Xylella fastidiosa* (*X.f.*) in the xylem vessels of stems, petioles and leaf blades. The disease progresses rapidly, causing severe water deficits in infected shoots (Goodwin et al., 1988) and vine death-often within two years. However the progression of the disease and the mechanism(s) by which the disease produces water deficits and death in infected tissues have not been well established.

The prevailing notion has been that vessels become occluded with bacteria or products of metabolism. However, we have shown that PD symptoms appear in grapevines prior to the development of a large *Xf* population. However, how the bacterium moves through and between vessels, whether vessels cavitate upon introduction of the bacterium by the insect vector or artificial inoculation, the nature and origin of the materials that occlude the vessels of infected vines, and the nature of the plant regulators that influence the vine's response to *Xf* are not known. We have continued our work testing the model (below) that proposes answers to these important questions about the development and progression of PD symptoms in grapevines.

X.f. introduction to vessels—>vessel cavitation—> initial water deficit—> X.f. population increase—> production of enzymes by X.f. (signals ?) —> cell wall digestion —> oligosaccharide signals —> ethylene synthesis rise—> a "wave" of vessel occlusion beyond the infection site —> global collapse of vine water transport—> leaf abscission—>vine death

OBJECTIVES

For this research period we have addressed a number of the elements of the PD development model.

1. What is the nature of the occlusions found in vessels of infected vines?
2. What is the impact of a vine's "water status" on the development of disease symptoms?
3. What is the evidence that *Xf* produces cell wall-degrading enzymes to facilitate the systemic spread of the bacterial population?
4. What is the role of ethylene in the development of vine responses to *Xf* presence?

RESULTS AND CONCLUSIONS

What is the nature of the xylem occlusions found in infected vines?

In prior reports we have reported that "gels" isolated from infected stems have sugar compositions suggesting that they are products of both *Xf* and plant metabolism. We have continued these studies, but it is difficult to isolate these occluding materials without co-isolation of bacterial and plant polysaccharides. Therefore, we have begun testing of immunohistochemical approaches for biochemical characterization of the occlusions. We have used anti-pectin antibodies linked to a fluorescent tag to "light up" the cell walls of tyloses in infected stems (Figure 1a). We have previously reported that tyloses form relatively early following vine inoculations with *Xf*. It is not a surprise that the tylose wall reacts; pectin is known to be an important polysaccharide in higher plant cell walls. Figure 1b shows that the same tagged antibody reacts with gels that occlude vessels and that the pectin gels co-localize with bacteria that are revealed by the use of an additional tagged antibody (Figure 1c).

The sequence of the *Xf* genome "predicts" that the bacterium can produce an extra-cellular polysaccharide with a structure very much like that of the bacterium *Xanthomonas campestris* (Figure 2a). Our analysis of polysaccharides extracted from infected grape stems suggests the presence of material containing the sugars of the predicted "fastidian gum" (Figure 2b). We are now using specific chemical and biochemical procedures to modify commercially available xanthan gum so that it has a structure like that predicted for the *Xf* gum. This will be used for the generation of antibodies that can be tagged (as above) and used to determine whether the occlusions in vessels of infected vines contain polysaccharides of bacterial origin.

What is the impact of a vine's "water status" on the development of disease symptoms?

In order to test the effect of a vine's relative water status on the development of PD symptoms, we subjected 'Chardonnay' (PD-susceptible) vines to three levels of water status (well-watered, moderately-stressed, and severely-stressed) by manipulating the watering schedules in the greenhouse. Vines were inoculated with *Xf* and standard physiological measurements of water stress were taken (to verify that the watering regimes had led to differing degrees of water stress) at intervals along with making visual assessments of PD leaf scorch symptoms. (At the end of the experiment we took stem and petiole samples of all of the vines. These will be tested for *Xf* presence soon.) The first indications of leaf scorch were seen 48 d after inoculation (Figure 3). Evaluations of symptoms were also made 77d and 91d after inoculation. The data indicate that the greater the water stress a vine experiences the quicker PD symptoms develop. Even after 91d, one-third of the well-watered vines showed no leaf symptoms. A poster at the 2003 PD Symposium will show additional data describing standard physiological measurements describing the vine stress caused by our watering regimes.

What is the evidence that Xf produces cell wall-degrading enzymes to facilitate the systemic spread of the bacterial population?

We have already reported that the "porosity" of the primary cell walls of the pit membranes that "separate" one vessel from its neighbors is much too small to permit passage of *Xf* from one vessel to the next, unless the pit membrane is damaged. Thus, systemic spread of bacteria introduced to a few vessels requires pit membrane degradation. Our model suggests that this is caused by cell wall-degrading enzymes, presumably produced by *Xf*. This idea is supported by the observation that the genome of the Brazilian and Temecula strains contain sequences that are similar to sequences known to encode the cell wall polysaccharide-degrading enzymes polygalacturonase (PG) and ß-1,4-glucanase. The idea is also supported by the report from the Meredith, Dandekar and Aguero PD project, that transgenic introduction of a PG Inhibitor Protein (PGIP) from pear fruits into grapevines reduced their susceptibility to PD.

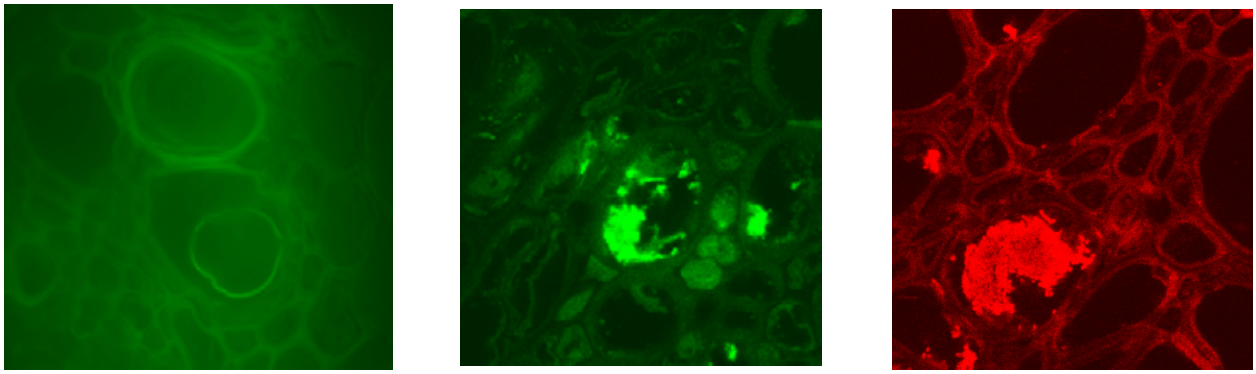
Accordingly, these *Xf* DNA sequences have been cloned. We are currently attempting to express these sequences in the bacterium *E. coli*. When we have expressed the glucanase and/or PG this will confirm that the bacterial sequences encode these enzymes and provide a source (the *Xf* gene-expressing *E. coli* cultures) of the enzymes for testing of other aspects of the cell wall model. Figure 4 describes the cloning and gene expression strategies we are testing.

What is the role of ethylene in the development of vine responses to Xf presence?

We have previously reported that treatment of grapevines with ethylene will trigger the occlusion of vessels with tyloses, just as occurs in PD-infected vines. Additional data supporting this point is provided in a poster presented at the 2003 PD Symposium from the Shackel and Labavitch, CDFA-funded project. We have developed chambers that will be used to monitor emanation of ethylene from healthy and infected vines. We will first test these chambers by measuring ethylene production by vines manipulated to force them to produce ethylene. We will then test them with inoculated vines. Our PD model places ethylene at a key point for the regulation of PD symptom development. If the model is accurate on this point, we will then examine the development of symptoms on inoculated vines that have been treated so that they are not able to respond to ethylene.

CONCLUSION

Continuing testing supports the proposed model of PD development in grapevines. Some steps predicted by the model have not yet been tested and confirmatory experiments are still necessary. Even without that work, however, it seems clear that a great deal of the problems caused in vines by introduction of *Xf* is caused by a vine response to the bacterium, rather than something specifically done to the vine by the bacterium.



a **b** **c**

Figure 1. Specific fluorescent antibodies that "light up" cell wall pectins were used to demonstrate the presence of pectin at the surface (e.g., cell wall) of expanding tyloses (1a) and in the gels that accumulate in vessels of PD-infected grapevines (1b). A second antibody (recognizing *Xf*) lights up an accumulation of bacteria in a vessel of an infected vine.

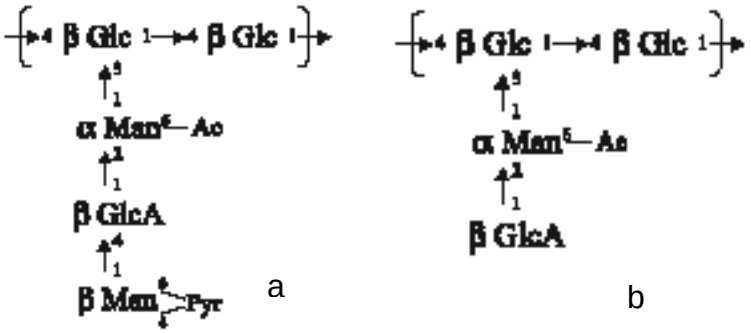


Figure 2. Chemical analysis of xanthan gum shows it to have a polymer backbone made up of ß-1,4-linked glucosyl residues and a "unit" structure consisting of 2 backbone residues, one of which bears a side chain of a terminal, pyruvylated mannosyll residue linked to a galacturonosyl residue linled to and acetylated mannosyl residue (2a). Analysis of the genes in the *Xf* genome suggests that the PD pathogen will make a polysaccharide (dubbed "fastidian" gum) lacking the terminal modified mannosyl residue. Our intent is to remove the terminal residue of xanthan gum and then raise antibodies to the modified polymer.

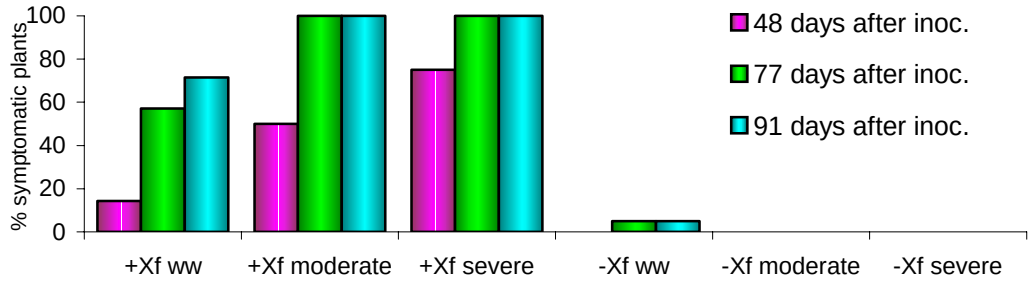
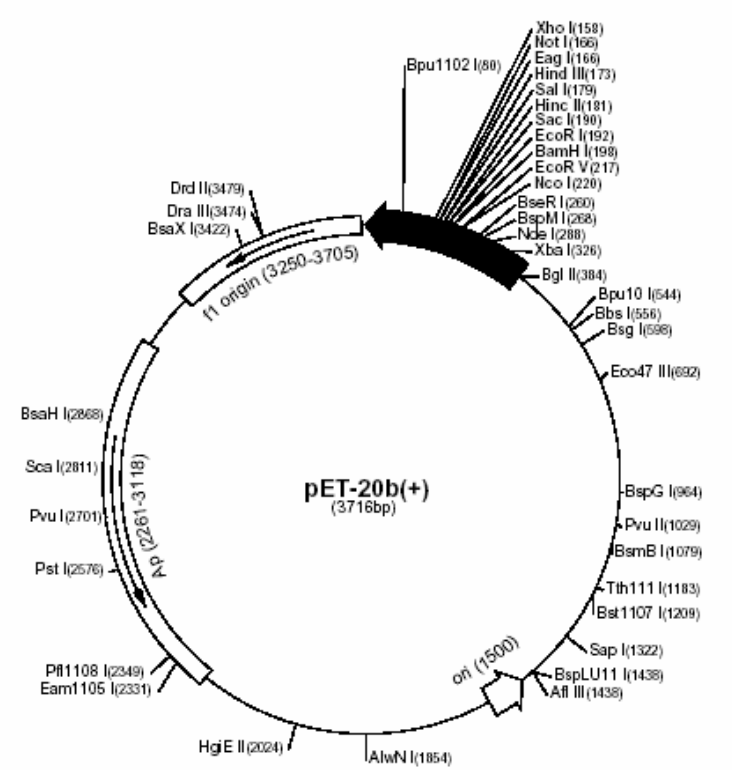


Figure 3. 'Chardonnay' and 'Cowart' grapevines in pots were watered according to three regimes, two of which were designed to cause the vines to experience water stress. Well-watered (ww) plants had an average leaf water potential of –0.6 MPa, moderately water-stressed vines (moderate) averaged –1.0 MPa, and the most water-stressed (severe) plants were –1.4 MPa. Vines were needle-inoculated with *Xf* and monitored at intervals (48, 77 and 91 d after inoculation) to determine the percent of vines showing leaf scorching and matchsticks. 'Cowart' vines showed no symptoms.



Novagen, EMD Biosciences, Inc.

Figure 4. The pET-20b (+) vector contains an N-terminal pelB signal sequence that directs the expressed protein to the periplasmic space, which increases the chances of proper folding. The pET-20b (+) vector also contains a C-terminal histidine tag that can be used in purifying the recombinant protein. This expression system is driven by the strong T7 promoter and controlled by IPTG (isopropyl-β-D-thiogalactopyranoside) induction.

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PARATRANSGENESIS TO CONTROL PIERCE'S DISEASE: ISOLATION AND ANALYSIS OF ANTI-*XYLELLA* SINGLE CHAIN ANTIBODIES

Project Leader:
David Lampe
Department of Biological Sciences
Duquesne University
Pittsburgh, PA 19282

Project Director:
Thomas A. Miller
Department of Entomology
University of California
Riverside, 92521

Collaborators:
Carol Lauzon
Dept. of Biological Sciences
California State University, Hayward
Hayward, CA 94542

Blake Bextine
Dept. of Entomology
University of California
Riverside, 92521

Don Cooksey
Dept. of Plant Pathology
University of California
Riverside, 92521

Frank Richards
Yale Medical School
New Haven, CT 06520

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ABSTRACT
We have used a two methods to attempt to isolate single-chain antibodies (scFv) specific for the surface of the Temecula strain of *Xylella fastidiosa*. A method using whole *X. fastidosa* cells to pan a phage display scFv library was successful and we recovered one very specific scFv that strongly interacts with Temecula and less so with the Tulare strain of *X. fastidiosa*. A method using a purified surface protein (mopB) from Temecula yielded two scFvs that interacted with that protein, but failed to interact with intact cells. Intended uses for these scFvs in a paratransgenesis method to control Pierce's disease will be discussed.

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 (Beard, Dotson et al. 2001), interference with HIV attachment to its target cells in the reproductive tracts of humans (Chang, Chang et al. 2003), and the elimination of persistent *Candida* infections from biofilms in chronically infected patients (Beninati, Oggioni et al. 2000). Paratransgenesis has also been applied to deliver cytokines mammalian guts to relieve colitis (Steidler, Hans et al. 2000; Steidler 2001). Thus, the method has wide applicability.

Alcaligenes xylosoxidans denitrificans (Axd) is bacterial species that can colonize the GWSS foregut and cibarium, as well as various plant tissues, including xylem. It is non-pathogenic in insects and plants and is not known to be a pathogen in 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. We have recently turned our attention to isolating factors that can specifically inhibit the transmission of *Xf* from GWSS.

We report here the isolation of a single chain antibody phage that is specific for the surface of *Xf* and appears to have some strain specificity. Other tests have shown that this antibody phage can inhibit the transmission of PD by sharpshooters under experimental conditions.

OBJECTIVES
1. Isolate single chain antibody (scFv) clones capable of binding to the surface of *Xylella fastidiosa* with high specificity either though the use of intact cells or purified surface proteins.
2. Test the strain specificity of these scFvs.

RESULTS
A. Single-chain antibodies (scFvs) as specific control agents of Xylella fastidiosa
We have spent the past year designing and implementing a strategy to isolate factors that are specifically targeted at *Xf* for use in a paratransgenesis approach to controlling PD. The greatest degree of specificity against PD is likely to be obtained using synthetic antibody constructs called single-chain antibodies, or scFvs. An scFv is composed of a single gene consisting of the variable regions of an antibody heavy chain and light chain fused together with a synthetic linker sequence. Genes for these scFvs are randomized at certain codons and are constructed in large libraries that can be "panned" with an antigen of interest to obtain specific binders.

B. Use of whole X. fastidiosa pv. Temecula to pan scFv libraries

Our target is the surface of *Xf* pv Temecula, therefore it made sense to use whole *Xf* to pan scFv libraries. We panned a commercially-available scFv phage library and obtained one very specific scFv that we call S1. This scFv does not interact with *Axd* or *Chryseomonas luteola*, two bacterial species that live in the gut of the GWSS and bacterial species that might be utilized in paratransgenesis. Interestingly, this particular scFv lacks a heavy chain region, thus the specificity is mediated only by the light chain. We do not yet know what structure or protein is bound by S1. S1 has been tested by Blake Bextine in GWSSs for its effect on disrupting the transmission of *Xf* to plants.

C. Use of genomic data and purified surface proteins

The S1 phage scFv was the only specific binder we obtained by screening whole cells, yet we would like to isolate multiple different scFvs in order to target different structures. This is possible by taking advantage of the available genomic and proteomic data for *Xf* to target specific surface proteins. The genomes of both the citrus variegated chlorosis (CVC) and Temecula (PD) strain of *Xf* have been sequenced (Simpson, Reinach et al. 2000; Bhattacharyya, Stilwagen et al. 2002). In addition, proteomic data is available for the CVC strain that documents which proteins are abundant and surface-exposed (Smolka, Martins et al. 2003). We targeted 5 surface exposed proteins (PilT, Type IV fimbriae, PilY1, hsf adhesin, and mopB) and used the Temecula genome sequence to identify homologues of them compared to the CVC strain.

We attempted to clone full-length genes for each target in *E. coli* fused to a maltose binding protein gene in order to aid in affinity purification. Only one of these constructs could be cloned (encoding mopB), probably because expression of the remainder of these are toxic in *E. coli*. A screen of the scFv library with purified Temecula mopB protein led to the isolation of two specific scFv clones. Neither of these, however, was able to bind to the surface of whole *Xf* pv Temecula cells in an ELISA, perhaps because the antigen that each scFv bound to was not exposed in whole cells. This remains a viable approach, but steps will be needed to express only parts of surface proteins that are actually surface-exposed in whole cells.

D. Strain specificity of scFvs.

We tested the S1 phage for its degree of strain-specificity. Below is a table based on a comparative ELISA using S1 and different *Xf* strains. Interestingly, some strain specificity appears to be present, which may reflect the known diversity of some *Xf* surface proteins (Bhattacharyya, Stilwagen et al. 2002).

CONCLUSIONS

We have isolated a scFv that has high specificity for the surface of the Temecula strain of *Xf*. This scFv is capable of interfering with the transmission of PD from GWSS to naïve host plants (pers. comm. B. Bextine). We are now working to move this scFv into candidate bacterial species for use in a paratransgenesis approach to controlling PD.

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PARATRANSGENESIS TO CONTROL PIERCE'S DISEASE: THE "SOCIAL LIFE" OF *ALCALIGENES XYLOSOXIDANS DENITRIFICANS*

Project Leaders:

Carol Lauzon
Department of Biological Sciences
California State University, Hayward
Hayward, CA 94542

Thomas A. Miller
Department of Entomology
University of California
Riverside, 92521

Collaborators:

Blake Bextine
Department of Entomology
University of California,
Riverside, 92521

David Lampe
Department of Biological Sciences
Duquesne University
Pittsburgh, PA 19219

Don Cooksey
Department of Plant Pathology
University of California
Riverside, 92521

Frank Richards
Yale Medical School
New Haven, CT 06520

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ABSTRACT

Characteristics of *Alcaligenes xylosoxidans denitrificans* (*Axd*), a bacterium associated with *Homalodisca coagulata* (Say) and several host plants of this sharpshooter, were examined because this bacterium is being considered for use as a paratransgenic vehicle for control of *Xylella fastidiosa* (*Xf*). *Axd* established in sharpshooter vectors of *Xf* but was not found in predators that *Axd*-fed sharpshooters. *Axd* did not establish on or within table grapes, or in a variety of red and white wines. The bacterium also did not establish in soil samples. *Axd* catabolized a variety of nitrogen substrates but did not produce certain extracellular enzymes considered to be virulence factors. An auxotrophic subpopulation of *Axd* that requires cysteine was isolated.

INTRODUCTION

Homalodisca coagulata (Say), the glassy-winged sharpshooter (GWSS), acquires and transmits the causative agent of Pierce's disease (PD), *Xylella fastidiosa* (*Xf*). While much attention has been put forth to characterize, describe, and understand the establishment of *Xf* within the sharpshooter, little effort has been directed toward the isolation of other microbial inhabitants within the alimentary canal of GWSS. If GWSS do possess a normal microbiota, or harbor transient microorganisms for some time period, then these microorganisms may inhibit or possibly facilitate the attachment of *Xf* with the sharpshooter. Determination of either scenario would add to what is currently known about PD transmission and could be useful for the implementation of a paratransgenic strategy to control PD.

Alcaligenes xylosoxidans denitrificans (*Axd*) is one bacterium that has been isolated routinely from GWSS Here we report on research over the past year that examines the use of *Axd* as a paratransgenic candidate for management of PD.

OBJECTIVES

1. Characterize the establishment of *Axd* in GWSS, blue green, and smoke tree sharpshooters.
2. Determine the survival and growth of *Axd* in fruits and wine.
3. Determine if *Axd* is passed horizontally to predators of GWSS.
4. Monitor the growth and survival of *Axd* in soil.
5. Survey *Axd* for auxotrophic subpopulations.

RESULTS

GWSS, captured in nature, were aseptically dissected for their alimentary canal organs, particularly, cibarial pumps, fore- and midguts. *Axd*, was the most frequently isolated bacterial species and subsequently was chosen as the first candidate for use in a paratransgenic strategy. We have spent the last year monitoring the activities of *Axd* and transformed strains of *Axd*, spanning the behavior and biology of *Axd* in host plants (both field and laboratory experiments), in sharpshooters, in predators that fed on sharpshooters and under different culture conditions. In summary, we have found that *Axd* and transformed strains of *Axd* establish in plant xylem of a variety of host plants and sharpshooters that feed on these plants. *Axd* and strains thereof do not, however, grow or grow well in soil with established microbial communities. Similarly, *Axd* and strains thereof do not grow in a variety of grapes or wines. *Axd* strains were also not found in predators that fed on sharpshooters. *Axd* strains lack the expression of typical virulence factors, such as extracellular enzymes and β- hemolysin. A cys^- population of *Axd* was isolated and this population, if deemed fit, would not establish in environments lacking cysteine.

CONCLUSIONS

Axd is a promising candidate for a paratransgenic approach to prevent, control, and/or manage Pierce's disease. *Axd* establishes well in vectors of *Xf* and host plant xylem but does not establish well in non-target environments that we have surveyed. If *Axd* cys^- are shown to be fit, the use of this strain would reduce and/or eliminate certain concerns associated with

the release of a genetically augmented bacterium in the environment. Nevertheless, it appears that the spread of *Axd* strains is limited by biotic and abiotic conditions associated with the host plant environment of Pierce's disease susceptible plants.

FUNDING AGENCIES
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CHARACTERIZATION AND IDENTIFICATION PIERCE'S DISEASE RESISTANCE MECHANISMS: ANALYSIS OF XYLEM ANATOMICAL STRUCTURES AND OF NATURAL PRODUCTS IN XYLEM SAP AMONG *VITIS*

Project Leader:
Hong Lin
Exotic & Invasive Disease & Pest Research Unit
USDA-ARS
Parlier, CA 93648

Cooperator:
Andrew Walker
Department of Viticulture and Enology
University of California
Davis, CA, 95616

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ABSTRACT
Understanding and utilizing natural defense mechanisms is a critical component of crop improvement. The ultimate solution to Pierce's disease (PD) problems likely relies on host resistance. This research proposal focuses on examining PD resistance in several grape species. Although some of resistant species have been tested, the mechanisms involving resistance are not well understood. It appears that PD resistance mechanisms vary. Some resistance mechanisms may be related to anatomical characteristics while others may be due to defense chemicals. The strategy that we are developing for this project will provide research tools to investigate PD resistance mechanisms.

INTRODUCTION
Pierce's disease (PD) caused by the *Xylella fastidiosa* (*Xf*) bacterium is a major disease problem in California. The extent to which PD affects the California grape industry has been dramatically increased by the recent introduction and establishment of a more effective vector, the glassy-winged sharpshooter (*Homalodisca coagulata*). Host plant resistance is a critical component of integrated crop management. Understanding the nature or basis of resistance mechanisms and utilizing resistance resources from native grape species offers a direct approach to combating PD.

Resistance to PD had been tested in many grape species (Mortensen et al., 1967, 1977) but the mechanisms involving in PD resistance have not been well characterized, hence, are not well understood. Given the fact that these species were derived from various genetic backgrounds and different origins, it is expected that the mechanisms of PD resistance may be different among grape species.

The hypothesis of this research proposal is that the mechanisms of PD resistance are due to chemical compounds (e.g. anti-microbial activity) present in xylem sap that suppress *Xf* or due to anatomical features of the xylem (e.g. pit membrane) that restricts *Xf*'s mobility or both. Plants produce a vast array of natural defense compounds. Most of these natural products are derived from secondary metabolic pathways. Compounds such as the isoprenoid, phenylpropanoid, alkaloid or fatty acid/polyketide, are continuously expressed in many plants that have proven effective against microbial attacks and insect/animal predation. Other types of compounds that are synthesized *de novo* in response to pathogen attack are known as phytoalexins. Stilbenes are examples of such compounds with low molecular weight phenolics occurring in a number of plant species including grape (Sparvoli et al., 1994). In grape, they act like phytoalexins, in response to against pathogens such as *Botrytis cinerea* (Jeandet et al., 1995), and *Rhizopus stolonifer* (Sarig et al., 1997).

One of the underlining aspects of this study is to determine if *Xf* resistance is confirmed by antimicrobial substances in the xylem sap, thus providing a new approach to controlling PD. Grafting with resistant rootstocks is commonly used to overcome pest and disease problems and nutrients and growth regulators move from the rootstock to the scion. Therefore, identifying and screening xylem antimicrobial compounds, and using these PD resistant plants as rootstocks may provide a unique opportunity for enhancing the expression of PD resistance without genetically modifying the scion. This last point is critical because it will be very difficult to produce economically competitive wine grape cultivars through classical breeding or genetic engineering, because of the conservative and international wine industry. If a rootstock that can confer PD resistance is produced the integrity of wine grape cultivars will be maintained.

OBJECTIVES
1. Evaluate if any structurally related factors are associated with PD resistance. Develop a novel multiple grafting technique to examine the correlation between pathogen movement and the features of anatomical structures. A quantitative PCR-based assay (*Xf*-specific Taq-Man PCR) will be used to measure the mobility of *Xf* and the size of the pathogen population in tissues. Scanning electron microscopy will be used to determine the comparative the anatomical structure of resistant and susceptible plants.
2. To characterize the chemical composition of xylem and identify the substances that may contribute to antimicrobial effects which prevent / suppress *Xf* colonization / multiplication. *In vitro* bioassay and chemical analysis will be used to determine the roles of these compounds.
3. To evaluate the transmissibility of the antimicrobial compounds from resistant plants and evaluate the performance of susceptible scions grafted on these resistant rootstocks.

RESULTS AND CONCLUSIONS

This project was funded in June, 2003. We selected 10 grape species (*Vitis aestivalis, V. candicans, V. champinii, V. labrusca, Muscadinia munsoniana, V. riparia, M. rotundifolia, V. rufotomentosa, V. shuttleworthii, V. simpsonii, V. smalliana, V. tiliifolia,* and *V. vulpina*) from different genetic backgrounds representing a wide range of resistance to PD. Currently, we are propagating these plants in greenhouse for later experiments.

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DIFFERENTIAL PLANT-INDUCIBILITY OF PUTATIVE VIRULENCE GENES BY *XYLELLA FASTIDIOSA* IN SUSCEPTIBLE AND RESISTANT PLANT HOSTS

Project Leader:
Steven E. Lindow
Department of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Cooperator:
Karyn Lynn Newman
Department of Plant and Microbial Biology
University of California
Berkeley, CA 94720

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ABSTRACT
Although many studies have addressed systemic movement of *Xylella fastidiosa* in the plant (4), we still lack a basic understanding of the mechanism by which *X. fastidiosa* cells, which initially are inoculated into relatively few xylem vessels, are able to spread throughout the xylem network of the host plant. Furthermore, we lack an understanding of why systemic movement by *X. fastidiosa* occurs in some host plant species but not in others. In other plant pathogens, including *Xanthomonas campestris*, which is closely related to *X. fastidiosa*, the expression of virulence genes such as those encoding gum synthesis, endoglucanase and pectinase activities, is required for systemic movement *in planta*. The genomic similarities between *X. fastidiosa* and *Xanthomonas* strongly suggest that similar virulence genes are required by *X. fastidiosa* for symptom formation in the plant (2). In some plant pathogens expression of virulence genes is dependent on certain plant factors. In this study we aim to determine the expression pattern of these virulence genes by *X. fastidiosa* during colonization of grapevines susceptible and resistant to Pierce's disease. Reporter genes will be placed under the control of regulatory regions of the genes to be studied, and these promoter-reporter constructs will be integrated into the *X. fastidiosa* genome to allow their stable expression *in planta*. Reporter gene expression will be monitored during different phases of disease progress to elucidate when expression of each gene occurs during disease progression. These data will enable us to construct a fairly accurate description of the course of virulence events leading to Pierce's disease after infection and to detect plant factors that affect virulence of *X. fastidiosa*.

INTRODUCTION
Traits in bacteria that contribute to virulence are often expressed only in the presence of a susceptible plant. In a variety of bacterial species, genes such as *hrp* genes involved in secreting virulence effector proteins into plant cells as well as those conferring the production of extracellular toxins or enzymes important in the disease process are not expressed in culture, but are rapidly expressed when bacterial cells are introduced into plants (1, 15). Similarly, many genes in the symbiotic nitrogen fixing bacterium *Rhizobium* are not expressed in culture but are expressed during the process of infection of roots, often in response to compounds leaked from the plant itself (12). While in many cases the plant factors that are involved in inducing expression of bacterial genes are unknown (5, 13), in several instances those plant compounds that modulate expression of bacterial virulence genes have been identified. For example flavanol glucosides such as quercetin and kaempferol as well as arbutin induce the expression of the *syrB* gene of *Pseudomonas syringae* that is required for production of the phytotoxin syringomycin (10, 14). Likewise, shikimic and quinic acids were found to induce production of toxin gene expression in *P. syringae* pv. *syringae* (7). Importantly, higher levels of plant-inducible gene expression were observed in those plant species that were most susceptible to these pathogens (6, 7). For example, the highest induction of *syrB* was found in extracts from the bark of the most susceptible cherry trees (6) and coronatine biosynthetic genes were induced to much higher levels when bacteria were introduced into host plants compared to non-host plants (7). Thus it seems clear that virulence genes are not constitutively expressed in plant pathogenic bacteria and that plants often inadvertently induce expression of such genes in the pathogens.

There is strong circumstantial evidence that *X. fastidiosa* expresses many of its virulence genes only when in plants. For example, polysaccharides that surround bacterial cells in xylem vessels are a prominent feature of Pierce's disease infections in grape, and the *X. fastidiosa* genome contains *gum* genes for the production of extracellular polysaccharide. However, such materials are produced in only VERY small amount in culture. This suggests that if, as is commonly thought, the polysaccharide in plants is of bacterial origin, than the *gum* genes must be induced when cells are in the plant. Likewise, there is little evidence of production of cellulases or other extracellular enzymes in X. *fastidiosa* cultures (J. Labovitch, personal communication). Preliminary studies have been made of gene expression in *X. fastidiosa* using DNA microarrays on membrane filters to assay the abundance of mRNAs corresponding to virulence genes (3). These expression arrays revealed that most of the several likely virulence genes such as those conferring production of cellulases, xylanases, pectinases, as well as regulators of other virulence factors were all expressed at a very low level in *X. fastidiosa* cultures (3). These results are consistent with the lack of evidence for virulence factors detected in cultured cells. At this time it is impossible to use such hybridization techniques to assess gene expression of bacteria while in plants. Instead it is possible to assess gene expression *in planta* using powerful and sensitive reporter genes such as *inaZ*, conferring production of ice nucleation protein, which is easily detected in "dirty" biological systems such as within plants, and when bacterial cell numbers are low. The thrust of this proposal will be to obtain direct estimates of virulence gene expression *in planta*.

One important question is whether resistance to Pierce's disease by certain plant hosts is due to *X. fastidiosa's* virulence strategy being unsuccessful in resistant plants or is attributable to the plant's ability to modify the behavior of the bacteria via host-specific induction of virulence genes. We hypothesize that in resistant plant hosts, *X. fastidiosa* is not expressing these virulence genes. Alternatively, *X. fastidiosa* may express virulence genes to the same extent in resistant and susceptible hosts but host plant anatomy may differ in its ability to resist the effects of *X. fastidiosa*-generated enzyme activity. This distinction will be important in developing resistant cultivars of grapevines.

To distinguish between these possibilities, we aim to use the genomic sequence of *X. fastidiosa* to identify the regulatory regions of putative virulence genes and to fuse these regions to reporter genes. We will monitor expression of the reporters in tolerant and susceptible grapevine cultivars and resistant alternate hosts as a measure of virulence gene expression. By comparing the level of expression of putative virulence genes by *X. fastidiosa* in resistant and susceptible plants we will learn whether resistance is a function of plant structure or *X. fastidiosa* behavior in different plants. In addition, we will gain an understanding of the pattern of expression of these genes during colonization. This work's contribution to a basic understanding of the mechanisms of *X. fastidiosa* systemic movement and plant resistance will be very useful for researchers attempting to find strategies for controlling disease in important agricultural plants. If, as we hypothesize, plant factors are involved in regulating virulence in a cultivar-specific or species-specific manner, it should be possible to target the production of such "inducers" of virulence in breeding programs or in transgenic plants in order to yield resistant grapevines.

OBJECTIVES

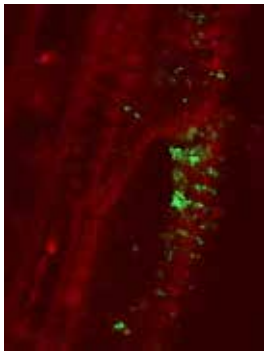
1. Construct *Xylella fastidiosa* strains that will report expression of putative virulence genes. This will be accomplished by fusing regulatory regions of putative virulence genes to the reporter genes *inaZ* and *gfp* and inserting these expression cassettes into a suicide vector, then integrating these constructs into the *X. fastidiosa* genome.
2. Determine putative virulence gene expression *in planta* and determine whether any correlations exist between expression and symptom development and/or population size. This will be accomplished by introducing the reporter strains into tolerant and susceptible grapevines and resistant alternate hosts and monitoring symptom development, *X. fastidiosa* populations and expression of InaZ activity (which will represent expression of putative virulence genes) in plant macerates at various times post-inoculation.
3. Obtain fine scale spatial information about virulence gene expression and determine whether there is a correlation between expression and colony size by using confocal laser scanning microscopy to locate *X. fastidiosa* colonies *in planta*, to measure their size and to monitor Gfp expression (which will represent expression of putative virulence genes) during timepoints determined to be critical in Objective 2.

RESULTS

Objective 1. Regulatory sequences of the putative virulence genes *gumB, engXCA,* and *pg* have been obtained by amplifying the 200-300 bp region upstream of the start codon from the *X. fastidiosa* Temecula genome by polymerase chain reaction. The promoters have been fused to two reporter genes, *InaZ* and *gfp[ASV]*.

The *InaZ* gene encodes a protein that nucleates ice formation in super-cooled water and is an excellent reporter system for monitoring gene expression in plant pathogenic bacteria (8). Advantages of this reporter are its ease of use (plant macerates are directly tested in a quick, simple, inexpensive ice nucleation assay), its extreme sensitivity (gene expression can be measured in as few as 10 cells) and the ability to quantify levels of gene expression. This sensitivity is crucial because virulence genes may be expressed only at very low levels or at specific times during colonization and sensitivity ensures that we will be able to detect even very low expression in a plant macerate. Likewise, we know populations are low in resistant plants and we need to monitor gene expression in such low populations. One drawback to this system is that only macerated tissue can be examined, however it will be ideal for elucidating overall gene expression characteristics, such as when expression occurs in relation to inoculation, what population levels are correlated with expression, and whether expression differs between resistant and susceptible hosts.

Figure 1. *X. fastidiosa* expressing green fluorescent protein observed in an unperturbed xylem vessel by CSLM.



The *gfp[ASV]* gene encodes an unstable variant of the green fluorescent protein. The unstable nature of the gfp will enable us to achieve the most accurate data on promoter activation conditions (9). *X. fastidiosa* has been successfully engineered to

express a green fluorescent protein constitutively (11). Fluorescent *X. fastidiosa* cells can be directly visualized in the plant using confocal laser scanning microscopy (CSLM). This type of microscopy captures images from deep within a sample, allowing visualization of unperturbed *X. fastidiosa* cells in intact xylem vessels (Figure 1). In addition, no fixation, washing or staining are needed and sample dissection is minimal, eliminating the potential for artifacts that plagues other types of microscopy used for *in planta* analysis of *X. fastidiosa*. This gfp expression is stable *in planta* and disease symptom formation and growth in the plant are the same for the gfp-expressing *X. fastidiosa* as for the wild type (11). In this study, *X. fastidiosa* will be engineered to express a green fluorescent protein under the control of virulence gene regulatory sequences. CSLM will be used to visualize *X. fastidiosa* cells in the plant and determine the pattern of virulence gene expression (which will correspond to green fluorescence). We will be able to visualize directly any cultivar-specific differences that were found using the InaZ system. This will be important for verifying whether differences detected are correlated with different patterns of growth in those plants or are due to a difference in host-specific gene induction.

These promoter-reporter cassettes have been introduced into plasmids designed to integrate into the *X. fastidiosa* chromosome (Figure 2). These plasmids have been electroporated into *X. fastidiosa* and we are currently screening

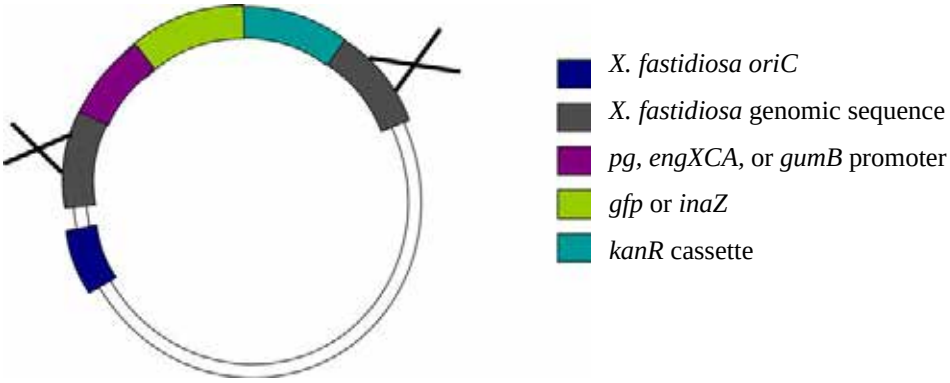


Figure 2. Design of suicide plasmids for integration of promoter-reporter and marker genes into the *X. fastidiosa* genome. Black "x" marks site of homologous recombination with the *X. fastidiosa* chromosome.

transformants for correct insertions. Since the project has only just recently started, we are performing initial aspects of the proposed work. The studies are proceeding according to schedule. The completion of this objective will result in the strains listed in Table 1.

Table 1. *X. fastidiosa* strains to be constructed

Strain	*Promoter*	*Reporter*	*Markers*	*Putative virulence activity*
Pg-ice	*pg*	Ice nucleation	Kan-2	pectinase
Pg-gfp	*pg*	Green fluorescence	Kan-2	pectinase
Eng-ice	***engXCA***	Ice nucleation	Kan-2	cellulase
Eng-gfp	***engXCA***	Green fluorescence	Kan-2	cellulase
Gum-ice	*gumB*	Ice nucleation	Kan-2	extracellular polysaccharides
Gum-gfp	*gumB*	Green fluorescence	Kan-2	extracellular polysaccharides

Objectives 2 and 3. These objectives will be addressed after the completion of Objective 1.

CONCLUSIONS

Conclusions will be drawn from future data that will be obtained during fulfillment of Objectives 2 and 3.

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EXPLORATION FOR FACULTATIVE ENDOSYMBIONTS OF SHARPSHOOTERS

Project Leader:
Alexander H. Purcell
Division of Insect Biology
University of California
Berkeley, CA 94720

Researcher:
Clytia Montllor Curley
University of California
Division of Insect Biology
Berkeley, CA 94720-3112

Cooperators:
Eoin Brodie (laboratory of Mary Firestone) and George Roderick
Dept. of Environmental Science, Policy and Management
University of California, Berkeley, CA

Chris Carlton
Dept. of Entomology
Louisiana State University
Baton Rouge, LA 70803

Russell Mizell
University of Florida
NFREC
Monticello, FL 32351-5677

Reporting Period: The results reported here are from work conducted from October 2002 to September 2003.

ABSTRACT
Glassy-winged sharpshooters (GWSS) were collected in California and several states in the southeastern US in 2002 and 2003 to search for pathogenic or beneficial bacteria of GWSS. Various tissues were examined for the presence of bacteria by PCR: hemolymph, eggs, and bacteriomes. Hemolymph could contain primary and secondary symbionts, either resident in hemolymph or in transit to other tissues. Eggs were expected to contain any transovarially transmitted microorganisms, described or otherwise, and bacteriomes were expected to contain at least the primary symbionts. A small subset of hemolymph and egg samples were cloned and sequenced based on unique digest patterns of their extracted 16s DNA. Cloned sequences were identified as belonging to the primary symbiont *Baumannia* (in eggs and hemolymph) and *Acinetobacter* and *Stenotrophomonas* (in hemolymph). *Wolbachia* was also cloned from hemolymph, and subsequently found in 62% (24/39) of egg, 8% (5/66) of hemolymph, and 76% (31/41) of bacteriome samples by PCR.

INTRODUCTION
Most Homoptera so far examined, including the leafhoppers (Cicadellidae), are hosts to endosymbiotic bacteria that live within the body cavity of the host insect (Buchner 1965; Douglas 1994; Moran and Baumann 2000). Some endosymbionts ("primary" symbionts), have a mutually obligate association with their host: they are housed in specialized host cells, bacteriocytes (= mycetocytes) within a bacteriome (collection of bacteriocytes); are transovarially (i.e., vertically) transmitted from mother to offspring, and cannot be cultivated outside of the host. In aphids, the primary symbionts (*Buchnera*) have been shown to provide the host with essential nutrients and are necessary for the proper development of the host (e.g., Douglas 1998). Little is known about the specific role of leafhopper primary symbionts. Suggestions include roles in osmoregulation, respiration, circadian rhythms (Schwemmler 1980) and nutrition (Douglas 1988). Recent work on the bacteriome-associated symbionts of GWSS described two bacteria, one of which has been partially sequenced and placed in the γ-Proteobacteria, (named *Baumannia cicadellinicola)*, the second being a Flavobacteria (Moran et al. 2003).

Primary symbionts are of great interest in terms of their effects on their hosts. However, of interest for this work are bacterial associates that are facultative (also referred to as "secondary"), i.e., that occur in some individuals or populations but not others, and that could be introduced into, or be augmented in, pest populations. Facultative bacterial associates have been described in a variety of Homoptera such as mealybugs (Fukatsu and Nikoh 2000), psyllids (Thao et al. 2000), aphids (Buchner 1965, Chen et al. 1996, Fukatsu et al. 2000, 2001, Sandstrom et al. 2001) and leafhoppers (Swezy and Severin 1930, Schwemmler 1974, McCoy et al. 1978, Purcell et al. 1986). The potential importance of these long-unrecognized microbial associates has become increasing clear from recent studies with aphids, where the effects of facultative symbionts varied from positive to negative, and affected host plant suitability (Chen et al. 2000, Leonardo and Muiru 2003), susceptibility to high temperatures (Chen et al. 2000, Montllor et al. 2002) and to parasitism (Oliver et al. 2003), often at dramatic levels.

We assume that similar bacterial symbionts and associated bacteria in leafhoppers such as sharpshooters are likely to be as common as in aphids and also to have large and important effects on the population biology of their hosts. Molecular tools have recently made their detection and characterization more feasible (Moran et al. 2003). A cultivable, facultative symbiont (called BEV) that occurs in the leafhopper *Euscelidius variegatus* was transmitted transovarially from mother to offspring (Purcell et al. 1986). BEV reduced fecundity by 80%, doubled development time, and increased mortality of its host (Purcell et al. 1986, Purcell and Suslow 1987). In addition to being transovarially transmitted, BEV was transmitted efficiently among *E. variegatus* leafhoppers (i.e., "horizontally") via feeding within plant tissues (Purcell et al. 1994).

Facultative symbionts could be convenient agents for introducing genetic materials into leafhopper populations. Advances in molecular biology have made the prospects of identifying, isolating, and manipulating even non-cultivable symbionts realistic. Essentially, symbionts that have a high rate of maternal transmission represent a type of cytoplasmically-inherited genome that may be more amenable to molecular manipulations than leafhopper chromosomal genes. Therefore it is of major importance to better understand these potentially useful bacteria in GWSS and related leafhoppers.

OBJECTIVES

1. Survey glassy-winged sharpshooter and other sharpshooters in California and the southeastern U.S. for facultative bacterial endosymbionts.
2. Determine by DNA sequencing the identity of any bacteria discovered.
3. Depending on type of microorganism and relative frequency in surveyed insects, select candidate symbionts to (a) attempt to culture, (b) determine whether they can be transmitted by injection of hemolymph from infected to uninfected GWSS or to other sharpshooter species, (c) determine whether they are transovarially transmitted, (d) determine whether they can be horizontally transmitted through plants and (e) determine whether any are beneficial or pathogenic to GWSS in terms of life history traits (growth, fecundity, longevity, parasitism).

RESULTS AND CONCLUSIONS

GWSS hemolymph samples from California and the southeast collected in 2002 and 2003 were extracted and examined by PCR for eubacterial 16s ribosomal DNA (Table 1).

Table 1. ***GWSS tissues sampled for bacterial DNA—collection location and date.***

LOCALITY	DATE	TISSUES[1] (NO. OF SAMPLES)	TOTAL NO. EXTRACTS
Bakersfield CA	Apr 2002 Aug 2002 June 2003	H (2) , B (3) H (25), B (14), E (10) H (18)	72
Riverside CA	Apr 2002 Oct 2002	H (3), B (5) H (9), B (2)	19
Baton Rouge LA	May 2002 Sept 2002	H (39), B (20), E (14) H (5), B (4)	82
Pearl River LA	June 2003	H (15), B (5),E (5)	25
Quincy FL	May 2002 Aug 2002	H (25), B (9), E (7) H (18)	59
Crestview FL	June 2003	H (24), B (8),E (19)	51
Tallahassee FL	June 2003	H (9), E (3)	12
Dothan AL	June 2003	H (9), E (3)	12
State Line AL	June 2003	H (6)	6
Martinville MS	June 2003	H (8), E (1)	9
McComb MS	June 2003	H (6), E (3)	9

[1]H=hemolymph, B=bacteriocytes, E=eggs

Forty-three percent of hemolymph samples from all localities tested positive for eubacterial 16sDNA by PCR. Twenty-six individuals of another four species of sharpshooters from California were also tested for bacteria in hemolymph, of which five (19%) were positive by PCR. DNA from a total of 12 GWSS tissue samples was chosen for cloning, and four produced multiple transformed *E. coli* colonies with 16s rDNA inserts. DNA from 11 of these colonies was chosen for sequencing. The most common sequence was identical to that of *Baumannia*, a recently described bacteriome-associated symbiont of the GWSS (Moran et al. 2003), which was cloned from hemolymph and eggs of GWSS from Louisiana and Florida (Table 2). Like other bacteriome inhabitants, *Baumannia* is presumably transovarially transmitted from mother to offspring via hemolymph (Buchner 1965). *Wolbachia*, a commonly found facultative symbiont of many insects, including GWSS (Moran et al. 2003), was also cloned from hemolymph of a California GWSS.

Table 2. ***Cloned bacterial DNA from GWSS tissue samples.***

COLLECTION LOCATION (SAMPLE / NO. CLONES SEQUENCED)	GWSS TISSUE	16S RDNA SEQUENCE IDENTITY OF INSERTS
Bakersfield (UC2/ 2)	hemolymph	*Wolbachia, Acinetobacter*
Louisiana State Univ (L20-8/ 5)	hemolymph	*Baumannia, Stenotrophomonas*
Crestview FL (CF7/ 2)	eggs	*Baumannia*
Pearl River LA (PRE/ 2)	eggs	*Baumannia*

DNAs from two additional bacteria not previously described from GWSS were also cloned from our samples (Table 2): *Acinetobacter* and *Stenotrophomonas* are aerobic γ-Proteobacteria, and not uncommon as environmental contaminants and nosocomial pathogens (e.g., Towner et al. 1991, Ribbeck et al. 2003). However, both have also been isolated from ticks and fleas (Murrell et al. 2003); and *Stenotrophomonas*, among other bacteria, was isolated from the guts of ants, where it was

presumed to provide nutrients and to be passed to offspring (Jaffe et al. 2001). *Stenotrophomonas* was also described as an endosymbiont of a fly (Otitidae), which failed to develop properly without its complement of bacteria (Wozniak and Hinz 1995).

After isolating *Wolbachia* from GWSS, we surveyed 146 of our extracts by PCR and detected this bacterium in 62% (24/39) of egg, 8% (5/66) of hemolymph, and 76% (31/41) of bacteriome samples by PCR. We will assess the frequency of the other bacteria we have detected in GWSS samples to date, and continue to look for additional facultative symbionts in extracted material and new collections. Finding a facultative symbiont of GWSS could impact the biological control of GWSS if such symbionts could be manipulated or eliminated from populations of GWSS.

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

Project Leader:
Alexander H. Purcell
Division of Insect Biology
University of California
Berkeley, CA 94720-3112

Researcher:
Rodrigo P.P. Almeida
Department of Plant and Environmental Protection Sciences
University of Hawaii at Manoa
Honolulu, HI 96822

Cooperators:
Steve Lindow
Plant and Molecular Biology
111 Koshland Hall
University of California
Berkeley, CA 94720-3102

Karyn Newman
Plant and Molecular Biology
111 Koshland Hall
University of California
Berkeley, CA 94720-3102

Reporting Period: The results reported here are from work conducted from October 2002 to October 2003.

ABSTRACT
Although it is known that *Xylella fastidiosa* (*Xf*) is located on the foregut of infected insects (infective nymphs loose transmissibility after molting and there is no latent period required for transmission), the exact location in the foregut from which the bacterium *Xf* is transmitted by sharpshooter leafhoppers is not known. We examined the spatial distribution of *Xf* in the precibarium of vectors that had been fed on infected grapevines. *Xf* cells attached on end to the insect's cuticle and were distributed throughout the precibarium, with few exceptions, on both pharynges. *Xf* was not present on top of the precibarial valve, but interestingly cells were observed within the valve's pit. In a transmission experiment with long inoculation access period, all infective insects transmitted *Xf* to healthy grapevines; individuals free of *Xf* on the precibarium did not vector the pathogen. An ancillary objective was to determine if the relatively low transmission rates (5-15% daily) by the glassy-winged sharpshooter (GWSS) were influenced by the plant tissue inoculated: stem or leaf. On grape, adult GWSS feed mainly on stems. In an initial experiment with low transmission rates (less than 7% per GWSS adult) there were no differences among treatments (stem only, leaf only, stem and leaf). This will be repeated with more insects per plant and longer exposure for inoculation, but these initial results suggest that feeding site on green tissues are not a major factor.

INTRODUCTION
The vector transmission of the bacterium *Xylella fastidiosa* (*Xf*) to plants is an essential step in the spread of Pierce's disease (PD). The process of transmission would seem to be simple. Virtually any xylem sap-feeding insect can be vector (Frazier 1966). There is no – or at most 30-60 minutes -- latent period, the time required between acquisition and inoculation. Because vectors stop transmitting immediately after molting, the bacteria must be transmitted from the foregut, whose lining is shed with molting (Purcell and Finlay 1979). The hypothetical model for transmission is that the bacteria attach to the foregut during feeding on *Xf*-infected plants, and some bacteria are detached during later feeding to inoculate other plants (Purcell et al. 1979).

But this simple view is deceptive. First of all, very few live cells of *Xf* -- certainly less than 200 per insect -- are needed for efficient transmission by the blue-green sharpshooter (BGSS) (Hill and Purcell 1995) or the glassy-winged sharpshooter (GWSS) (Almeida and Purcell 2003). This makes determination of the infective status of insect vectors very difficult because even highly sensitive methods for *Xf* detection do not detect the bacterium in all transmitting insects. The small number of *Xf* needed for efficient transmission implies that the area of the foregut involved in transmission is very small, thus saturated by small numbers of *Xf*, which implies that large mats of *Xf* often seen in transmitting sharpshooters are superfluous for transmission.

The attachment (and subsequent detachment) of *Xf* to the vector foregut is thought to be a key in its vector transmission to plants (Purcell et al. 1979) and has provided an incentive to better understand this phenomenon. How these tiny bacteria remain attached to the insect foregut in such a fast-moving fluid environment of 5 to 10 cm/second is still unknown. The use of the cell signaling mutants (Newman and Lindow, in this Proceedings) has opened new ways to examine experimentally the vector transmission process. Although not an objective of this related project, it is important to learn why cell-cell signaling by *Xf* is so important to transmission.

A second transmission phenomenon we propose to evaluate using GWSS is whether access to leaf petioles, young stems, or the bases of stems affects vector transmission efficiency in grape. GWSS adults prefer feeding on stems of grape test plants, but nymphs and young adults tested on leaves (including leaf petiole) were much more efficient at transmitting than were adults (Almeida and Purcell 2003). Leaf age had a relatively small but statistically significant effect on transmission by BGSS (Purcell 1981). The procedures to test this are simple and straightforward. It is important to know how various locations on grape affect transmission performance in order to incorporate vector transmission rates in estimates of economic thresholds for control of GWSS.

OBJECTIVES

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

RESULTS

The morphology of the precibarium (a canal between the food canal of the stylets and the sucking pump formed by the combined pharynges) of BGSS has been previously described (Backus and McLean 1983, Brlansky et al. 1983, Purcell et al. 1979). The channel was divided into distal and proximal regions by A flap-like valve located on the epipharynx. On the epipharynx we observed the 10 D-sensilla and 8 P-sensilla first identified by Backus and McLean (1983). The 2 H-sensilla were also observed. The proximal section of the precibarium, posterior to the valve, had sutures on the epipharynx that were not present on the hypopharynx.

Xf cells were observed on both pharynges, always attached to the insect's cuticle at each bacterium's narrow end. Probably because of the long incubation period given to insects after pathogen acquisition, we observed only cell mats rather than isolated cells/colonies. The hypopharynx had homogenous mats, with only one area always free of *Xf* (Figure 1). Based on measurements of both pharynges, this is the area where the precibarial valve or pit probably interlocks with the hypopharynx. In some cases, cells were observed only proximally to the precibarial valve; if present distally, they were always observed proximally. Cells were never found on top of the precibarial valve or the area on the epipharynx with sutures (Figure 2). Cells were often found within the precibarial valve's pit. The proximal end of the precibarium was also a location where *Xf* attached. *Xf* was also observed in the groove at the distal end of the cibarium. We also dissected 15 GWSS adults that had 4 days acquisition access period on infected plants and were transferred as a group to mugwort for 2 weeks. Only one individual was observed with *Xf*, distributed in the same manner as on GWSS.

Nineteen of the 25 insects used in our transmission experiment were adequately dissected to evaluate presence or absence of *Xf* on the precibarium. Ten insects were positive, and all of them transmitted the bacterium to grapevines. The other 9 individuals were *Xf*-free and did not transmit to plants. Location of *Xf* was the same as described above. Because of the long incubation period used, we cannot infer the location where *Xf* probably detached to inoculate the healthy plants. *Xf* transmission does not require a latent period, thus no bacterial multiplication is required for transmission. Transmission experiments using short acquisition and inoculation access periods are being conducted to determine if we can consistently locate initial *Xf* colonization areas in the foregut of transmitting insects.

CONCLUSIONS

1. Using long acquisition and inoculation access periods, we found a good correlation between vector transmission to plants and the occurrence of *Xf* on the precibarium of an efficient vector (BGSS).

2. There are specific areas on the precibarium of both pharynges where *Xf* is absent in *Xf*-colonized sharpshooters. One of these is on the distal extremity of the precibarial valve. Another is where the valve should seat against the hypopharynx. A third is the medial region of the proximal precibarium on the epipharynx. These areas might represent areas where the detachment of *Xf* cells occurs during the inoculation phase of vector transmission so that the bacteria can be expelled during feeding.

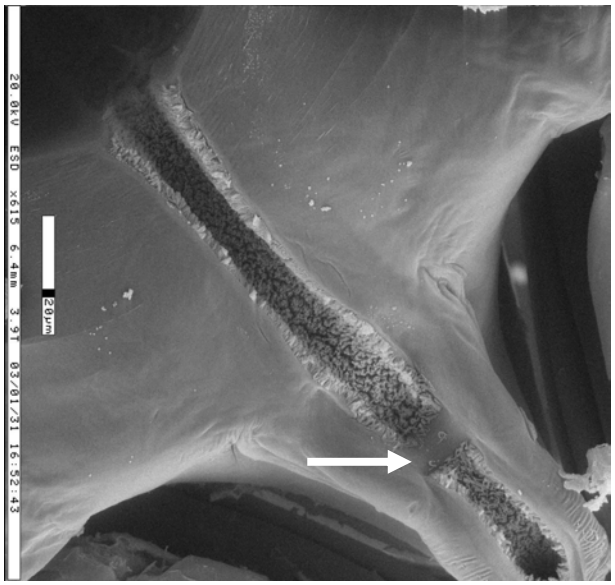

Figure 1. Hypopharynx of BGSS with *Xf* cell mat. Arrow indicates region where *Xf* was always absent.

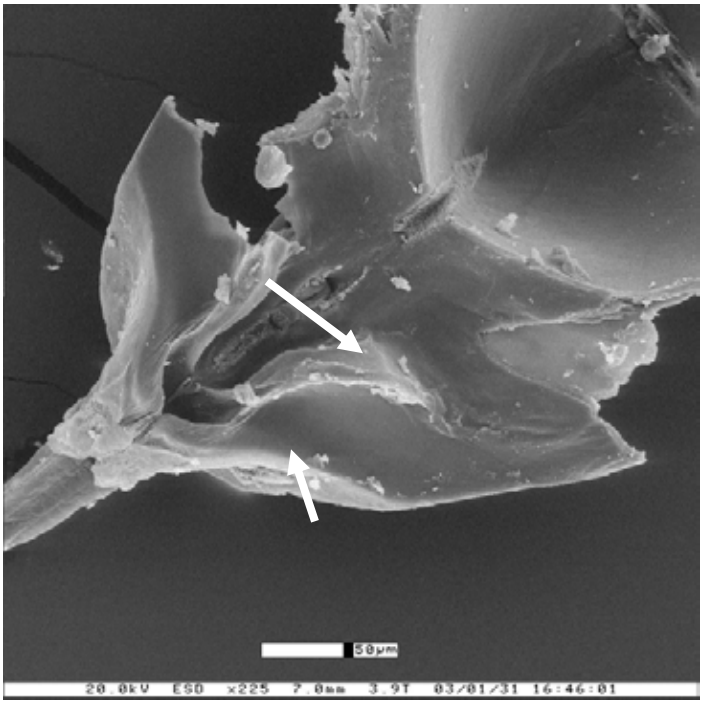

Figure 2. Epipharynx of BGSS with *Xf* cell mat. Arrows: a) precibarial valve, with no *Xf* attached on top of the flap-like structure; b) proximal region of precibarium canal free of *Xf*.

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MECHANISMS OF PIERCE'S DISEASE TRANSMISSION IN GRAPEVINES: RELEVANCE OF HYDRAULIC ARCHITECTURE

Project Leaders:

Thomas L. Rost
Section of Plant Biology
University of California
Davis, CA 95616

Mark A. Matthews
Dept of Viticulture and Enology
University of California
Davis, CA 95616

Joshua F. Stevenson
Section of Plant Biology
University of California
Davis, CA 95616

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ABSTRACT

The arrangement of vascular tissue within the nodes of Chardonnay grapevine (*Vitis vinifera*) shoots was studied as an investigation of potential pathways of infection for the bacterium that causes Pierce's disease (*Xylella fastidiosa*). Grapevine stem anatomy of the current year's growth was observed with both light and scanning electron microscopy and xylem conductance was observed by following traces of stains within the vascular tissue. The pattern of vascular divergence to lateral organs is described and implications for the spread of Pierce's disease within the grapevine shoot are discussed.

INTRODUCTION

The spread of *Xylella fastidiosa* (*Xf*) bacteria or bacterial products within the grapevine likely leads to Pierce's disease (Hopkins and Mollenhauer 1973). A thorough understanding of the hydraulic architecture is necessary to predict both the effect of localized xylem blockage on distal or basal organs and the pathways for movement of bacteria or phytotoxins within grapevine shoots. The general vegetative anatomy and the primary vascularization of grapevine have been summarized (Fournioux and Bessis 1973, Pratt 1974, Fournioux and Bessis 1974, Fournioux and Bessis 1979, Mullins et al. 1992). Although patterns of grapevine hydraulic architecture have been proposed, confirmation of this descriptive work is needed.

OBJECTIVE

Analyze the vascular arrangement of the grapevine in the context of the spread of Pierce's disease within the plant from the site of inoculation to a systemic presence.

RESULTS

Grapevine nodes were serially sectioned (Figure 1). In the most basal section below the node, a complete ring of secondary xylem (wood) is present bounding a regular parenchymatous pith. At this location, less than a centimeter from the node proper, there are no visually distinct leaf traces. In the next distal section, five leaf traces are distinct and have begun to diverge from the stele; two dorsal traces, two ventral traces, and a lateral trace (Figure 1B). The number of leaf traces at each node was variable; ranging from four to eight traces, with five being the most common. Anastomosis of leaf traces may begin while the trace is still in the stem cortex before it enters the petiole. As a consequence of leaf trace divergence from the stele a leaf gap in the vascular cylinder is created. Once the leaf traces have diverged from the stele in an axial orientation, their pathway quickly bends perpendicular from the vertical axis and they become radially oriented. Within a few millimeters after the leaf traces have diverged into the petiole, the parenchymatous gaps in the stele are no longer present and no leaf traces are distinct for further distal nodes. Large portions of the lateral regions of the node are void of conductive tissue (gap) due to vascular divergence into the tendril on one side and to the developing summer lateral shoot and compound bud on the other side (Figure 1E). Within one lateral gap, vascular differentiation occurs between the developing summer lateral shoot and each of the compound buds to the existing axial hydraulic network. Integration of the developing xylem of the summer lateral shoot to the axial system occurs through differentiated branch traces connecting the base of the summer lateral to the xylem of the main shoot (Figure 1F). The common summer lateral shoot and compound bud gap and the tendril gap close in the stele within a few millimeters distal to these respective organs (Figure 1H). Additionally, a sclerified parenchymatous diaphragm present in the node has been replaced by a regular unsclerified parenchymatous pith. Acropetal to the node, the stele is again complete with no gaps in the stele or distinct traces present.

A transverse section through the shoot shows an early stage of leaf trace divergence similar to that depicted in Figure 1B (Figure 2). There is a difference in vessel diameter between the lateral and dorsiventral sectors of the grapevine stem; laterally sectored vessels typically have narrow diameters and dorsiventral vessels have wider diameters. From this particular sample, the dorsi-ventral sector vessel diameters were 65 ± 21 microns (mean $\pm$ sd), whereas lateral sector vessel diameters were 31 ± 12 microns. In this case there are five leaf traces; two in the dorsal sector, two in the ventral sector, and one in a lateral sector. The leaf traces are identifiable by tracheary elements of a comparatively narrow diameter. The leaf traces each originate from a single lamella, and as with all the lamellae of the stem, are delimited by very tall rays. The exact lammelar location of trace origin is variable for all traces from node to node, with the lateral trace(s) sometimes originating nearly from the ventral side. The leaf traces diverge into the petiole at nearly a right angle to the axial system. Each pair of dorsal and ventral leaf traces may appear to fuse (Figure 2 upper right) or remain distinct (Figure 2 lower right).

Figure 1. Representative images from serially sectioned node (left). Images B, E, F, and H correspond to sections through locations (D1, D2–dorsal traces, V1, V2-ventral traces, L-lateral trace, TG-tendril gap, CG-common lateral shoot and compound bud gap, T-tendril, P-petiole, Pi-unsclerified pith, SL-summer lateral, arrow in F indicates branch traces, arrow in H indicates closing lateral gap).

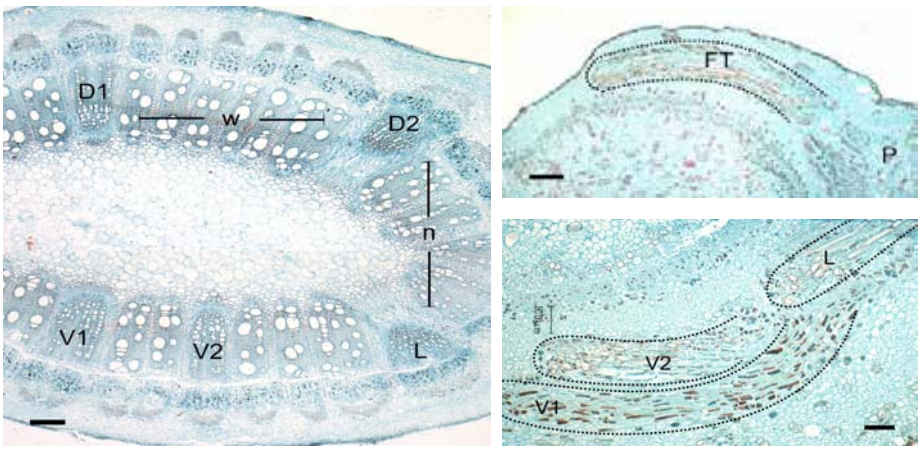

Figure 2. Cross sections displaying diverging leaf traces, stem vessel dimorphism, and subjective fusion of traces (D1, D2–dorsal traces, V1, V2-ventral traces, L-lateral trace, w-dorsal sector with wide vessels, n-lateral sector with narrow vessels, FT-dorsal traces that appear fused, P-petiole)

Multiple stains were used as tracers to follow potential pathways of water and bacterial movement through the node allowing each set of dorsiventral traces and subsequent anastamoses to be followed independently (Figure 3). As the stains moved through the node region, no stains were observed across the sclerified parenchyma diaphragm indicating an absence of medullary vascular connections. The stains also allowed visualization of trace divergence from the stele over a distance of roughly a millimeter and the leaf gap that was created.

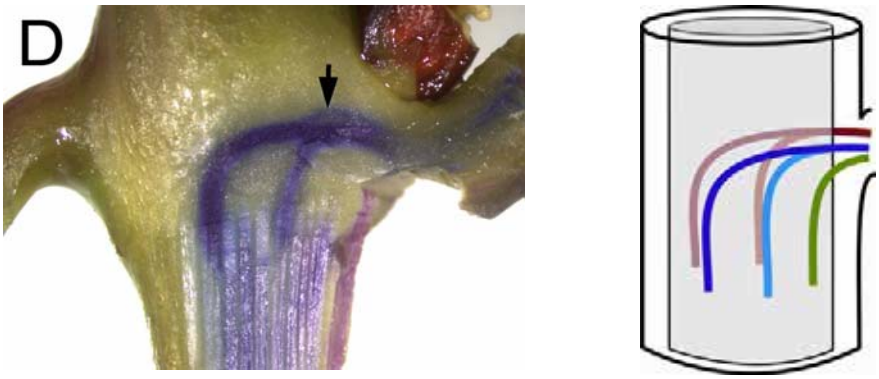

Figure 3. Stain tracer initiated in stem tissue following the conductive stream within leaf traces into the petiole (left). Model of leaf trace divergence into petiole based on serial sectioning and stain tracers (right). Arrow indicates distinct leaf trace.

Tissue macerations were made of samples dissected from a stem internode (wood and pith), stem node (wood and diaphragm), leaf trace, petiole, young summer lateral, and tendril. Vessel elements in the stem typically had scalariform

lateral wall pitting and simple perforation plates verified by resin casting and electron microscopy (Figure 4). Vessel elements with helical secondary walls and simple perforation plates were predominant in the leaf trace and petiole macerations, but were also visible in all other samples likely representing the primary xylem component of these tissues. Narrow tracheary elements commonly bordered wide vessels and are likely vasicentric tracheids (Metcalf and Chalk 1950). These vasicentric cells possessed tracheid-like qualities including delicate spiral thickenings and bordered pits, however, absence of perforations could not be verified.

CONCLUSIONS

Examination of the vascular structure of the node of *Vitis vinifera* Chardonnay grapevine confirmed many aspects of prior investigations, however, no evidence was found that the traces of each leaf are distinct for four internodes before they leave the stele (Fournioux and Bessis 1979), or that trace fusion necessarily occurs between pairs of dorsal and ventral leaf traces (Fournioux and Bessis 1974). Additionally, variability in number of leaf traces present at each node presented here was not suggested in previous published reports (Fournioux and Bessis 1979).

No distinct traces were observed in serial sections either immediately before a node, or immediately after a node. Leaf traces of fully expanded leaves were visibly and conductively distinct only a few millimeters before divergence from the stele. Leaf gaps closed within a few millimeters of trace divergence, and after gap closure no distinct traces for successive nodes were observed. If traces are not visibly distinct across even one node, there is little support for the idea that traces are distinct for four nodes of mature tissues. This is a significant finding for the study of the spread of Pierce's disease within a grapevine as bacteria must move through leaf traces to colonize the leaf lamina and vice versa. If leaf traces are distinct for four nodes then *Xf* present in a specific leaf would have to be directly inoculated, inoculated within a trace that supplies that leaf, or enter a trace four nodes basal to a leaf from adjacent stem xylem. If leaf traces are only distinct for a short length many more sources of inoculation are possible.

Fusion between mature traces is subjective based on the juxtaposition of two or more adjacent traces. Dorsal and ventral traces were observed from the point of single traces diverging from the stele and progressing into the base of the petiole in both transverse and tangential planes. Frequently these traces did not appear to fuse at all, and when the case for fusion could be argued it was likely that traces were simply juxtaposed with little or no ground tissue between them. If leaf trace fusion does not, or rarely occurs then bacterial colonization of the petiole and perhaps subsequently the leaf lamina may be segregated depending on the location of the source of bacteria from the stem. If specific leaf traces supply specific regions of the leaf lamina then a lack of fusion between traces may make a uniform dispersion of bacteria throughout the leaf unlikely.

The stem of the grapevine can be described as sectored based on the consistent location of large parenchymatous gaps, separate from leaf gaps, in the vascular cylinder. These gaps are created by the regular divergence of xylem into the tendril on one side, and into the summer lateral shoot and compound bud on the other. The result of the regular repeating gap pattern creates 4 sectors in the stem; a dorsal sector, a ventral sector, and two opposite lateral sectors (Figure 4). The consequence of having the large gaps lacking vascular tissue in the lateral sector(s) of each node is that long distance conductance beyond one internode may only occur in the dorsal and ventral sectors of the stem. Consequently, depending on the location of sharpshooter feeding, bacteria introduced within dorsal and ventral sectors would be more likely to move to distal regions of the shoot than bacteria introduced to lateral sectors. Additionally, the wider vessels of the dorsi-ventral sectors would provide a lower resistance pathway for long distance movement of bacterial aggregations.

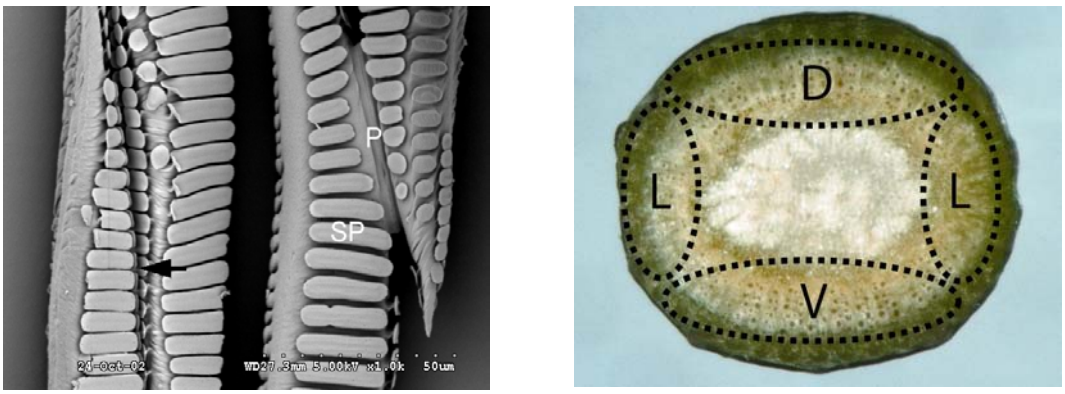

Figure 4. Resin-casts of the interior surface of vessel elements showing frequent scalariform pitting (left) and proposed stem sectoring based on regular patterns of lateral vascular divergence. SP-scalariform pit, P-simple perforation, D-dorsal sector, V-ventral sector, L-lateral sectors.

The characteristics of the tracheary elements within grapevine wood and primary tissues may contribute to the level of susceptibility to PD. The relatively wide vessels found within stem wood possess simple perforation plates with scalariform intervascular pitting (Metcalf and Chalk 1950). Simple perforation plates likely provide a low resistance pathway for bacterial cells between consecutive vessel elements allowing the bacteria to move relatively unimpeded through a single vessel. Resistance to bacterial movement would occur at the end of a vessel within the terminal vessel element. The very

wide scalariform lateral wall pits (Figure 4) within the terminal vessel elements create a large pit membrane surface area which may be weaker and susceptible to bacterial breach by digestion, or physical damage due to the physical stress of cavitation and refilling cycles within the vessel. The combination of these traits may allow bacteria to move through the stem system very quickly until the vessel lamellae in which they are located diverges into a lateral organ. Once bacteria are in a leaf trace or petiole, much narrower and shorter vessels and tracheids may act to filter bacteria from the conductive stream.

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CHARACTERIZATION OF RESISTANCE TO PIERCE'S DISEASE IN *MUSCADINIA ROTUNDIFOLIA* AND THE POTENTIAL FOR CONFERRING RESISTANCE TO GRAFTED *VITIS VINIFERA* SCIONS

Project Leaders:
Andrew Walker
Jonathan Ruel
Department of Viticulture and Enology
University of California
Davis, CA 95616

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ABSTRACT
Muscadinia rotundifolia is a wild grape native to the Southeast U.S. In field trials, as well as in the wild, this species exhibits strong resistance to Pierce's disease (PD). Incorporating this resistance into *Vitis vinifera* production systems in California will require identifying prime breeding material and increasing our understanding of the mechanism of resistance. The first objective of this study was to determine if resistance to *Xylella fastidiosa* (*Xf*) and PD varies among wild *M. rotundifolia* populations across a natural gradient of disease pressure. Greenhouse trials are underway using material collected in Florida, Georgia and Tennessee. The second objective was to test the common belief that some *M. rotundifolia* cultivars are more *Xf* resistant than others are. Again, trials are currently underway. Thirdly, we want to test if *Xf* resistance in *M. rotundifolia* is consistent when challenged with different *Xf* strains from Florida and California. Lastly, we want to examine the potential for conferring *Xf* and PD resistance to *V. vinifera* scions by grafting them to resistant *M. rotundifolia* rootstocks. This project is ongoing and only very preliminary results are available now. One such result is that even the least resistant *M. rotundifolia* selections are much more resistant than the *Vitis vinifera* cultivars. We have also confirmed that supposed graft incompatibility between the two species can be overcome using green grafting techniques. These grafted plants are entering disease trials now.

INTRODUCTION
Use of resistant plant material is a proven approach to solving crop pest and disease problems. This approach can provide a robust solution that, among other advantages, avoids the potential negative environmental impacts associated with a chemical control strategy. Unfortunately, in the case of Pierce's disease, it appears that all the common cultivars of *V. vinifera* are susceptible to the disease (Raju and Goheen 1981). Historically, breeders and researchers have looked to the wild grape species of the southeastern U.S. for potential sources of PD resistance (Loomis 1958, Mortensen et al. 1978). PD pressure is very high in this region and many of the local species are indeed resistant or tolerant. In particular, *M. rotundifolia* appears to have exceptional resistance and is planted extensively in Florida (Loomis 1958, Hopkins et al. 1974). Efforts have been made to create PD resistant bunch grapes by crossing *M. rotundifolia* with *V. vinifera*. Initial success was limited because of genetic incompatibility, but efforts continue and some fertile hybrids are available (Lu et al. 2000). Such hybrids are also being used in work aimed at identifying the genes responsible for the resistance (Krivanek and Walker 1999, 2000). The current project is aimed at increasing our understanding of how PD resistance (or tolerance) functions in M. rotundifolia with the thought that such knowledge will improve our chances of incorporating resistance into *V. vinifera*. We will also explore the potential for conferring this resistance to *V. vinifera* via grafting.

OBJECTIVES
1. Determine if resistance to *Xylella fastidiosa* (*Xf*) and Pierce's disease varies among wild *M. rotundifolia* populations across a natural north/south gradient of disease pressure.
2. Test the common belief that some *M. rotundifolia* cultivars are more *Xf* resistant than others.
3. Determine if *Xf* resistance in *M. rotundifolia* is consistent when challenged with different *Xf* strains from Florida and California.
4. Determine if there is any potential for conferring *Xf* and PD resistance to *V. vinifera* scions by grafting them to resistant *M. rotundifolia* rootstocks.

RESULT
Variation in resistance in wild M. rotundifolia
We completed a collection trip in June 2003 that covered the range of PD pressure from Florida (high) to Tennessee (low). Replicated potted cuttings from 11 collection sites are now in a resistance trial in the greenhouse (n = 138, including controls). This experiment serves as an indirect test of the hypothesis that *Xf* resistance in *M. rotundifolia* reflects an evolved response to the disease. Results indicating that resistance tracks disease pressure would suggest that resistance did evolve in response to the disease. We would therefore expect the mechanism to be something specific such as an induced defense that results from some chemical elicitor. If resistance does not appear to be an evolutionary response, we might expect a more general mechanism such as basic differences in xylem structure or xylem sap composition.

Variation in resistance in M. rotundifolia cultivars

Past research and 'common knowledge' suggest that some *M. rotundifolia* cultivars are actually quite susceptible to PD (Hopkins et al. 1974, Mortensen et al. 1977). Unfortunately, the studies cited suffered from poor experimental design lacking proper replicates and controls. In another greenhouse trial, we are examining *Xf* resistance in 2 reportedly susceptible cultivars ('Pride' and 'Lucida') and two reportedly resistant cultivars ('Southland' and 'Carlos'). This trial is comprised of 47 potted vines. For comparison, we included the highly susceptible *V. vinifera* ('Chardonnay') and the less susceptible *V. vinifera* ('Chenin Blanc'). Meeting this objective will help us narrow our search for the mechanism of resistance by identifying candidates for comparisons of resistant and susceptible genotypes within *M. rotundifolia*. Preliminary results indicate that even the least resistant *M. rotundifolia* are much more resistant than both *V. vinifera* cultivars.

Resistance to different Xf strains

Xylella fastidiosa and PD are widespread across the warm regions of the Americas and occur in a vast number of host plants including many crops. Genetic work has shown that the *Xf* strains that cause diseases in different crops differ genetically (Chen et al. 1992, Hendson et al. 2001). We also know that different *Xf* strains differ in their ability to produce PD symptoms in grape (Hopkins 1985). We would like to know how the resistance in *M. rotundifolia* holds up across a number of different strains. Identifying selections with broad and robust resistance will be of obvious benefit to the breeding programs aimed at producing resistant grape varieties. This objective is on hold while we await the approval of our APHIS permit application to import *Xf*.

Xf resistance from rootstocks?

The wine grape industry would prefer a solution that allows them to keep pure *V. vinifera* scions. Thus, positive experimental results with this objective would have obvious practical implications and indicate that resistance may be derived from some mobile secondary metabolite. In fact, success with this approach will depend entirely on the mechanism of resistance. Some reasons to be hopeful include two other cases of rootstocks conferring disease resistance/tolerance to scions in grapes: with crown gall (Sule and Burr 1998) and with fanleaf virus (Walker et al. 1991). Furthermore, rootstock choice has been shown to affect xylem chemistry, leafhopper feeding, and the concentrations of *Xf* in peach (Gould et al. 1991). This past summer, we used green-grafting techniques to produce replicated combinations of *M. rotundifolia* selections and *V. vinifera* var. 'Chardonnay' (n = 27 potted vines). To increase the chance of success with this objective, we also created a number of grafts using other *Xf* resistant species such as *V. girdiana* and *V. arizonica* as rootstocks (n = 16 potted vines). These plants will enter a resistance trial including inoculation with *Xf* in November.

CONCLUSIONS

The results of this study will increase our understanding of the mechanism of PD resistance in wild grapes. On a more applied level, the project will identify prime material to be incorporated into breeding programs. Furthermore, testing for graft-conferred resistance from rootstocks could lead to a novel solution to the PD problem in wine grape viticulture.

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Section 1B: Detection and Field Monitoring Methodologies for the Glassy-winged Sharpshooter and Xylella fastidiosa

PRESSURE CHAMBER EXTRACTION OF XYLEM FLUID: IMPROVING BACTERIAL DETECTION IN PLANTS AFFECTED BY *XYLELLA FASTIDIOSA*

Project Leader:
Blake Bextine
Department of Entomology
University of California
Riverside, CA 92521

Project Director:
Thomas A. Miller
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted from June 1, 2002 to May 31, 2003.

ABSTRACT
Xylella fastidiosa is the xylem-limited bacterium that causes Pierce's disease of grapevine and oleander leaf scorch. Detection of this pathogen prior to symptom development is critical for improved management of the pathogen. ELISA and PCR are currently used for routine detection of the pathogen; however, both detection methods are limited by low titer or patchy distribution of the bacterium within a host plant. In the study reported here, we directly compared *X. fastidiosa* detection in whole-tissue samples to xylem fluid samples from grapevine and oleander. Collection of xylem fluid samples improved sensitivity of pathogen detection by ELISA (41.0%) compared to whole-tissue samples (20.5%) in asymptomatic grapevine. Additionally, pathogen detection in asymptomatic grapevine by PCR was also improved when xylem samples were tested (66.7%) compared to whole-tissue samples (23.1%). There were no differences in frequency of detection of *X. fastidiosa* in symptomatic grapevines by ELISA or PCR dependent upon sample collection method. Assays of xylem fluid samples did not improve detection of *X. fastidiosa* in symptomatic or asymptomatic oleander compared to assays of whole tissue. Finally, in a direct comparison of ELISA and PCR, we found no significant differences in frequencies of positive grapevine or oleander samples detected.

INTRODUCTION
The xylem-limited bacterium, *Xylella fastidiosa*, has been identified as the causal organism of several economically important diseases in California (Freitag 1951). Pierce's disease of grapes, the most notable of these diseases, is caused when the pathogen interrupts the translocation of water and nutrients through the xylem of affected plants (Purcell 1997). *X. fastidiosa* also causes leaf scorch and declines in elm, sycamore, oak, maple, oleander, and almond (Purcell and Hopkins 1996).

X. fastidiosa collection methods hamper all detection techniques used for pathogen management and study. The most common method of collection, extraction of the bacterium directly from tissue (leaves, shoots, or stems) has several limitations, such as low cell numbers collected and high amounts of plant DNA and organic matter that can interfere with ELISA or PCR, hindering early detection and often resulting in false negatives (Blua, personal communication). Improving the consistency of detection will enhance the study of the interaction between host plants and the pathogen. A better bacterial collection technique would benefit commonly used sensitive detection techniques. Several techniques have been used to collect xylem sap for identification of bacteria. Vacuum extraction has been used to identify bacteria in xylem sap of grapevine (Bell et al. 1995, Guo and Lu 2001).

Using the pressure bomb technique, we hoped to increase the consistency of *X. fastidiosa* detection in plants. This improved technology would allow pathogen diagnostics to be quantified and will improve the detection techniques already being used by concentrating the titer of bacteria being detected. Our intent was to improve bacterial detection in plants used for transmission tests; however, pressure chamber extraction of xylem fluid is applicable to detection of other endophytic bacteria in other plants. We plan to expand the method to detect other bacteria, those with anti-*Xylella* properties, in the vascular tissues of plants that can be used for paratransgenesis.

OBJECTIVES
The goal of the research proposed was to improve the efficiency and consistency of bacterial detection in plants. The technique developed in this research study will be applicable to other plant/pathogen systems, improving detection of pathogens in a simple and cost effective manner. The specific objectives of this work were to:

1. Improve *Xylella fastidiosa* detection methods in oleander and grapevine by extracting DNA from xylem fluid samples rather than whole tissue samples.
2. Develop assay for extraction of bacteria to improve sensitive molecular techniques currently being used for detection of pathogens and endophytic bacteria.

RESULTS AND CONCLUSIONS

Grapevine xylem was relatively easy to collect under pressure from a Scholander pressure bomb with a pipette. It exuded from the cut stem as a clear fluid. In contrast, the collection of xylem fluid from oleander was relatively more difficult to collect using the Scholander pressure bomb because it exuded as a froth, making collection difficult to keep sterile. The froth indicated cell collapse within the stem; therefore samples collected were most likely not pure xylem fluid.

Use of the Scholander pressure bomb to collect samples was more efficient than whole-tissue samples for detecting *X. fastidiosa* in asymptomatic grapevines. Both whole-tissue and xylem fluid samples were collected from 30 grapevines to be tested by ELISA and PCR on August 27, 2002 (10 visually symptomatic and 20 visually asymptomatic) and an additional 30 grapevines were sampled on October 23, 2002 (11 visually symptomatic and 19 asymptomatic). For data analysis, results from these two collection dates were pooled. Statistical analysis of samples pooled across dates did not reveal differences between collection technique in symptomatic plants detection using ELISA (χ^2=0.099 df=1, *p* value=0.7530) or PCR (χ^2=1.867 df=1, *p* value=0.1718). However, *X. fastidiosa* detection in xylem fluid samples of asymptomatic plants was significantly better than whole-tissue extract by ELISA (χ^2=12.045 df=1, *p* value=0.0005) and PCR (χ^2=14.978 df=1, *p* value=0.0001).

Use of the Scholander pressure bomb to collected samples was not more efficient then whole-tissue samples when analyzed by ELISA or PCR. Ninety-four oleanders were sampled on April 22, 2002, 56 symptomatic and 38 asymptomatic, and 30 oleander samples were taken on November 8, 2002, 15 symptomatic and 15 asymptomatic. As in the grapevine samples, results from these two collection dates were pooled for data analysis. In symptomatic plants detection by ELISA using either collection method was not significantly different (χ^2=0.201 df=1, *p* value=0.6539). In symptomatic plants tested by PCR, whole-tissue extraction was significantly more sensitive than xylem fluid samples (χ^2=10.327 df=1, *p* value=0.0013). In asymptomatic plants there was no significant differences between collection methods by ELISA (χ^2=1.941 df=1, *p* value=0.1630) or PCR (χ^2=2.192 df=1, *p* value=0.1387).

There were no significant differences between the ELISA or PCR method of detection in grapevine (χ^2=1.35 df=1, *p* value=0.245) or oleander (χ^2=0.115 df=1, *p* value=0.734).

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FUNDING

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

Project Leaders:
Blake Bextine
Matthew J. Blua
Richard Redak
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported her are from work conducted from September 2002 to September 2003.

ABSTRACT
Dependable detection of *Xylella fastidiosa* (*Xf*) in glassy-winged sharpshooters (GWSS) is imperative for understanding *Xf* epidemiology and optimizing grapevine protection strategies. In this study, we have developed methods for extracting *Xf* DNA from GWSS vectors and optimized a SYBR green I based real-time PCR detection protocol that is fast, consistent, and inexpensive. The Qiagen DNeasy Tissue kit (Qiagen Inc., Hercules, CA, USA) was the most efficient kit tested in our studies, having a lower detection limit of 500 cells in the presence of insect tissue. The considerably faster pre-extraction method of repeatedly flushing the foregut with lytic buffer with vacuum pressure prior to extraction using Qiagen DNeasy Tissue kit was not significantly different than whole-tissue maceration. Storage of GWSS samples at -4°C did not compromise *Xf*-detection capabilities.

INTRODUCTION
New epidemics of Pierce's disease of grapevine induced by *Xylella fastidiosa* (*Xf*) in southern California are associated with the invasive vector species *Homalodisca coagulata*, the glassy-winged sharpshooter (GWSS). These epidemics have stimulated investigations of plant protection tactics that depend on reducing the interaction between infectious GWSS and grapevines. Deployment of these tactics would be optimized by the ability to monitor the dispersion of *Xf* in infectious GWSS. The ability to detect *Xf* in GWSS vectors would also allow us to describe its movement in time and space at both local and regional levels.

PCR protocols have been developed to detect *Xylella fastidiosa* (*Xf*) in plants (Minsavage et al. 1993, Oliveira et al. 2002, Schaad et al. 2002). Unfortunately, when methods are adapted for insects they tend to be inconsistent. Extraction of DNA from *Xf* in insect vectors may be difficult for several reasons. First, *Xf* exists at low titers in vectors (Nome et al. 1980). The large amount of insect DNA present in the extract could interfere with extraction of *Xf* DNA by saturating the capacity of the extracting process, thus diluting the DNA of interest. Second, the bacterium adheres to sites in the foregut of the insect. This region of the insect's alimentary canal is chitonized and may be difficult to expose to DNA extraction. Third, potential DNA inhibitors are commonly found in insects. It has been suggested that pigments contained in the insect's eyes might inhibit PCR, and removal of the eyes prior to extraction has improved detection (Gispert, unpublished data). Removal of the eyes is tedious and time consuming and therefore not suitable for extracting DNA from large numbers of GWSS samples.

The selection of a DNA extraction protocol depends on the system in which it is being applied. Because no one extraction protocol fits every detection system, factors such as performance of the kit, price, time investment, and maximum number of samples extracted must be considered. In our recent studies, we compared three standard methods and 11 commercially available kits for relative efficiency of *Xf* DNA extraction in the presence of insect tissue. All of the protocols tested were proficient at extracting DNA from pure bacterial culture (1x105 cells) and all but one protocol successfully extracted sufficient bacterial DNA when in the presence of insect tissue. DNA extraction kits were found to be amenable to large sample sizes by being relatively inexpensive and time-efficient.

OBJECTIVES
The over-all goal of this project is to develop a method of detecting *Xf* in GWSS that would allow us to conduct epidemiological studies and optimize plant protection. To this end, our objectives are:
1. Develop a DNA extraction protocol that is optimal for *Xf* DNA recovery from GWSS.
2. Develop a real-time PCR-based detection system that is sensitive, rapid, and cost-efficient.

RESULTS
Extraction kit comparison
The Qiagen DNeasy Tissue kit detected *Xf* 50 cells without insect tissue present (Table 1). When the sample dilution series was tested with insect tissue, 500 cells was the lowest concentration detected (a 10-fold decrease). Although the immunomagnetic separation method returned similar results without insect tissue, it only allowed detection of *Xf* at $\geq$1600 cells with insect tissue (Table 1). The Fermentus DNA Purification kit only detected the concentration of 1600 cells without insect tissue and did not detect any dilution with insect tissue present.

Table 1. Comparison of three DNA extraction methods by PCR results from samples spikes with dilutions of *X. fastidiosa* culture with or without insect background. n=5 for each kit and sample combination

cells/ml	DNeasy Tissue kit (Qiagen Inc.)		Immunomagnetic separation[a]		Genomic DNA purification kit (Fermentus Inc.)	
	With GWSS[b]	Without GWSS[c]	With GWSS[b]	Without GWSS[c]	With GWSS[b]	Without GWSS[c]
5000	++++	++++	+++	+++++	-	++
1600	++	+++	+	++++	-	L
500	L	++	-	++	-	-
50	-	L	-	L	-	-
0	-	-	-	-	-	-
PCR + control	++++		++++		++++	
PCR – control	-		-		-	

*The density of the PCR products on the agarose gels was related to the relative intensities of the bands compared to one another. The number of + illustrated the relative brightness of the band, L indicates a very faint band, and - indicates no PCR product.

[a]Immunomagenetic separation (Dynal Biotech and Agdia Inc.).

[b]Extract of one GWSS head added to sample.

[c]No insect background in sample.

Real-time (RT) PCR (Sybr Green I)

Real-time PCR was performed in a Rotor Gene 3000 (Corbett Research, Australia) using iQ SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA) in 20µl reactions with 5µl of extracted DNA in H_2O. For detection of *Xf*, we used the *Xf*-ITS primer set (Schaad et al. 2002), which was originally designed to by used with a molecular probe system; XfF1 (5' AAA AAT CGC CAA CAT AAA CCC A 3') AND XfR1 (5' CCA GGC GTC CTC ACA AGT TAC 3'). After an initial denaturing step of 3m at 95°C, the reactions were cycled 40 times under the following parameters: 95°C for 20s, 53°C for 20s, 72°C for 20s. At the end of the PCR, temperature was increased from 72 to 99°C at a rate of 1°C/45s, and fluorescence was measured every 45s to construct the melting curve. PCR product produced in positive samples had a specific melting temperature of 86.1° compared to the melting temperature of the primer/dimmer (80.9°), so the *Xf*-positive samples were easy to separate from negative samples (Figure 1). A non-template control (NTC) was run with each assay and the negative control.

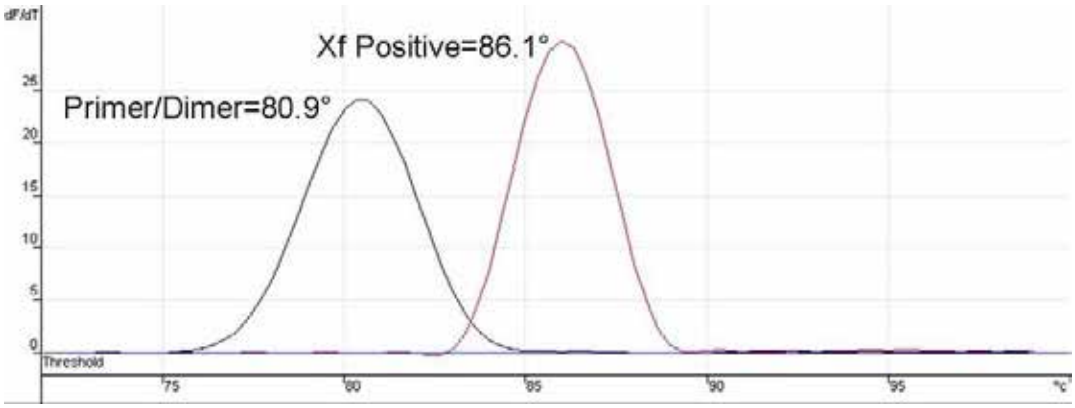

Figure 1. Melting curve analysis with two amplicons produced by *Xf* positive or primer/dimmer. The melting peaks separate by size similar to bands on an electrophoresis gel.

Vacuum infiltration as a pre-extraction method for detecting Xf in GWSS. We developed a method of extruding *Xf* from GWSS that does not require maceration of insect tissue, the most tedious step in the DNA extraction process. Using a method of repeatedly flushing the foregut with lytic buffer by changing vacuum pressure, *Xf* DNA was sufficiently collected for detection by RT-PCR. In a direct comparison, the proportion of *Xf*-positive GWSS was not significantly different using the vacuum extraction method (71.2%, n=139), compared to whole-tissue maceration (76.3, n=139) (chi-square=0.91). We believe this method works well for several reasons. First, because insect tissue is not macerated, less insect DNA and PCR inhibitors should be released. Second, by repeatedly applying vacuum pressure and releasing it, lytic buffer is continually flushed through the foregut of the insect allowing for better recovery of *Xf*. Vacuum extraction was amenable to large numbers of samples. Using a 96-well plate, 96 samples can be processed at the same time, compared to one sample at a time with maceration.

Effects of freezing on detection of Xf from GWSS

Storing GWSS samples at -4° for 10 d prior to extraction and RT-PCR detection did not affect *Xf* detection (ANOVA, LSD, p=0.001). In a direct comparison, 85.1% of GWSS having DNA extracted immediately after removal from *Xf*-infected plants (n=48) tested positive, compared to 77.1% of GWSS stored at -4° for 10 d and 81.3% of GWSS that were stored in mineral oil at -4° for 10 d.

CONCLUSIONS

We have made substantial progress in our goal of developing an efficient and sensitive method to detect *Xf* in GWSS vectors. First, we have successfully used a commercially available DNA extraction kit to detect a lower detection limit of 50 *Xf* cells without a GWSS head background and 500 *Xf* cells with a background. This kit is consistent with our goal of making our method cost-efficient and amenable to a large sample size. Second, we have developed the used of RT PCR technique to simultaneously detect and quantify *Xf* DNA. Third, we have developed a means of rapidly extracting multiple samples simultaneously.

Our future research directions will focus on lowering detection limits by examining additional primer sets, and crude dissections of GWSS head to reduce interfering DNA and PCR inhibitors. After optimizing our detection method we will examine the relationship between grapevine inoculation by individual GWSS and the ability to detect *Xf* in them. Understanding that relationship will allow us to study details of *Xf* epidemiology and optimize plant protection.

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FUNDING AGENCIES

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RELATIONSHIPS BETWEEN TOTAL POPULATION COUNTS OF GLASSY-WINGED SHARPSHOOTERS AND NUMBERS OBTAINED FROM VARIOUS SAMPLING METHODS

Project Leaders:
Matthew J. Blua
Richard Redak
Department of Entomology
University of California Riverside
Riverside, CA 92521

Cooperators:
David H. Akey
USDA-ARS
Cotton Research Laboratory
Phoenix, AZ

Carlos E. Coviella
Department of Entomology
UC Riverside
Riverside, CA 92521

REPORTING PERIOD: These results are from work conducted between October 2002 through October 2003

ABSTRACT
We seek to understand the way in which commonly used sampling methods work, in relation with real population densities. We used short citrus experimental trees and commercial-size ones, in order to encompass most of the size range of plants that are important hosts to GWSS. At the University of California, Agricultural Operations, Riverside, California, we conducted two series of experiments in citrus with natural GWSS populations. The trees were 2 m tall navel oranges that have been blocked, shaped, and skirted for uniformity within the block, as well as commercial-sized citrus trees. We compared visual counts (adults and nymphs), beat-net (adults, early nymphs and late nymphs), and yellow sticky cards (adults), with the absolute GWSS density for each tree using the tent-fogging methodology. We used fluorescent colored dust to mark 100 GWSS released inside the tents to determine the percentage recovery. These surveys were conducted every other week from June 2002 through September 2003. The results showed significant correlations between the methods and real population densities, with R^2 values that ranged from 0.19 to 0.84. The results how that correlations are better from small trees and bushes, but also that for commercial-size trees most of the methods can explain close to 50% of the real population size. We seek now to include temperature, wind, rain, and cloud coverage data to a multivariate analysis in order to explore whether that would improve the correlations, and thus to better assess the predictive value of the most commonly used sampling methods for GWSS.

INTRODUCTION
Most of our knowledge of GWSS dispersion has been obtained with relative sampling methods in vineyards and citrus orchards. Currently, sampling methods are being used to determine timing of pesticide treatments and to judge their efficacy (e.g., General Beale project, Bakersfield). This use implies that the sampling method used relates in a known way to population density. Unfortunately, this is not the case, and some important questions are raised. If a given treatment against GWSS results in "zero counts" by beat sampling, does that necessarily indicate that there are no GWSS in the area due to the treatments, or could some GWSS be left alive but at density below the detection threshold of the monitoring tool? Could an unknown low density of GWSS be enough to vector PD within or between treated areas? Does the relationship between population sampling precision and accuracy change seasonally?

OBJECTIVES
The goal of our research is to correlate the numbers of *Homalodisca coagulata* (glassy-winged sharpshooter, GWSS) obtained by various sampling methods currently used in GWSS population monitoring with the population density of GWSS in the flora sampled.

RESULTS
We ran the regressions for both datasets separately. The aim was to see whether the dataset from small trees, which are a good model for bushes, show similar correlations that those of commercial-sized trees, for these two extremes in size encompass most of the types of GWSS hosts.

Obtaining relative estimates and absolute counts from short experimental trees.
The dataset analyzed covers from July 2002 through September 2003 (21 dates). Nymph data from small experimental trees showed significant correlations between total counts and beat net and timed visual counts. These regressions had an R^2 = 0.82, and 0.84 respectively (Table 1). Adult data from the same small experimental trees showed significant correlations between all the methods tested. The regression of sticky card data on total counts, for instance, explains close to 50% of the variance of sticky card data. Beat net and visual counts are less accurate when compared with actual population densities per tree (Table 2).

Table 1. P-values and R^2 (in parenthesis) data for the regressions for juvenile GWSS counts from data from short experimental citrus trees.

Juvenile GWSS	Total counts	Sticky card	Beat net	Timed counts
Total counts	---			
Sticky card	X	---		
Beat net	< 0.0001 (0.82)	X	---	
Timed counts (visual)	< 0.0001 (0.84)	X	<0.0001 (0.71)	---

X: No nymph data was collected from sticky traps.

Table 2. P-values and R^2 (in parenthesis) data for the regressions for adult GWSS counts from data from short experimental citrus trees.

Adult GWSS	Total counts	Sticky card	Beat net	Timed counts
Total counts	---			
Sticky card	0.0002 (0.50)	---		
Beat net	< 0.0001 (0.48)	0.0001 (0.57)	---	
Timed counts (visual)	< 0.0001 (0.35)	0.01 (0.26)	< 0.0001 (0.73)	---

Obtaining sampling and total counts from commercial-sized citrus

The dataset analyzed from commercially-sized citrus trees covers from April through July 2003 (13 dates). For these citrus trees, there was no correlation between any of the methods tested for juvenile GWSS. Only the data from beat net samplings supported analysis. Data from timed counts returned zeros for most dates and could not be analyzed. No nymph data was collected from sticky traps. The adult dataset showed significant correlations, but very low R-squared values (Table 4). This results suggest that the prediction value of all this methods seems thus to be low in terms of real adult GWSS densities per tree.

Table 3. P-values and R^2 (in parenthesis) data for the regressions for adult counts from data from commercially-sized trees.

Adult GWSS	Total counts	Sticky card	Beat net	Timed counts
Total counts	---			
Sticky card	0.0052 (0.48)	---		
Beat net	< 0.0001 (0.46)	0.002 (0.59)	---	
Timed counts (visual)	0.0056 (0.19)	0.0070 (0.49)	0.0036 (0.20)	---

CONCLUSIONS

These results show that the sampling systems commonly used to measure GWSS populations can explain close to 50% of the adult GWSS field population density on commercial citrus trees. The estimations based on smaller host plants are in excess of 80%. Beat net and visual counts seem to be reliable methods to assess juvenile GWSS densities at least in small trees and bushes. It is interesting that particularly for adult GWSS, when tested against total counts all the methods showed very close results for small experimental trees and commercial-size trees. These methods will continue to be the basis for GWSS population assessment, but some caution should be kept in mind, when drawing conclusions beyond the accuracy of the measurements. In particular, sticky trap data seems to be constrained not only by whether conditions, but also by the traps' insect load, that reduces trap efficiency (see project's last year report). We are seeking to improve our estimations of the methods' accuracy, by including wind speed, temperature, rain and clod cover in a multivariate model.

FUNDING AGENCIES
Funding for this project was provided by the University of California Pierce's Disease Grant Program. This project received a one-year no cost extension, and we will continue to gather the same data through July 2004 to increase the accuracy of these estimations.

A MONOCLONAL ANTIBODY SPECIFIC TO GLASSY-WINGED SHARPSHOOTER EGG PROTEIN: A TOOL FOR PREDATOR GUT ANALYSIS

Project Leaders:

James Hagler
Western Cotton Research Lab
USDA-ARS
Phoenix, AZ 85040

Kent Daane
Division of Insect Biology
University of California
Berkeley, CA 94720

Heather Costa
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:

Valerie Fournier
Division of Insect Biology
University of California
Berkeley, CA 94720

David Akey
Western Cotton Research Lab
USDA-ARS
Phoenix, AZ 85040

Matthew Blua
Department of Entomology
University of California
Riverside, CA 92521

Russell Grove
Exotic & Invasive Dis. & Pests Lab
USDA-ARS
Parlier, CA 93648

Jesus de Leon
Beneficial Insects Research Unit
USDA-ARS
Weslaco, TX 78596

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

ABSTRACT

Egg-specific monoclonal antibodies (MAbs) have been developed to the glassy-winged sharpshooter (GWSS). Younger GWSS eggs yield stronger ELISA reactions than older eggs. The indirect ELISA format was not effective for detecting GWSS egg antigen in whole body or gut dissected earwig specimens. Further ELISA optimization tests are underway to increase the efficiency of the ELISA procedure. A survey of the predator complex inhabiting citrus in Riverside, CA indicates that the earwig, *Forficula auricularia* is the most common predator.

INTRODUCTION

Effective control of the glassy-winged sharpshooter (GWSS) will require an areawide integrated pest management approach (AW-IPM). A major component of AW-IPM is the exploitation of the pest's natural enemies, which, when utilized to their greatest potential, can increase the effectiveness of other control tactics. Unfortunately, very little information exists on GWSS's predaceous natural enemies. Evidence of predation of GWSS eggs and adults has been observed in the field (JH pers. obs.); however, the composition of the predator complex, and the relative impact of each predator on GWSS mortality is unknown. A major obstacle is the difficulty of studying predators in their natural environment. Unlike parasitoids, predators rarely leave evidence of attack. Laboratory experiments can be used to evaluate the suitability of particular prey and the rates of predation. However, lab studies seldom translate to field situations. Direct field observations are sometimes used to identify predators of key pests, but the small size and cryptic nature of predators and GWSS make direct observations difficult. Furthermore, direct field observations are time consuming, labor intensive, and disruptive to the normal predator foraging process. Microscopic analysis of predator gut contents have been used but the process is not suitable for predators that liquefy prey contents for consumption. These difficulties have resulted in a deficiency of information on the impact that predators have on suppressing key insect pests. A proven "indirect" technique for measuring predation is use of protein-based immunological assays employing pest-specific monoclonal antibodies (MAbs) (Hagler et al. 1992, Hagler and Naranjo 1994-a,b). To this end, we have developed egg-specific MAbs to GWSS (Hagler et al. 2002). The MAb assays provide an avenue to qualitatively assess the impact of predator species on populations of GWSS eggs.

OBJECTIVES

Our ultimate goal is to identify the composition of the GWSS predator complex and to identify the relative impact each predator species has on GWSS using a pest-specific ELISA. Prior to examining the gut contents field-collected predators for GWSS remains, a few ELISA optimization studies are needed. Here we report on tests conducted to determine GWSS-specific ELISA responses to: (1) individual GWSS eggs of known age, and (2) predators that have consumed GWSS eggs. These studies are needed prior to conducting ELISAs on field-collected predators. We also report on the abundance of potential GWSS predators found in citrus.

RESULTS

ELISA response to GWSS eggs of known age

GWSS eggs were assayed by the egg-specific indirect ELISA described by Hagler et al. (2002). Data indicate that the ELISA reactions were greater for younger eggs than older eggs. (Figure 1).

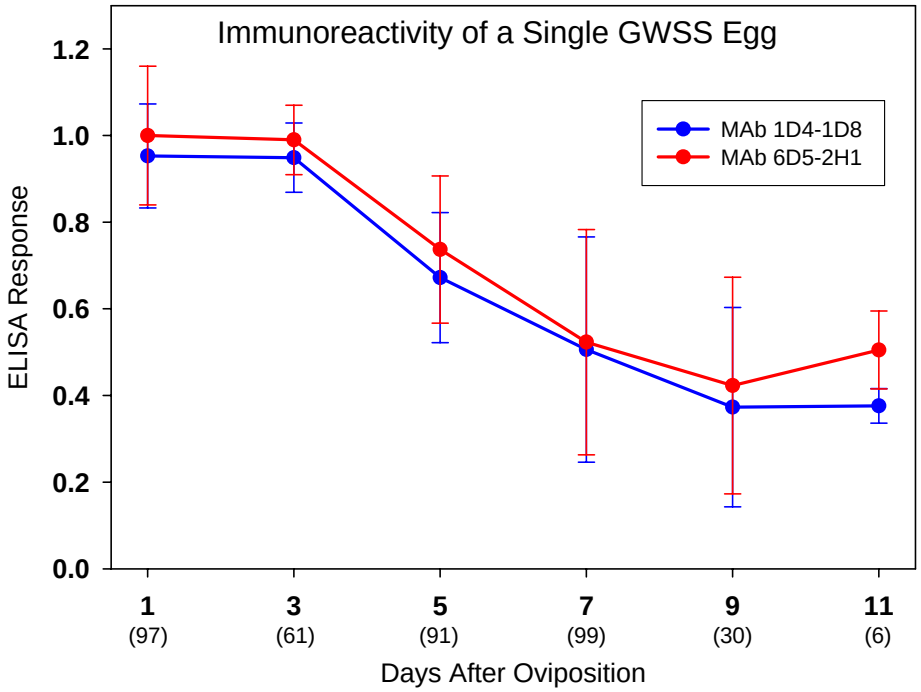

Figure 1. Mean (±SD) ELISA reaction yielded by two GWSS egg-specific MAbs to a single egg of known age. The numbers in parenthesis below the x-axis are the sample sizes for each time interval.

ELISA response to earwigs that consumed GWSS eggs

Individual adult earwigs, *Forficula auricularia* (Dermaptera: Forficulidae) were placed in Petri dishes with a single GWSS egg mass. An individual earwig was allowed to feed continuously on the egg mass. Once an individual ceased feeding it was removed from the Petri dish and frozen. Each earwig was then analyzed by ELISA for the presence of GWSS egg antigen. Two groups of earwigs were examined by ELISA: (1) whole-body specimens, and (2) gut dissected specimens. Data indicate that none of the whole-body specimens and only 10% of the gut dissected specimens yielded a positive ELISA reaction (Figure 2). Previous work with other insect species has shown that the indirect ELISA format is less sensitive than the sandwich ELISA format for detecting prey remains in large predators (Hagler et al. 1997, Hagler and Naranjo 1997, Hagler 1998). We are developing a sandwich ELISA for use in detecting GWSS remains in earwig guts. Additionally, we are testing the indirect ELISA on other predator species (e.g., *Chrysoperla carnea*, *Collops vittatus*, *Hippodamia convergens*, etc.).

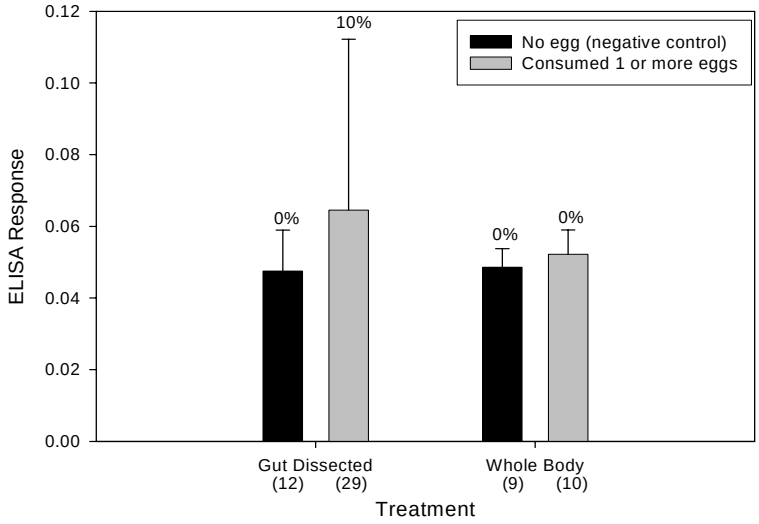

Figure 2. Mean (±SD) response of the GWSS-specific ELISA to earwigs that consumed one or more GWSS eggs. The number above each error bar is the percentage of individuals from each treatment that yielded a positive ELISA reaction for the presence of GWSS egg antigen. The numbers in parenthesis below the x-axis are the sample sizes for each treatment.

Arthropods collected from citrus

Total counts of arthropod species inhabiting the citrus canopy in Riverside, CA were obtained from whole-tree samples collected by D. Akey (pers. comm.). Briefly, 3 entire trees were sampled every other week during GWSS outbreaks. The area under each tree was covered by ground cloth and the tree canopy was covered with a waterproof tent. Each tree was fogged with pyrethrum and PIP. After 3 h, the tent was removed, the tree was shaken for 5 min, and the arthropods on the ground cloth were counted. The densities of GWSS nymphs and adults are reported by Akey et al. in this volume. The abundance of the other arthropods collected from trees is given in Figure 3. By far, the most abundant predator species encountered was the earwig, *F. auricularia*. Laboratory feeding trials are underway to determine earwig feeding activity on GWSS eggs (egg consumption rate, handling time, etc.). Various species of beetles, ants, and spiders were also collected from the citrus trees. Gut content ELISAs will be performed on each predator as soon as the ELISA procedure has been optimized.

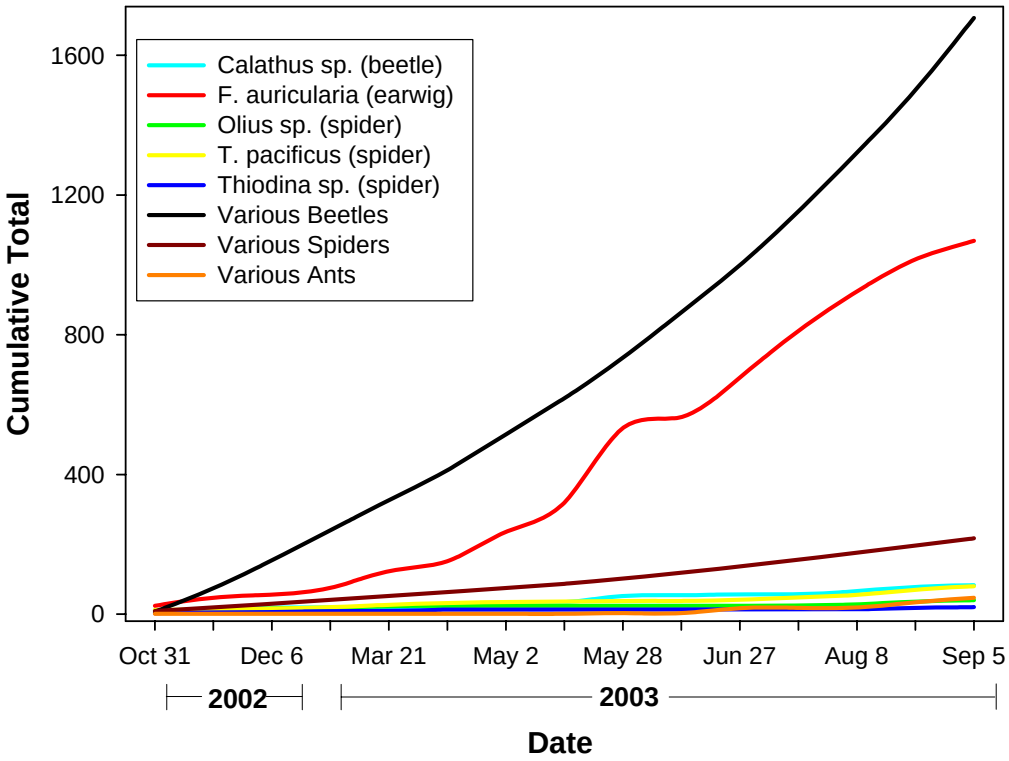

Figure 3. The cumulative population of arthropods collected from citrus trees in Riverside, CA.

CONCLUSIONS

An understanding of the key natural enemies of GWSS will contribute to an areawide IPM approach for GWSS control. Once key predators are identified they can be better exploited for conservation and augmentative biological control programs.

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FUNDING AGENCIES
Funding for this project was provided by the University of California Pierce's Disease Grant Program, and the USDA Agricultural Research Service.

DEVELOPMENT OF TRAPPING SYSTEMS TO TRAP GLASSY-WINGED SHARPSHOOTER (*HOMALODISCA COAGULATA*) ADULTS AND NYMPHS IN GRAPE

Project Leader:
Raymond L. Hix
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:
Michael R. McGuire
Western Integrated Cropping Systems
USDA-ARS
Shafter, CA 93263

Gary Puterka
USDA-ARS
Appalachian Fruit Research Station
Kearneysville, WV

Reporting Period: The results reported here are from work conducted from August 2002 to October 2003.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) *Homalodisca coagulata* is native to the southeastern United States (Young 1958) where it is a known vector of various strains of the bacterium *Xylella fastidiosa*. Since its introduction into California, it has become established in large numbers in certain areas. Pierce's disease (PD) has been a problem in California for more than 100 years, but the GWSS is a more efficient vector of *X. fastidiosa* because it is a stronger flier than native California sharpshooters, and it can feed on the xylem of seemingly dormant woody stems.

The wine industry in Temecula, California has been seriously impacted by PD losing about 30% of its vineyards to date. The combination of PD and GWSS in California poses a serious threat to the grape industries. About 98,000 acres of table grapes are currently cultivated in California with 11,000 acres of table grapes in the Coachella Valley (Riverside County). The Coachella Valley has a history of PD and there is currently a high population of glassy-winged sharpshooters. The combination of the bacterium and new vector creates a serious disease threat to grape in the area. A similar situation has occurred in Kern County, California as well.

One of the crucial components and cornerstones of integrated pest management is the monitoring for the presence and density of a pest. Proper detection methods allow for optimum integration of biological, cultural, physical, chemical and regulatory measures to manage a pest. Yellow sticky traps have been used extensively in the southeastern U.S. for monitoring leafhoppers including GWSS in peach (Ball 1979) and citrus (Timmer et al. 1982). However, the reliability of these methods to detect the GWSS in California is questionable, and traps specifically designed for GWSS do not currently exist. To compound the situation, current methods are not standardized. For example, different sizes and shades of yellow sticky traps are being used in monitoring programs. The AM designation on certain traps actually refers to the apple maggot for which the trap was designed. Furthermore, the relationship of trap catches to actual populations of GWSS in grape or citrus are currently unknown.

Trap designs based on the behavior and biology of the insect in question have a much higher chance of success than relying on trial and error of traps designed to monitor other insects. Female GWSS secrete and deposit brochosomes on the forewings just prior to egg laying (Hix 2001). These spots are then scraped off during egg lying. Furthermore, white spots are secreted before each egg mass is laid, and female GWSS can only produce rod shaped brochosomes after mating. It is therefore feasible to relate preovipositional females with white spots and residues to egg masses in associated vegetation analysis. The white spots are very visible on females caught in traps (Hix 2001). Many leafhopper species produce brochosomes, but only females are known to produce the rod shaped brochosomes (Rakitov 2000). As reported here in 2001, data from the intercept traps and colored plates clearly indicated that GWSS are attracted to yellow as well as orange. Attraction to these colors was statistically significant and demonstrated that even though the AM type trap may have a reliability issue, it is clearly not a "blunder trap."

OBJECTIVES

This research addresses:

1. Which hue of yellow is the most attractive to GWSS;
2. What is the field longevity of a trap before weather and photo degradation impact trap reliability;
3. How does trap catch relate to populations of GWSS in citrus and grape;
4. GWSS spectral sensitivity;
5. How does temperature affect trap catch;
6. The feasibility of using certain wavelengths of light to enhance trap catch of GWSS in vineyards and associated orchards;
7. Develop and evaluate sticky barriers to trap and detect GWSS nymphs within a vine or tree canopy.

RESULTS AND CONCLUSIONS

Ten trap colors were field tested in 2002 to repeat tests from 2001. Colored Solo® plates and Seabright™ yellow panel traps (a Pherocon® AM clone) were deployed in citrus groves with known high GWSS populations. Traps were checked weekly and visual count of egg masses, nymphs, and adults were made. The yellow and orange Solo plates were very successful in catching adult GWSS. Yellow plates caught statistically more GWSS than Seabright yellow panel traps while orange traps usually caught more than the Seabright yellow panel traps (Figure 1). The interesting thing is that the yellow plates were more reliable at catching GWSS at low population levels than the Seabright yellow panel traps. Square and circle traps were made using the same yellow material and glue (Stickem Special™ Hold Fast formulation) to test GWSS response to 2-dimensional shapes to determine if that is why plate routinely catches more GWSS. On 1 August 2003 the square trap mean was 139.9 (± 33.8 SEM) compared to the circle trap mean 151.3 (± 22.8 SEM). Since the difference wasn't due to shape, two glue types (Stickem Special Regular and Stickem Special Hold Fast formulations) were tested on Seabright yellow panel traps and Solo yellow plates. The Solo yellow plates caught significantly more GWSS than the Seabright yellow panel regardless of glue type (Figure 2). In addition, four trap sizes made from the same material and glue were evaluated. The larger traps caught more GWSS than traps of smaller area (Fig.ure3), but the 499 cm^2 trap caught more GWSS per cm^2 than the larger and smaller traps. In summary, size and color are the two most important factors in sticky trap design as long as a suitable glue is used. The two stickiest glues available were used in the study. Stickem Special Regular and Stickem Special Hold Fast formulation glues are currently in use in the GWSS monitoring components of the various areawide management programs in California. Apparently, an attribute of color other than hue-angle is responsible for the differences in trap catches for the Solo yellow plate, the Seabright yellow panel traps, or Pherocon AM traps.

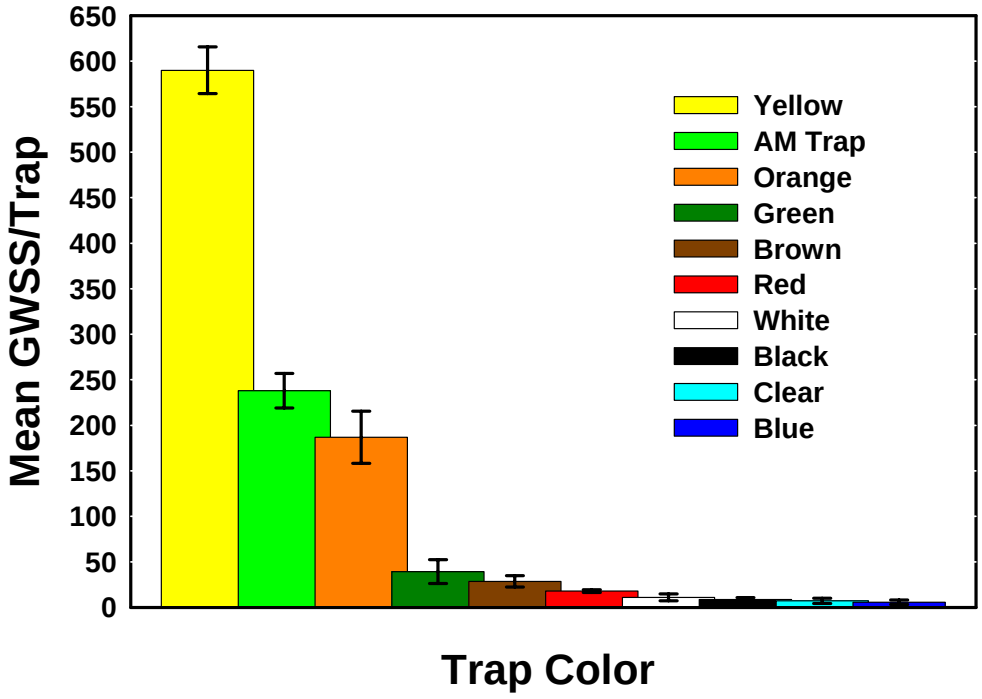

Figure 1. Mean Number (± SEM) of GWSS Trapped on Colored Solo® Plate Traps (one side of plate) at the Peak Trap date of 17 Aug 2002. . There were 5 of each trap color.

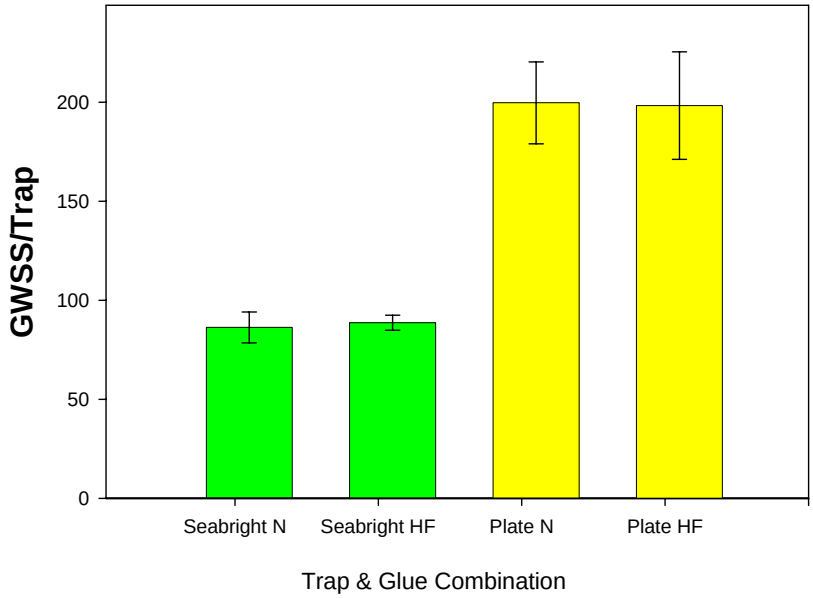

Figure 2. Means (± SEM) for Seabright yellow panel traps and Solo yellow plates (one side only) with Stickem Special™ Regular (N) and Stickem Special Hold Fast (HF) formulations. Reps = 5.

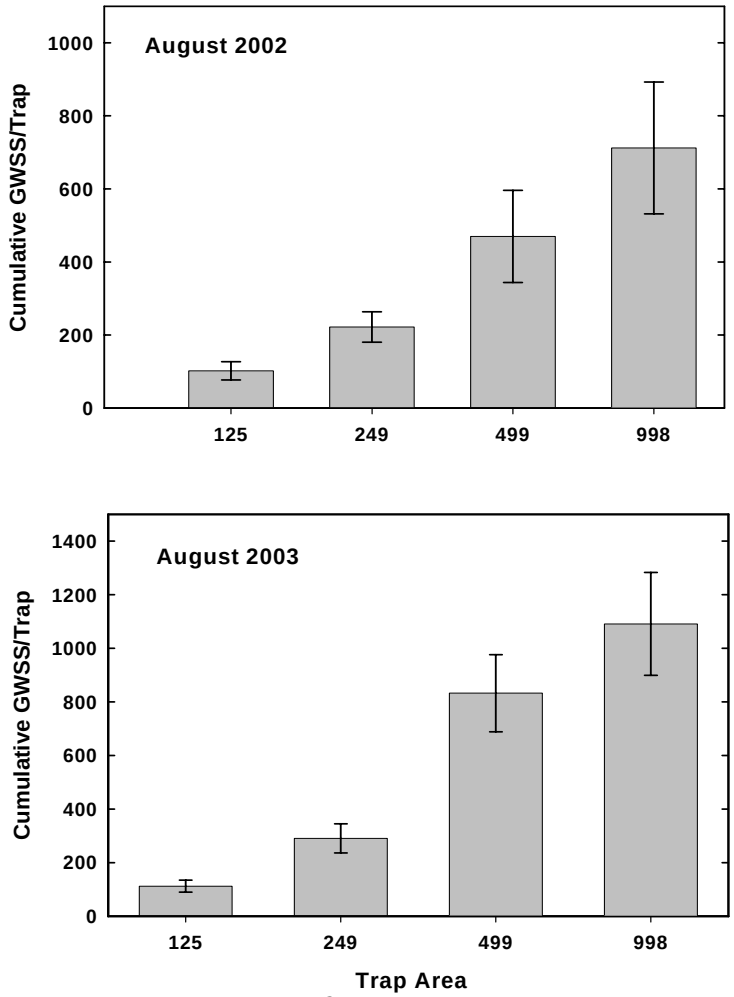

Figure 3. Comparison of trap sizes in cm^2. Cumulative means for a 4 week period in August. N = 5 ± SEM.

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FUNDING AGENCIES

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

SAMPLING, SEASONAL ABUNDANCE, AND COMPARATIVE DISPERSAL OF GLASSY-WINGED SHARPSHOOTERS IN CITRUS AND GRAPES: SAMPLING PROGRESS REPORT

Project Leaders:
Steve Naranjo
Steven Castle
USDA-ARS
Phoenix, AZ

Nick Toscano
Department of Entomology
UC Riverside

Reporting Period: The results reported here are from work conducted from October 2002 to October 2003.

ABSTRACT
The spatial distribution of nymphal and adult glassy-winged sharpshooter (GWSS) was studied in citrus orchards in Riverside, CA using a bucket sampling method. On average, about 2.4 times as many GWSS were collected in the upper half of the tree canopy compared with the lower half and about 1.6 times as many were collected on the south side of trees compared with the north side. The coefficient of variation (CV=SD/mean) was nearly 2 times lower in samples taken from the upper half of the canopy compared with the lower half, but there were no differences in the CVs among different compass directions. These findings were used to refine the sample unit for sampling GWSS in citrus. Sticky trap catches of GWSS adults were highly correlated with on-plant GWSS populations within a given year but the relationship was variable between years. Based on the bucket sampling method we present density-dependent sample size and sample cost estimates and a preliminary sequential sampling plan for estimating relative population density of GWSS in citrus. We have applied this sampling program towards estimating the incidence of *Xylella fastidiosa* in GWSS adults. A progressive increase in the proportion of adults positive for *X. fastidiosa* occurred from the time of adult emergence in late June, 2002 through April, 2003. The mean titer of *X. fastidiosa* in heads and thoraxes also increased progressively through this period, suggesting that the potential for vectoring *X. fastidiosa* may rise as the spring generation of adults ages.

INTRODUCTION
Decision-making in knowledge-based pest management depends upon sampling methods that provide reliable information on pest densities and distributions. Practical sampling methodology must balance sampling precision with simple and cost-effective collection techniques. In 2001 and 2002 four glassy-winged sharpshooter (GWSS) sampling methods were evaluated in citrus orchards. These included hand (bucket and beat net) and gasoline-powered (D-Vac and A-Vac) samplers. The bucket sampler was the most versatile and easiest to use with its extendable pole allowing access to foliage 4-6 m above ground. Samples obtained with the bucket sampler were also cleaner than those obtained with other methods and therefore required less handling during sample processing. All methods showed similar patterns of population change over time, but based on quantitative analyses the bucket sampler and the beat net were generally the least costly over the largest range of densities of both nymphal and adult stages. Studies in 2003 focused on quantifying the spatial distribution of GWSS nymphs and adults within citrus tree using the bucket sampler. These studies will help refine the sample unit and further reduce the cost of sampling. A preliminary sequential sampling plan is presented for the precise estimation of relative population density of GWSS. We also continued to examine the relationship between the abundance of GWSS on plants to adult GWSS capture on yellow sticky traps placed within orchards. Such studies will help define the utility of commonly used sticky traps as a method for monitoring GWSS abundance.

It is well recognized that the major threat of GWSS populations is the potential for vectoring *X. fastidosa* to uninfected grapevines in commercial vineyards. One practical application of a sampling plan would be to precisely estimate densities of GWSS within an orchard or vineyard and then determine what proportion are positive for *X. fastidiosa*. Accurate identification of individuals positive for *X. fastidiosa* is an essential part of an overall appraisal of the risk posed by a particular population. Work began in April, 2002 exploring ELISA, PCR, and culturing techniques for the detection of *X. fastidiosa* in GWSS. Sampling and evaluation of the proportion-positive among southern California populations of GWSS has been concentrated thus far in Riverside, owing to resident populations that remain sufficiently large year-round for continuous sampling. A temporal profile of the incidence of *X. fastidosa* in GWSS populations will provide information on when titers of *X. fastidiosa* are highest, and perhaps when dispersing adults are most inoculative. This approach, when integrated into an efficient sampling program providing reliable estimates of GWSS densities, may help to protect grape vineyards and other susceptible crops by recognizing when they are most vulnerable to transmission of *X. fastidiosa* by GWSS.

OBJECTIVES
The overall objective of the research project is to develop statistically-sound sampling methods and plans for estimating density and inoculum potential of GWSS for research and management applications.

RESULTS
Spatial distribution and sampling. To better define the spatial distribution of GWSS nymphs and adults in citrus trees we used the bucket sampler to draw sample units from 8 distinct locations on the tree corresponding to upper and lower halves of the canopy at each of four compass directions (NE, NW, SE, SW). The sample unit consisted of 10 thrusts of a bucket

sampler (a 5 gallon plastic bucket attached to an extendable pole and fitted with a funnel at the bottom that directed insects into a collection cup) into the tree canopy at the specified location. Samples were collected from 14 sites in 2003 that consisted of 6 orchards, each sampled on several dates between early June and late August. Within each orchard sample units from each of the 8 locations on the tree were collected at 20 sites. To minimize disruption, we collected only 2 sample units on opposite sides of any single tree (e.g. SE-upper and NW-lower). All samples were collected at UC-Agricultural Operations, Riverside, California. Analyses of each site individually and all sites pooled indicated clear directional and canopy height patterns in the distribution of both nymphal and adult GWSS (Figure 1). On average about 2.4 times as many GWSS were collected in the upper half of the tree canopy compared with the lower half and about 1.6 times as many were collected on the south side of trees compared with the north side. These patterns held when samples were collected in the afternoon. More importantly from a sampling perspective, the relative variation in counts also showed clear patterns (Figure 2). The coefficient of variation (CV=SD/mean) was nearly 2 times lower in samples taken from the upper half of the canopy compared with the lower half. There were no significant differences in the CVs among the different compass directions. These overall results suggest that samples should be taken from the upper half of the canopy. Based on coefficients variation, there is no particular advantage relative to compass direction; however, population densities would be expected to be different between the northern and southern portions of the tree and one or the other position should be used to standardize sample collection.

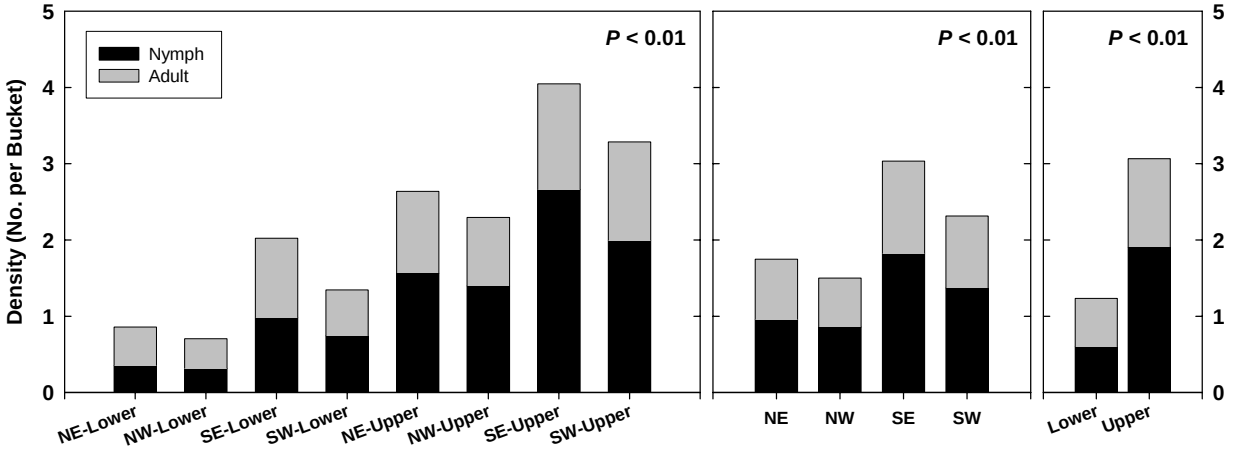

Figure 1. Spatial distribution of nymphal and adult GWSS on citrus trees based on pooled data from 14 sites during 2003. *P*-values denote the results of mixed-model ANOVA.

On 6 sampling dates in 2002 and 8 dates in 2003 we placed yellow sticky traps (Pherocon AM, 22.8 x 27.9 cm) within orchards to capture GWSS adults. Traps were attached to 1 m wooden stakes, placed between rows, and left exposed for 72 hours. We estimated on-plant densities of GWSS (mainly adults) with the bucket method in these same orchards at the time of trap placement. On-plant counts were highly correlated with trap catches in each year; however, regression analyses indicated that the relationships between plant densities and trap counts were very different between years. Samples in both years were collected between late June and early October in the same orchards. These preliminary findings suggest that the prediction of field populations of GWSS from trap catches may be problematic. A similar problem was demonstrated by Naranjo et al. (1995) using sticky traps to predict population densities of sweetpotato whitefly in the cotton system.

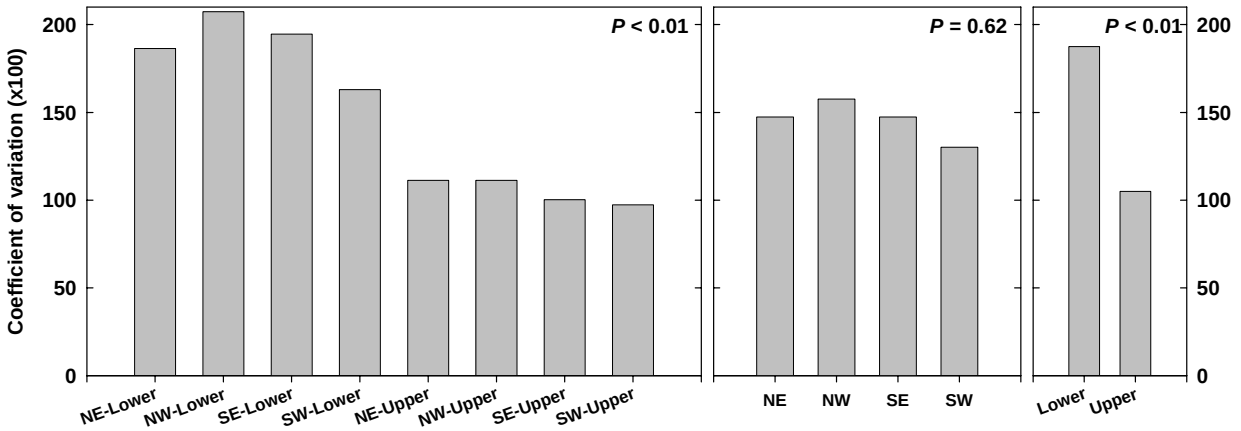

Figure 2. Relative variation (CV = SD/mean) of GWSS counts (nymph + adult) from samples on different portions of citrus trees based on pooled data from 14 sites during 2003. *P*-values denote the results of mixed-model ANOVA.

Based on bucket sampling data collected from 2001 to 2003 we calculated density-dependent sample size and sampling cost curves for two levels of statistical precision (Figure 3). The sample size curve is given by $n = am^{b-2}/D^2$, where n is sample size, D is statistical precision (SE/mean), m is mean density, and a and b are parameters from the Taylor power law (Taylor 1961) relating the sample variance to the sample mean. Sample cost was estimated as the product of the total time need to collect and process a single sample unit and sample size. Sample size and sample cost increase with higher levels of precision and decline as density increases. For example, at a precision of 0.25 less than 10 sample units would be needed to estimate relative densities over 10 GWSS per bucket. Sample costs plateau regardless of precision because of the increased time required to count increasing numbers of GWSS. Because population density is unknown at the time of sampling, we formulated a preliminary sequential sampling plan (Figure 3) using the method of Green (1970). In a sequential plan the need for additional sample information is assessed following the collection of each sample unit so that no more sample units than necessary are collected.

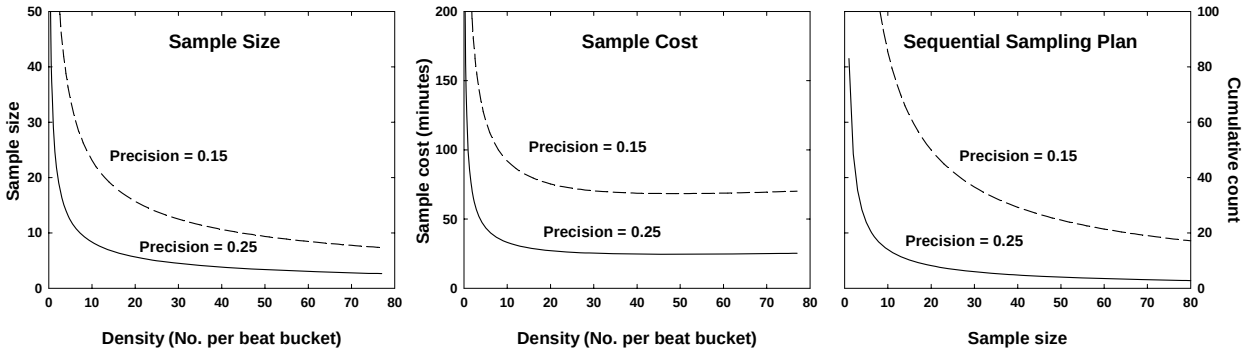

Figure 3. Sample size, sample cost, and a preliminary sequential sampling plan for the bucket method at two levels of statistical precision (SE/mean).

Incidence of *X. fastidiosa* in GWSS populations. Primary spread of *X. fastidiosa* in grapevines occurs when vectors such as GWSS move into vineyards and transmit *X. fastidiosa* at some unknown level. To estimate what proportion are positive for *X. fastidiosa* and possibly inoculative, GWSS adults were collected periodically from the sampling orchards and frozen for detection of *X. fastidiosa*. PCR, ELISA and media culturing techniques were employed, but quantitative ELISA conducted with negative controls and GWSS-based standards provided the most informative results. A greenhouse colony of GWSS started from eggs and maintained on *X. fastidiosa*-free plants served as the source of negative controls (Figure 4a) and "clean" adults that, when homogenized in known concentrations of *X. fastidiosa*, provided a standard curve (Figure 4b) for estimating *X. fastidiosa* titers in field-collected GWSS (Figure 5).

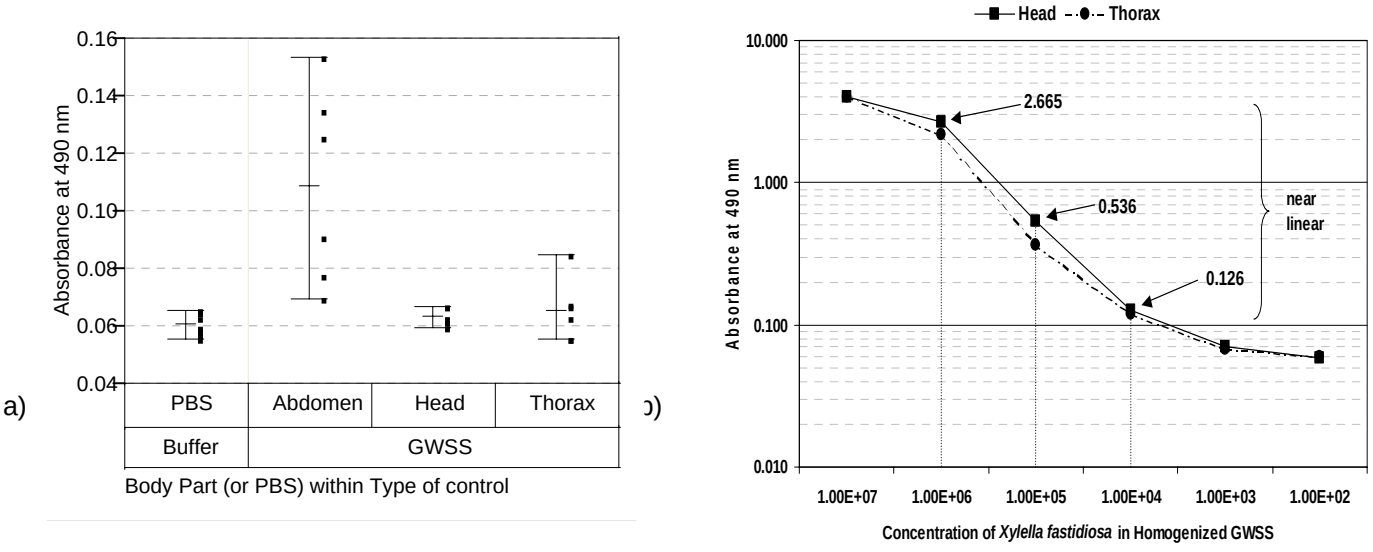

Figure 4. ELISA results for (a) GWSS adults (n=6) reared from eggs on *X. fastidiosa*-free plants grown in a greenhouse. The mean response for each set of points is indicated by the horizontal dash near the midpoints of the vertical lines (range). $Absorbance_{490}$ values for the head and thorax only were not significantly different from the PBS buffer control. A standard curve using *X. fastidiosa*-free GWSS adults was produced (b) and used to estimate the concentration of *X. fastidiosa* in field-collected GWSS adults represented in Figure 5.

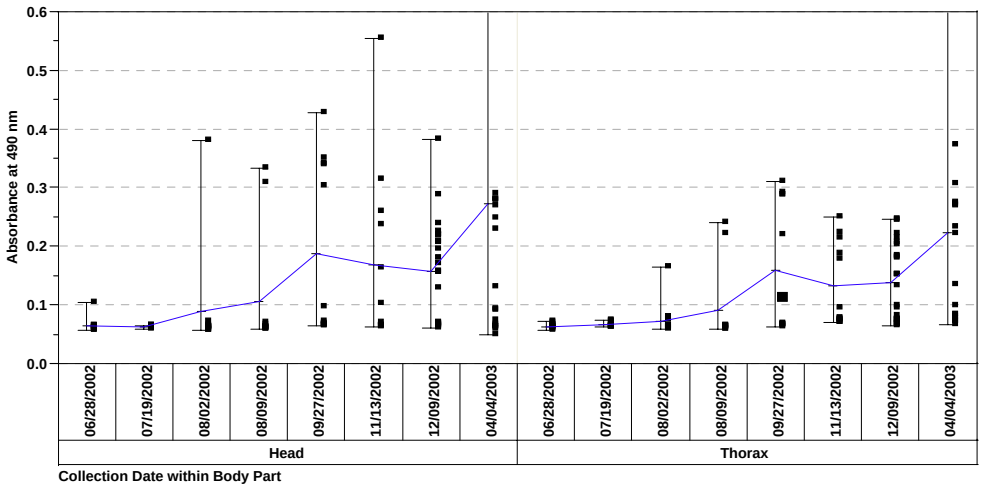

Figure 5. Progressive incidence of *X. fastidiosa* in GWSS adults collected in Riverside, California between June '02 and April '03. An identical pattern of increasing mean titers (represented by the traversing line) of *X. fastidiosa* in both head and thorax segments can be observed, but with consistently higher mean levels in the head.

CONCLUSIONS

Sampling is a fundamental component of the study of population dynamics and central to the development of robust strategies for pest management. The results of our research have focused on the development of an efficient method for estimating densities of GWSS in citrus. Based on considerations of precision and cost we have identified a bucket sampler as an efficient sampling method. Further study of the spatial distribution of GWSS within citrus trees has helped to refine the sample unit and further reduce sampling costs. We present a preliminary sequential sampling plan that will enable researchers and pest managers to precisely estimate the relative density of GWSS at a minimal cost. Further work will be needed to independently test the validity of this sampling plan. For pest management, additional research will be needed to define problematic population densities requiring control. Coupling information on the incidence of *X. fastidiosa* in GWSS will provide better definition of threshold densities.

FUNDING AGENCIES

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

RAPID BIOLUMINESCENT MONITORING OF INFECTION BY THE PIERCE'S DISEASE AGENT

Project Leader:
Glenn M. Young
Department of Food Science and Technology
University of California
Davis, CA 95616

Cooperator:
John Labavitch
Department of Pomology
University of California
Davis, CA 95616

Reporting Period: This report summarizes the goals of this project. Funding for this project initiated October 1, 2003.

ABSTRACT
This report summarizes the goal of a new project focused on bioluminescent quantification and imaging as an approach to monitor *Xylella fastidiosa* infection of grapevine. This research will be applied to addressing how *X. fastidiosa* grows within and spreads through xylem tissue of infected plants.

INTRODUCTION
Xylella fastidiosa has a sophisticated biphasic lifestyle that defines the Pierce's disease infectious cycle; it lives exclusively within the xylem tissue of susceptible plants and in the foregut of insect vectors (2, 3). The complex nature of bacterial-host interactions that take place during the Pierce's disease infectious cycle precludes a standard dogmatic approach to rapidly investigate preventative measures to control this devastating disease. Current approaches toward understanding the progression of Pierce's disease are limited by the length of time required to evaluate disease progression. Symptoms of Pierce's disease often appear only after many weeks or months of grapevine infection with *X. fastidiosa* (2, 3). At earlier stages of infection, *in planta* observations of infection are limited to procedures that are quite time consuming, often are destructive and necessitate large sample sizes to correlate experimental observations with disease pathology. Our goal is to develop a new approach to probe host-pathogen interactions that take place during Pierce's disease of grapevines.

Procedures currently used to detect *X. fastidiosa* infection of grapevine include bacteriological isolation of bacteria from infected plant tissue, the detection of bacterial antigens in plant tissue by ELISA and detection of *X. fastidiosa* nucleic acids by PCR. Each of these techniques provides valuable information, but are limited because they are somewhat expensive, time consuming and require significant numbers of samples to provide statistically significant results. The goal of this study is to develop bioluminescent techniques to extend the current limits of Pierce's disease investigation. The use of bioluminescent technology can overcome many of the current experimental limitations faced by researchers and has some additional benefits. First, strains of *X. fastidiosa* engineered to be bioluminescent can be monitored directly and their numbers can be quantified with the use of a luminometer or a photon sensitive camera coupled to a computer. This time-efficient and cost-effective method of monitoring *X. fastidiosa* infection of grapevines will provide valuable information about the progression of Pierce's disease at pre-symptomatic stages of infection and may reduce the number of plants that need to be infected to complete an experiment. Second, the use of bioluminescent strains of *X. fastidiosa* may allow quantification of growth and spread of bacteria in host tissues. We will evaluate the potential use of *in vivo* bioluminescent imaging (IBI) of whole plants as a non-destructive approach to monitor *X. fastidiosa* growth and spread during infection of grapes. IBI has previously been used to monitor cellular activities in plants and is a proven concept that has already been used by plant physiologists (1, 4). In this project, we present the application as a novel approach to analyze Pierce's disease. When used to its full potential, IBI represents a nondestructive approach that can provide insight at multiple time points over the course of hours, days and weeks from the point of *X. fastidiosa* entry into the plant through systemic spread of the bacteria in xylem tissue. If successful, IBI can be further exploited to rapidly evaluate protective methods and intervention procedures to limit Pierce's disease. In addition, this experimental approach may allow us to rapidly evaluate current and newly developed cultivars of grapes for resistance to *X. fastidiosa* infection.

IBI measures light emitted by specially engineered strains of *X. fastidiosa* from sources within living tissues. The emission of light by the bacteria does not cause any deleterious effects to biological samples (1). Thus biolminescent strains of *X. fastidiosa* offer us the opportunity to take a novel approach that will impact our understanding of Pierce's disease. The success of this research project should help expedite the treatment discovery and development process, thereby offering partners vested in the Pierce's Disease Program the potential to save both time and money in taking prevention strategies into the field. In addition, IBI provides sensitive spatial resolution of the location of infecting bacteria within the plant providing insight on the development of plant infection not achievable with other approaches. Imaging of bacterial infections within living plants provides the opportunity to further our understanding of the processes leading to symptoms of Pierce's disease.

OBJECTIVES

The primary goal of this research project is to use bioluminescent quantification and imaging as an approach to monitor *Xylella fastidiosa* infection of grapevine. This research will be applied to addressing how *X. fastidiosa* grows within and spreads through xylem tissue of infected plants. More specifically, this approach will allow us to evaluate the hypothesis that *X. fastidiosa* growth within xylem tissue coincides with the restriction of xylem fluid transport.

1. Engineer virulent strains of *X. fastidiosa* that produce luciferase.
2. Monitor bioluminescence of Lux^+ strains of *X. fastidiosa* during *in vitro* growth in a defined medium and xylem fluid.
3. Examine growth and spread of bioluminescent *X. fastidiosa* during infection of grapevines.

RESULTS AND CONCLUSIONS

This is a new project that received funding in October of 2003. Results from this project will be available during this next year.

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FUNDING AGENCIES

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

Section 2B: Glassy-winged Sharpshooter Biology, Including Plant-Insect Interactions

SAMPLING, SEASONAL ABUNDANCE, AND COMPARATIVE DISPERSAL OF GLASSY-WINGED SHARPSHOOTERS IN CITRUS AND GRAPES: DISPERSAL PROGRESS REPORT

Project Leaders:
Jacquelyn L. Blackmer
James R. Hagler
WCRL-USDA-ARS
Phoenix, AZ 85040

Reporting Period The results reported here are from work conducted from December 1, 2002 to November 4, 2003.

ABSTRACT
Environmental variables and host-plant quality influence insect population dynamics and the timing and extent of their dispersal. An understanding of how these factors influence glassy-winged sharpshooter (GWSS) development and movement is needed to better predict the spread of Pierce's disease and to aid area-wide management strategies. We investigated how plant factors (i.e., amino acids, osmolality, xylem pressure) and environmental parameters (i.e., wind speed, temperature, relative humidity, barometric pressure) influenced sharpshooter population dynamics and movement in a citrus grove setting. Number of egg masses and adults were counted on branches that were sampled for xylem sap. Collection date, tree, and cardinal direction were noted, and xylem pressure, and amino acids (total, essential and amides) were measured. In conjunction with xylem sap collections, movement of sharpshooters was monitored with yellow and clear sticky traps at 4-h intervals during the day and throughout the night. During replicated sampling periods, 40 times more sharpshooters were trapped on yellow sticky traps in comparison to clear sticky traps and the majority, regardless of sex, were trapped between 1000 and 1400 h. Higher trap catches were associated with increasing temperatures above 18ºC, but were not significantly associated with changes in wind speed, relative humidity or barometric pressure. Trap catches varied significantly over the trapping season, but did not differ due to trap location, indicating that there was no strong edge effect for GWSS. Relative to xylem sap collections, xylem pressure and amides varied due to collection date and time of day, and xylem pressure was positively correlated with trap catches. Osmolality, total amino acids, essential amino acids, and percent amides had no apparent relationship with trap catch. GWSS egg counts varied significantly due to collection date and cardinal direction, with the majority of eggs observed on the east and south sides of the trees.

INTRODUCTION
Insect dispersal can be influenced by numerous factors, such as increasing population densities, reproductive status, biased sex ratios, host breadth, declining host quality and changing environmental conditions (Denno 1979, 1985; Taylor 1985; Denno et al. 1991; Blackmer and Phelan 1991; Blackmer and Byrne 1993a,b, 1999; Blackmer and Cross 2001). A better understanding of how these factors influence the movement of GWSS will be crucial in the management of this pest and the spread of Pierce's disease (PD).

OBJECTIVE
Determine the effects of host-plant quality and environmental variables on GWSS movement as an aid to predicting insect and disease spread.

RESULTS
In Fillmore, CA movement of GWSS in a citrus orchard was measured relative to time of day, environmental parameters, and xylem flux. Temperature, relative humidity, barometric pressure, wind speed, and wind direction were monitored at the center of the site with a portable weather station. Sticky traps were changed and xylem sap was collected at 4-h intervals from 0600 to 2200 h (8 samples per time interval on five collection dates). Xylem sap was extracted from 25-cm-long orange stems with a pressure chamber (Scholander et al. 1965). An equal number of samples were taken from the four cardinal directions from each of four trees for each sampling interval for a total of 200 samples. We used a Fiske Model 110 micro-sample osmometer to determine osmolality of the xylem sap, and a Beckman 7300 Analyzer to detect amino acids.

Trap Catch
Significantly more sharpshooters were trapped between 1000-1400 h than at any other time interval during the 24-h-sampling periods; very few sharpshooters were trapped at night ($F=14.36$; df=4, 128; $P<0.0001$). Yellow traps captured significantly more sharpshooters than the clear control traps (85.6 ± 24.3 versus 2.1 ± 0.88, respectively). However, the patterns of recapture relative to time of day and week were similar regardless of trap color ($R^2=0.44$, $P<0.0005$). Equivalent numbers of males and females were captured relative to collection date, time of day, and trap color. Trap catch varied over the five weeks of the sampling period, and was higher from mid-July to early August when compared to collections made in late August to early September ($F=16.82$; df=4, 44; $P<0.0001$). Trap catch relative to position in the field was not significantly different ($P=0.34$), indicating that there was no strong edge effect. Of the environmental parameters monitored, only temperature explained a significant amount of the variability in trap catch in the citrus setting ($R^2=0.58$, $P< 0.0001$). Sharpshooters were rarely trapped when temperatures fell below 18°C.

Xylem Collections

Twenty amino acids were detected, with aspartic acid, serine, asparagine, glutamic acid, arginine, and proline being predominant (comprising 80-90% of the samples). Total amino acids, essential amino acids, percent amides, xylem pressure, and osmolality varied significantly due to collection date ($P < 0.005$ for each). Total amino acids, percent amides and xylem pressure also varied significantly due to time of day ($P < 0.005$), but only xylem pressure and percent amides varied in a consistent manner from week-to-week (Figure 1). Xylem pressure and percent amides peaked at approximately 1400 h. Cardinal direction from which the samples were taken had no effect on amino acid concentrations, or xylem pressure, and only a slight effect on osmolality ($P < 0.05$); highest readings were on the eastern side of the trees.

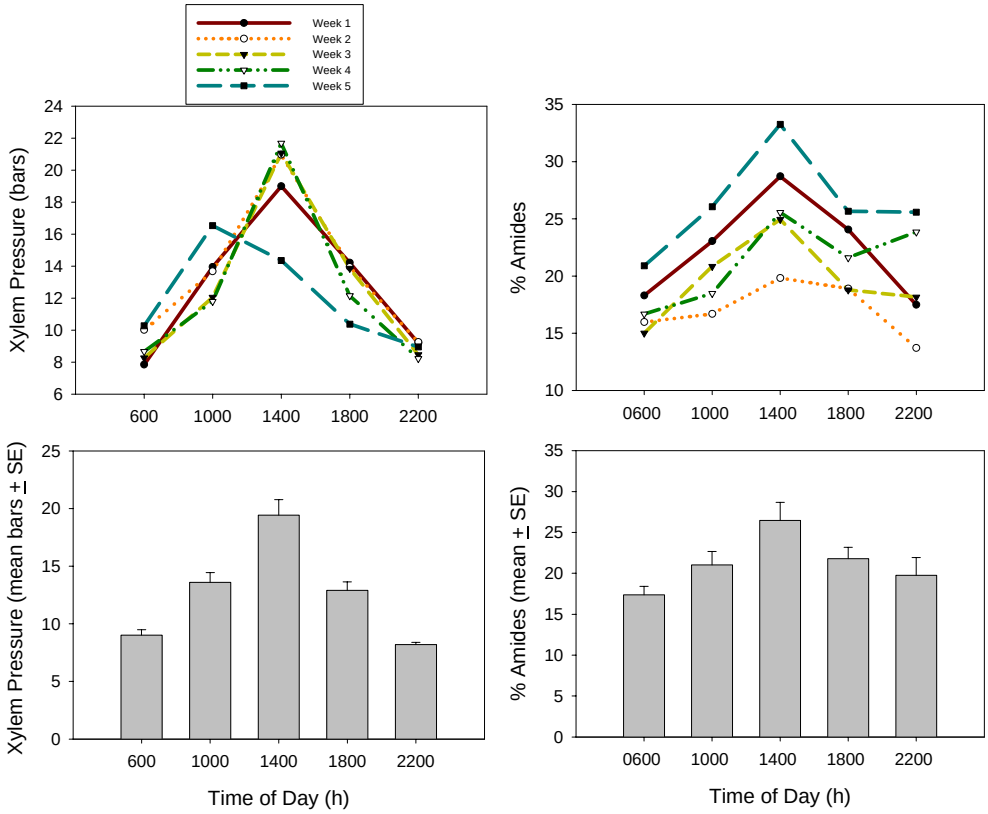

Figure 1. Weekly and mean (± SE) xylem pressure and amides (% ± SE) from orange cuttings relative to time of day, Fillmore, CA.

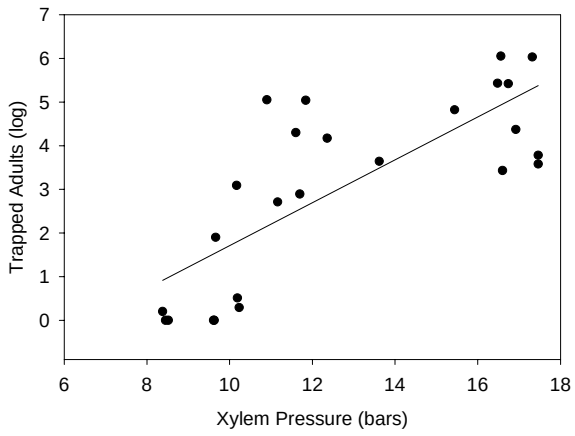

Figure 2. Association between xylem sap pressure (bars) and number of adult GWSS trapped on yellow sticky traps in an orange grove, Fillmore, CA.

Xylem Dynamics and Insect Movement

The number of adults and eggs found on the sampled orange branches varied significantly due to collection dates ($P< 0.0001$); number of eggs also varied due to cardinal direction ($P< 0.0001$). Fewer egg masses were found in mid-July than on other collection dates, and most egg massees were found on the east ($x = 12.4$) or south ($x = 10.2$) sides rather than the west ($x = 6.5$) or north ($x =5.2$) sides of the trees. The number of egg masses was not associated with the number of adults, and was only weakly associated with osmolality ($R=0.16$; $df= 1,198$; $P=0.025$) and essential amino acids ($R=0.2$; $df=1,198$; $P=0.003$). The number of adults trapped on yellow sticky traps was only associated significantly with xylem pressure readings ($R = 0.77$; $df=1,23$; $P<0.001$; Figure 2).

CONCLUSIONS

- Trap catch was influenced by trap color, collection date, and time of day. In the citrus setting, trap catch increased with temperatures above 18ºC. Previously, we found that in a more open setting, flight activity was suppressed by temperatures below 17ºC and by wind speeds above 3 m s^{-1} (Blackmer et al. in press).
- Xylem parameters varied considerably due to collection date, and total amino acids, percent amides and xylem pressure varied due to time of day. However, the number of adults trapped on yellow sticky traps was only associated with xylem pressure. Higher pressures at midday may limit the insects feeding efficiency and lead to opportunistic movements that result in the exploitation of alternate hosts.
- The east and south sides of the citrus trees were preferred sites for oviposition as indicated by higher numbers of egg masses. This preference was weakly associated with higher osmolality and a greater concentration of essential amino acids.

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

Project Leaders:
Heather S. Costa
Donald A. Cooksey
Department of Entomology
University of California
Riverside, CA 92521

Cooperator:
Blake Bextine
Department of Entomology
University of California
Riverside, CA 92521

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

ABSTRACT
In this project we are testing the effects of feeding substrate on the acquisition and retention of *Xylella fastidiosa*. We are using two strains of *X. fastidiosa* that are present in California: a Piece's disease (PD) strain that infects grape, and an oleander leaf scorch (OLS) strain that infects oleander. For the initial phase of this project, we began by comparing the relative proportion of insects that tested positive after acquisition of a given strain of *X. fastidiosa* when they were maintained on a plant species that was either a host or non-host of that strain. We are now in the process of testing individual insects using strain-specific PCR methods to detect the presence the pathogen. In the first three replicates using the OLS strain, the proportion of insects testing positive after acquisition was similar regardless of whether the insects were fed on a host or a non-host of the OLS strain. Analysis of samples that have been collected in additional replicates will allow us to determine if this pattern is consistent, and whether or not the same type of pattern is observed with insects carrying the PD strain. Additional experiment will be conducted to test the effects of varying the feeding substrate on transmission rates of each strain.

INTRODUCTION
Several genetically distinct strains of the bacterial plant pathogen, *Xylella fastidiosa*, have been identified in the United States. Some of these strains infect grapevines and cause symptoms of Piece's disease, while others do not infect grape (Hopkins et al. 1989, Chen et al 1992, da Costa et al. 2000, Henderson et al. 2001). The key factors that contribute to host plant specificity of *X. fastidiosa* strains are not known, however, it is likely that differences in xylem nutritional content among plant species, and differences in the nutritional requirements of each strain, play some role in host plant specificity. This concept is supported by the fact that the strains grow at different rates when cultured i*n vitro* on media with different nutritional contents (Davis et al. 1980, 1981, Hartung et al. 1994, Yonce and Chang 1987).

The glassy-winged sharpshooter is capable of acquiring and transmitting several different strains of *X. fastidiosa* from a variety of host plants (Purcell and Hopkins, 1996, Purcell et al. 1999, Costa et al. 2000). It has been assumed that *X. fastidiosa* present in the mouthparts of the insect vector obtain nutrition from the host plant xylem as it passes through the insect. Thus, one might expect that the ability of a *X. fastidiosa* strain to survive in the insect's mouthparts, and be successfully transmitted, would be dependant on the xylem content of the plant host on which the insect feeds.

In this project, we are using two strains of the pathogen that are present in California to test the effects of feeding substrate on the acquisition and retention of *X. fastidiosa*: a Piece's disease (PD) strain that infects grape, and an oleander leaf scorch (OLS) strain that infects oleander (Purcell and Hopkins 1996, Blua et al. 1999, Purcell et al. 1999). These two strains have different host ranges; the PD strain does not infect oleander, and the OLS strain does not infect grape. Thus, one might expect an OLS strain *of X. fastidiosa* would be retained longer in insects feeding on oleander than in insects feeding on a non-host of the oleander strain. Likewise, a PD strain would be retained longer in insects feeding on grapevines than they would on a non-host of the grape strain.

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

4. Test the effects of antibacterial materials on acquisition and transmission of *X. fastidiosa* by GWSS.
5. Test the effects of variation in substrate pH and free ion availability on the acquisition and transmission of *X. fastidiosa* by GWSS.

RESULTS AND CONCLUSIONS

Progress on Objective 1

We are still in the early stages of this project, and are reporting here on progress made on Objectives 1 and 2 during the first 3 months of study. For the initial phase of this project, we began by comparing the relative proportion of insects that tested positive after acquisition of a given strain of *X. fastidiosa* when they were maintained on a plant species that was either a host or non-host of that strain.

Grape plants (*Vitis* spp.) infected with a Pierce's disease strain of *X. fastidiosa*, and oleander plants *(Nerium oleander*) infected with an oleander leaf scorch strain were used as sources of inoculum. The strain of *X. fastidiosa* infecting plants was confirmed by PCR. Groups of GWSS adults were caged on either an OLS infected oleander plant, or a PD infected grapevine for 2 days. Samples of insects from infected source plants were tested using PCR to determine the initial proportion of insects that tested positive for the respective strains. Insects from each source plant species were split into two groups, and moved to an uninfected plant of the same species as the source plant (oleander or grape), or to a non-host of either strain (chrysanthemum) (Figure 1). Samples of insects were collected at 1, 3, and 7 days (or longer if adults remained) after transfer to uninfected hosts and frozen.

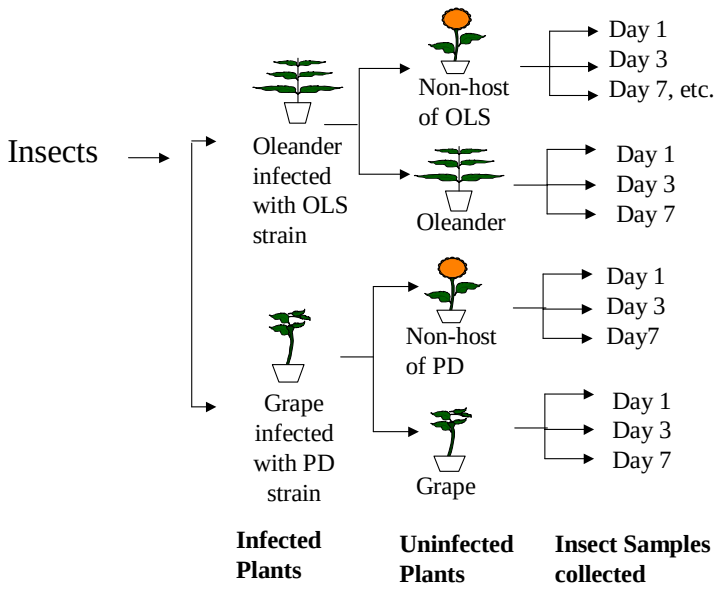

Figure 1. Diagram of experimental design for testing retention times of the OLS and PD strains of *X. fastidiosa* when insects were maintained on a either a host or non-host plant of each strain after acquisition.

The experiment was replicated multiple times with oleander and grape strains until at least 30 insects in each test category were collected. The first 4 replicates of the experiment did not have enough insects surviving to 7 days or longer to allow adequate comparison, thus, an additional 4 replicates for each strain were done until adequate numbers survived 7 days or longer.

We are now in the process of testing individual insects that were collected using strain-specific PCR methods to detect the pathogen. We extracted *X. fastidiosa* DNA from individual insects using a commercially available DNA extraction kit (e.g. Qiagen DNeasy Tissue Kit). All samples were macerated in disposable, mesh-lined sample bags (Agdia, Inc.) that allowed straining before processing to remove large particles of insect material. Individuals were placed in bags with 200 µl of PBS, and homogenized using a ceramic pestle on the outside of bags resting on a hard surface. Strained liquid (180 µl) was transferred from the bag to a 1.5 mL microcentrifuge tube, and the kit directions for insect DNA extraction from the DNeasy Tissue Kit handbook supplied with the kit were followed.

Amplification was performed using XF1968-L/XF1968-R or XF2542-L/XF2542-R primers to specifically amplify the target strain (Cooksey et al. unpublished). These primer sets produce different sized bands for OLS and PD strains of the pathogen.

An example of results from three replicates of experiments using the OLS strain from oleander (210 insects tested) is shown below (Figure 2). In the first three replicates using the OLS strain, the proportion of insects testing positive after acquisition

was similar regardless of whether the insects were fed on a host or a non-host of this strain. Analysis of samples collected from the additional replicates will allow us to determine if this pattern is consistent, and whether or not the same type of pattern is found with insects carrying the PD strain.

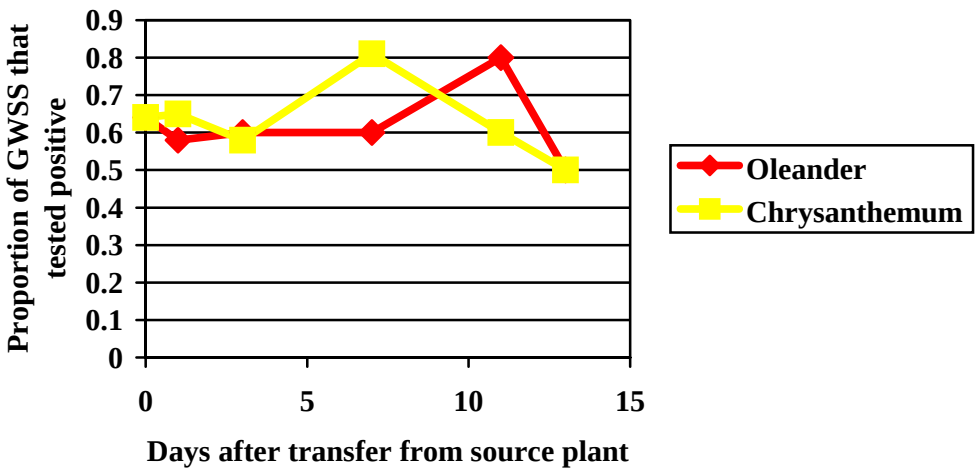

Figure 2. Graph summarizing three replicates of experiments testing retention of the OLS strain of *X. fastidiosa* by the GWSS when insects were maintained on a host (oleander) or a non-host (Chrysanthemum) of the OLS strain after acquisition from oleander.

Progress on Objective 2

To test if substrate can influence the ability of insects to acquire or retain a particular strain of *X. fastidiosa* more directly, we will use a pathogen delivery system that will allow us to either maintain or manipulate the feeding substrate as desired. We have just begun the process of culturing each isolate we will be using, and building up sufficient cultured material to allow us to begin experiments of Objective 2. In these trials, isolates of each strain will be delivered to insects through a cut stem delivery system developed by Bextine et al. (2002) that will allow us to manipulate the substrate carrying the pathogen as desired.

CONCLUSIONS

The first objective of this proposal was to compare retention times of *X. fastidiosa* when infected glassy-winged sharpshooter (GWSS) were subsequently fed on plants that were either hosts or non-hosts of the strain they carried. We are still in the process of analyzing the results of these experiments, however, if we should continue to see a pattern where the retention of a host-specific strain does not decline in insects feeding on a non-host of that strain (as observed in initial studies with the OLS strain), this could mean that xylem content of the feeding substrate is not critical in determining which strains of the pathogen are retained by insects. If on the other hand, we begin to find that retention of a *X. fastidiosa* strain decreases when insects feed on non-host of that strain, this could suggest that the xylem content of the feeding substrate plays a significant role in determining which strains of the pathogen are retained by insects. Additional studies will be conducted to test the effects of varying insect feeding substrate on the transmission rates of each strain, because the frequency of detection of the pathogen in insects may not always indicate the frequency of transmission.

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FUNDING AGENCIES
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POPULATION GENETIC STRUCTURE OF THE GLASSY-WINGED SHARPSHOOTER DETERMINED BY ISSR-PCR DNA FINGERPRINTING

Project Leader:
Jesse H. de León
USDA- ARS
Beneficial Insects Research Unit
Weslaco, Texas 78596

Cooperators:
Walker A. Jones
USDA- ARS
Beneficial Insects Research Unit
Weslaco, Texas 78596

David J. W. Morgan
Cal. Dept. Food and Agriculture
Mount Rubidoux Field Station
Riverside, California 92501

Reporting Period: The results presented here are from work conducted from FY2002 to FY2003.

ABSTRACT
In the present study compound Inter-Simple Sequence Repeat (ISSR) primers containing CA/GT-repeat motifs in their sequences were utilized to estimate the population genetic structure of *Homalodisca coagulata* (Say). Eighteen populations from throughout the U. S. and a population from Tahiti, French Polynesia were analyzed. The eighteen U. S. populations were arbitrarily assigned to three regions- southeastern (SE), southwestern (SW) (Texas), and western (W) (California) regions. A total of 62 and 91 neutral polymorphic markers were identified with p-15 and p-13, respectively. Exact tests for population differentiation indicated significant differences in marker frequencies among the 18 populations; in addition, significant differences were also observed within each region. Analyses of molecular variance (AMOVA) showed a significant partitioning of gene diversity among regions, 11% with p-15 and a lower value of 3% with p-13. The majority of the variance, however, was distributed within populations, 83% and 88% with p-15 and p-13, respectively. Values of G_{ST} (8-11%) and θ (7-10%) for among region variation were of comparable magnitudes to the AMOVA results. A dendrogram based on Reynolds coancestry distance performed with p-15 clustered the U. S. populations into two main groups, with the southeastern populations in one cluster and the southwestern and western populations in another cluster. Within the western region, dendrograms performed with p-13 and p-15 showed in both cases that the Edison and Bakersfield populations clustered as outliers. The present results estimate, for the first time, the population genetic structure of *H. coagulata* and suggest that a subset of insects in California may have their origins in the southwestern region (Texas); furthermore, these results are suggestive of more than one founding event in California.

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*, a bacterium that causes Pierce's disease in grapevines (*Vitis vinifera* and *V. labrusca*) (Hopkins and Mollenbauer 1973). *H. coagulata* are native to the southern United States, from Florida to Texas and they are also distributed in Northern Mexico (Turner and Pollard 1959, Nielsen 1968, Brlansky et al. 1983). Within the last 10 years, *H. coagulata* have established in southern California where they pose a serious threat to the wine and table grape industry (Sorensen and Gill 1996). Recently, we developed DNA markers for *H. coagulata* for the purpose of estimating genetic variation in natural populations (de León and Walker 2003). These DNA fingerprinting procedures permit detection of DNA variation in Simple Sequence Repeats (SSR) without the need to isolate and sequence specific DNA fragments (reviewed in Karp and Edwards 1997). Many classes of microsatellite repeat motifs have been identified, though the class most abundant in eukaryotic genomes is the CA-repeat. The presence of these repeat motifs in high copy number and their dispersion throughout the genome of all eukaryotes tested has been demonstrated by earlier studies (Tóth et al. 2000). Therefore, because of their high density, oligonucleotides complementary to these CA-repeat motifs can be used as single primers to target a significant portion of the genome and reveal highly polymorphic banding patterns (Zietkiewicz et al. 1994).

OBJECTIVES
The objectives of the present study were to:

1. Estimate genetic variation within and among populations
2. Estimate the population genetic structure
3. Ascertain if our method was sensitive enough to determine the origin of *H. coagulata* present in California.

RESULTS AND CONCLUSIONS
ISSR-PCR was utilized and we demonstrated and compared the ability of two compound ISSR primers p-13, A(CA)7(TA)2T and p-15, T(GT)7(AT)2 to generate polymorphic markers and to estimate geographic variation in 18 natural populations (529 individuals) of *H. coagulata* from the U. S. and a population from Tahiti, French Polynesia (15 individuals). Different approaches to estimate population differentiation- Exact tests (Raymond and Rousset 1995), ΦST (Excoffier at al. 1992), GST (Nei 1987), θ (Weir 1990, 1996), and dendrograms based on genetic distance (Reynolds et al. 1983) (UPGMA method) were than applied and compared.

A total of 62 and 91 neutral polymorphic markers were identified with compound ISSR primers p-15 and p-13, respectively in the 19 populations of *H. coagulata* (Table 1). Within the U. S., percentage polymorphic loci was 100% for each region with both ISSR primers and the highest polymorphic ratio (number of polymorphic loci per number of insects) was seen in the southwestern populations. Exact tests for population differentiation indicated that significant differences in marker frequencies existed among the 18 populations from the U. S. (χ^2 = 664; df = 138; *P* = 0.0000; p-15 and 1,279; df = 202; *P* = 0.0000; p-13) and in addition, exact tests showed marker frequency differences within regions, with the western populations showing the highest values with both primers.

Tables 2 and 3 present results from different approaches used to apportion variation into within- and among-population levels. AMOVA analyses showed a statistically significant partitioning of gene diversity among regions, 11% (Φ_{CT} = 0.114; df = 2; *P* = 0.001) with p-15 and a lower value of 3% (Φ_{CT} = 0.027; df = 2; *P* = 0.001) with p-13 (Table 2). Significant differentiation was also distributed among populations within regions, 6% (Φ_{SC} = 0.064; df = 15; *P* = 0.001) with p-15 and 9% (Φ_{SC} = 0.088; df = 15; *P* = 0.001) with p-13. The majority of the variance, however, was distributed within populations, 83% (Φ_{ST} = 0.171; df = 511; *P* = 0.001) with p-15 and 88% (Φ_{ST} = 0.113; df = 511; *P* = 0.001) with p-13. Table 3 shows a comparison of other genetic differentiation estimates, G_{ST} and θ. Excellent agreement was seen between G_{ST} and θ values for the within population variances, 89.0 and 90.1% and 92.0 and 93.0% with p-15 and p-13, respectively. Within each region, little genetic differentiation was seen with G_{ST} and θ results with either primer, though the western region populations demonstrated slightly higher G_{ST} (0.0492; p-15 and 0.0843; p-13) and θ (0.0403; p-15 and 0.0631; p-13) values; furthermore, the indirect estimate of gene flow, Nm based on G_{ST}, demonstrated slightly lower values (9.66; p-15 and 5.43; p-13) with both primers, indicating that the western region was slightly more differentiated, in accord with the Exact tests above. Overall, gene flow was greater among populations within regions than among regions. Taken together though, these overall results indicate moderate genetic differentiation of *H. coagulata* populations from the U. S., but the fact that most of the genetic variation is distributed within populations may be an indication of strong microgeographical differentiation.

A dendrogram based on Reynolds et al. (1983) coancestry distance performed with p-15 is shown on Fig. 1A. Two main clusters were formed with the southeastern region populations (cluster B) separated from the southwestern and western region populations (cluster A). Within cluster A, clusters or subgroups are formed with Edison and Bakersfield populations forming the second separate cluster (d). Two more clusters are seen within cluster c. Western or California populations are distributed within three separate clusters within the main cluster A. Southeastern populations formed two clusters within cluster B, with Tifton and Cairo, GA residing in one subgroup. Results performed with p-13 showed a similar pattern of clustering but with some variation, in that case Weslaco and Monte Alto, TX populations were clustered within the southeastern populations, though the western and southeastern region populations were still clustered separately (data not shown). In order to see a clearer picture of the western region populations, analyses were performed separately from the rest of the U. S. populations with p-15 and p-13 and are demonstrated on Figs. 1B and 1C, respectively. With both ISSR primers two main clusters (A and B) were formed. Some variation in clustering of the populations is seen within cluster A between the two primers; however, results show that in both cases, the Edison and Bakersfield populations were clustered (B) as outliers from the rest of the California populations. In the western region, Edison and Bakersfield are more geographically isolated from the rest of the western populations and appear to be more differentiated.

Table 1. Analyses of H. coagulata from the U. S. analyzed by region.
No. P, number of polymorphic loci; Polymorphic ratio (number of polymorphic loci per number of insects); %P, percent polymorphic loci (POPGENE program); Exact tests (χ^2) (results over loci) (TPFGA program) for population differentiation (Raymond and Rousset 1995). Marker frequencies were based on Lynch and Milligan's (1994) Taylor expansion estimate. df = degrees of freedom (for Exact tests); ***, *P* = 0.0000 (overall *P*-value).

Region	No. Insects	No. P	Polym. ratio	%P	χ^2		df
p-15:							
SE	169	34	0.20	100	201.90	***	72
SW	120	54	0.45	100	178.83	***	112
W	240	46	0.19	100	311.76	***	102
All	529	62	0.12	100	664.39	***	138
p-13:							
SE	169	67	0.40	100	415.92	***	126
SW	120	84	0.70	100	403.77	***	178
W	240	83	0.35	100	640.83	***	174
All	529	91	0.17	100	1,279.04	***	202

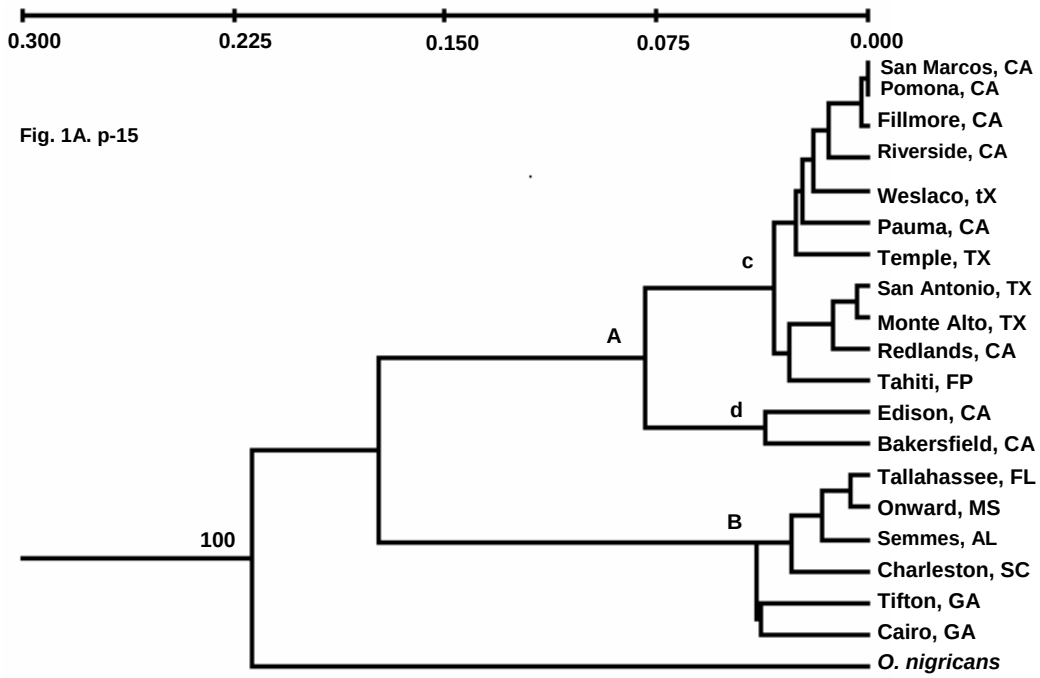
Table 2. Analyses of molecular variance (AMOVA) (GenAlEx program) for *H. coagulata* populations from the U. S. Statistics include: df, degrees of freedom; SS, sum of squares; MS, mean squares; Est. var., estimated variance; and %D, distribution of total variance. **, $P = 0.001$.

Source	df	SS	MS	Est. var.	Φ- statistics	%D
p-15:						
amg regions	2	93.43	46.71	0.244	$\Phi_{CT} = 0.114$ **	11
amg pops./regions	15	80.24	5.35	0.122	$\Phi_{SC} = 0.064$ **	6
within pops	511	904.0	1.77	1.769	$\Phi_{ST} = 0.171$ **	83
p-13:						
amg regions	2	75.28	37.64	0.128	$\Phi_{CT} = 0.027$ **	3
amg pops./regions	15	239.7	15.98	0.403	$\Phi_{SC} = 0.088$ **	9
within pops	511	2124.3	4.16	4.157	$\Phi_{ST} = 0.113$ **	88

Table 3. Estimates and comparison of G_{ST} (POPGENE) and θ (TFPGA) values for *H. coagulata* populations from the three regions of the U. S. G_{ST} (mean), coefficient of gene differentiation; θ (mean), theta is analogous to F_{ST}; and Nm, gene flow (POPGENE).

Region	G_{ST}	θ (SD)	Nm	Region	G_{ST}	θ (SD)	Nm
p-15:				**p-13:**			
SE	0.0426	0.0321 (0.008)	11.24	SE	0.0615	0.0551 (0.008)	7.63
SW	0.0426	0.0376 (0.014)	11.22	SW	0.0465	0.0449 (0.010)	10.25
W	0.0492	0.0403 (0.016)	9.66	W	0.0843	0.0631 (0.026)	5.43
All	0.1101	0.0989 (0.048)	4.04	All	0.0799	0.0668 (0.012)	5.75

Figure 1. Dendrograms based on Reynolds coancestry distance (TFPGA). Relationships showing the 19 geographic populations of *H. coagulata* performed with p-15 (A). *Oncometopia nigricans* (23) are included as an outgroup. Western region (California) populations were analyzed separately with p-15 (B) and p-13 (C). Genetic distances are indicated above the dendrograms and bootstrap support values (greater than 40%) are indicated at the nodes.



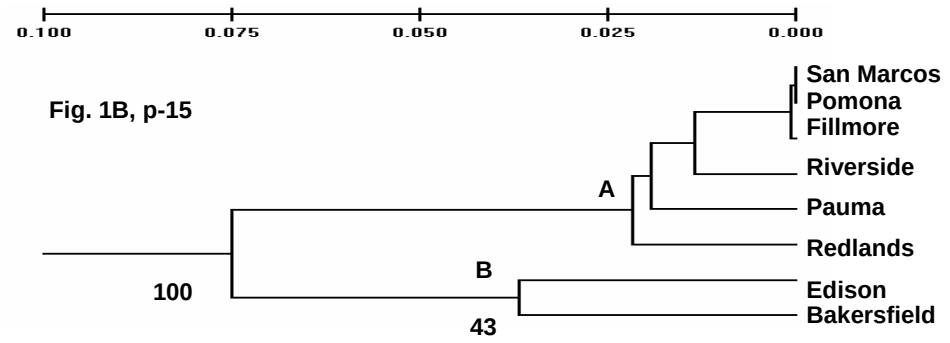


Fig. 1B, p-15

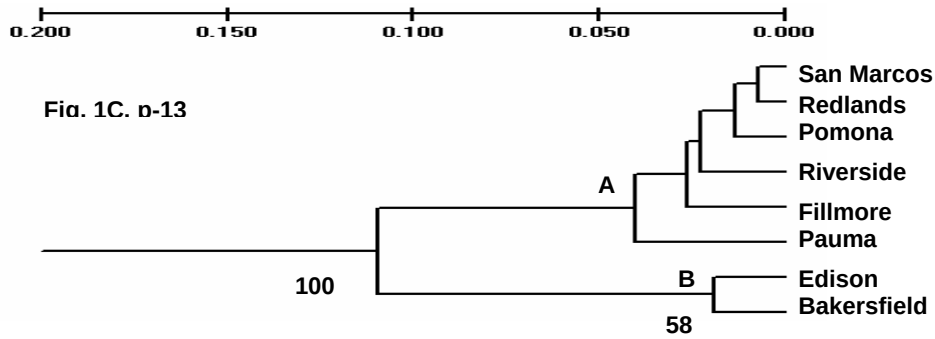


Fig. 1C, p-13

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

Project Leader:
Thomas P. Freeman
Electron Microscopy Laboratory
North Dakota State University
Fargo, ND 58105

Cooperators:
Roger A. Leopold
Dennis R. Nelson
James S. Buckner
USDA-ARS
Biosciences Research Laboratory
Fargo, ND 58105

Thomas J. Henneberry
Western Cotton Research Laboratory
Phoenix, AZ 85040

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

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

RESULTS
Ultrastructural studies have contributed to a more complete morphological understanding of the mouthparts of the glassy-winged sharpshooter (*Homalodisca coagulata*). Our first objective, mouthpart description, has been met. We have identified and described the morphology of specific sharpshooter mouthpart structures important for understanding stylet movement and penetration. A detailed description of these structures can be found in Leopold et al., 2003.

Mechanosensory-like sensilla cover most of the terminal segment of the labium, and are found along the margin of the labial groove in all three labial segments. A pair of short sensilla, located at the very tip of the labium, is often found in contact with the extended stylets (Figure 1). Triangular spines occur singly, in straight-line clusters, or in palmate patterns on the exposed labial surface (Figures 1, 2). The unexposed surfaces of the labial groove lateral and basal to the stylets are covered with numerous multi-lobed palmate structures (Figure 3) that appear to be in constant contact with the mandibular stylets. Larger palmate structures are located at the terminal end of the labial groove (Figure 4). These structures are located below the stylets, with only the tips of the palmate lobes apparent when the stylets are extended.

Mandibular stylet morphology varies somewhat with stage of development (Leopold et al., 2003; Freeman et al., 2002). A series of cup-shaped flanges are located along the medial surface of these stylets, with small papillae located between the flanges. Slender fingerlike projections with pointed tips are found on the ventral side near the tip of adult mandibular stylets. The projections become flattened and tab-like proximally. Each mandibular stylet has a single unbranched dendritic canal from base to tip (Figure 5).

The paired maxillary stylets interlock with each other along their length (Figure 5), except for a short distance at the apex (Figure 6). These joints, similar to mortise-and-tenon joints, keep the stylets together, forming a food canal and salivary canal, and also allow extension of either stylet individually (Figure 6). A single dendritic canal in the base of each maxillary stylet (Figure 6) branches toward the tip of the stylet (Figure 7), with neurons (Figure 8)

reaching the short row of small dentitions along the edge of each maxillary stylet (Figures 6, 9) and extending further to the very tip of the stylet.

Fractured stems reveal the course of the sheath from the flange on the outer epidermal wall well into the host-plant tissues (Figure 10). These sheaths can be dissected out of stem or leaf tissue more or less intact and examined using scanning

electron microscopy (Figures 11, 12). Ultrastructural changes related to stylet penetration of individual cells are difficult to determine. Stylet penetration ruptures cell walls, with the salivary sheath material masking much of the ultrastructure of damaged cells. The length of the sheath, the density of the sheath material, and the requirement for ultrathin sections make transmission electron microscopy arduous. Confocal scanning light microscopy provides an opportunity to examine intact salivary sheaths and assess cellular damage using optical sections and 3D reconstruction.

Salivary sheaths (Figures 13-18) formed by all developmental stages from newly hatched sharpshooter nymphs to adults were examined from 14 different host plants. Sheaths varied from unbranched (Figures 13, 16) to highly branched (Figures 13-15), and terminated in various host-plant tissues. Approximately 65% of salivary sheaths formed by adult sharpshooters terminated in host-plant xylem tissue. Parenchymal cells of the cortex or medullary ray were the next most frequent location for termination. Only a few sheaths terminated in phloem tissues or in the pith of host plants. Nymphs and second instars, with shorter stylets, preferred to feed on the mid- and lateral veins of leaves, or on very small vascular bundles along the leaf margins, rather than on stems.

Salivary sheaths commonly were found in very close proximity to one another (Figures 13, 14). It has yet to be determined whether these multiple sheaths are formed by a single sharpshooter, moving only slightly from one feeding position to another; or if each sheath is formed by a different individual. Branched salivary sheaths were found in both vascular and parenchymatous tissues. It is unclear if branched salivary sheaths represent failed attempts to locate vessel elements in the xylem tissue, or if penetrated parenchyma cells provide a nutritive advantage to the sharpshooter. Preliminary studies have shown that the sharpshooters may produce drops of exudate even without reaching the water-conducting vessel elements, suggesting that parenchymatous cell contents may be ingested.

Salivary sheaths have been found in contact with vessel elements with no indication of actual cell-wall penetration (Figure 16), and without salivary sheath material in the cell lumen. It may be possible for the sharpshooter to remove water from the vessel through the pits in the wall without actually penetrating the cell wall. However, there is clear evidence that more commonly the wall of the vessel element is ruptured by the penetrating stylets (Figures 17, 18). The large volume of salivary sheath material in the lumen of many vessel elements (Figures 15, 17, 18) is sufficient to restrict or block completely the translocation of xylem fluid within a single vessel element. The occlusion of numerous vessel elements could result in the deterioration or death of the plant even in the absence of pathogenic bacteria.

CONCLUSIONS

This ultrastructural study has provided and will continue to provide data necessary to completely understand the anatomy and morphology of the sharpshooter related to stylet penetration, salivary sheath formation, feeding behavior, transfer of the bacterium *Xylella fastidiosa*, and the nature of Pierce's disease.

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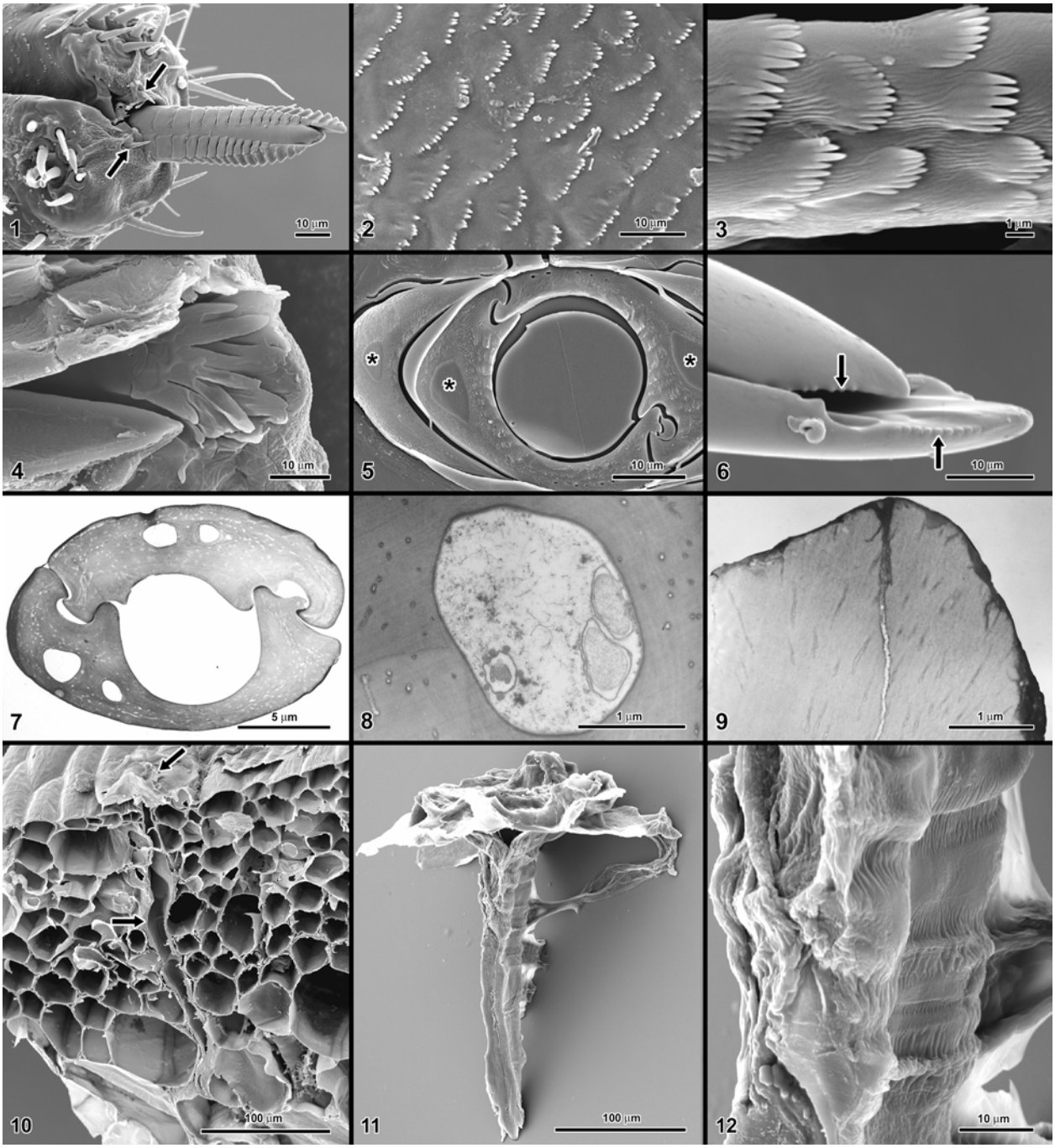

Figure 1. Sensilla (arrows) at the tip of the labium in contact with the mandibular stylets.
Figure 2. Peg-like spines in straight line row on the exposed surface of the labium.
Figure 3. Multi-lobed structures located on the unexposed surface of labium.
Figure 4. Palmately lobed structures found at the apex of labium.
Figure 5. Cross-sectional view of the interlocked maxillary stylets and dendritic canals (*).
Figure 6. Tips of the maxillary stylets showing row of denticles (arrows).
Figure 7. TEM section of maxillary stylets each with a pair of dendritic canals.
Figure 8. Dendritic canal with neurons near the tip of a maxillary stylet.
Figure 9. Section through a denticle at the tip of the maxillary stylet.
Figure 10. SEM micrograph showing a flange and salivary sheath within the host plant.
Figure 11. Salivary sheath dissected out of a host plant.
Figure 12. External surface of a salivary sheath removed from a host plant.

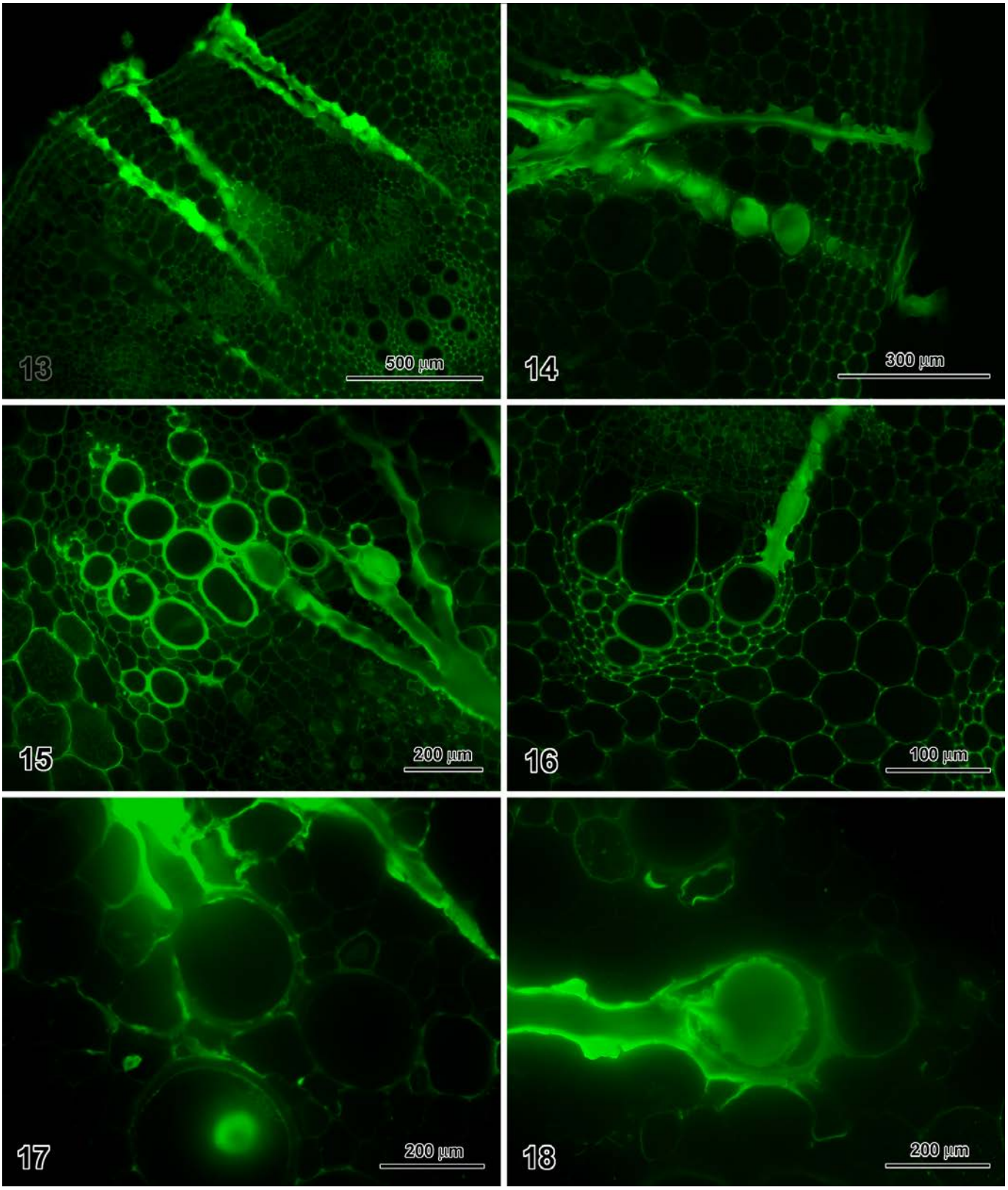

Figure 13. Cross-section of stem with multiple salivary sheaths.
Figure 14. Overlapping branched salivary sheaths in a plant host stem.
Figure 15. Two branches of a salivary sheath terminate in vessel elements of the xylem.
Figure 16. Salivary sheath terminated at the edge of a vessel element without penetration.
Figure 17. Vessel element with the wall ruptured by sharpshooter stylets.
Figure 18. Vessel element plugged with salivary sheath material.

GLASSY-WINGED SHARPSHOOTER IMPACT ON ORANGE YIELD, FRUIT SIZE, AND QUALITY

Project Leader:
Raymond L. Hix
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:
Mary Lu Arpaia
Department of Botany & Plant Science
University of California /KAC
Riverside, CA 92521

Carol Lovatt,
Department of Botany & Plant Science
University of California
Riverside, CA 92521

Beth Grafton-Cardwell
Department of Entomology
University of California/KAC
Riverside, CA 92521

Phil Phillips
UC Cooperative Extension, Ventura County
Ventura, CA 93003

Reporting Period: The results reported here are from work conducted from July 2002 to October 2003.

INTRODUCTION

It is currently unknown what impact the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, has on fruit yield, size and quality as well as tree vigor. The goals of this project are to determine the usefulness of management of GWSS to prevent yield loss, fruit size reduction, and degraded fruit quality. This information is paramount before we can even begin to incorporate these into conventional IPM programs. First we have to know what impact GWSS has on citrus, and second we need to know how to use the currently available materials against the GWSS in IPM programs to prevent potential losses without disrupting citrus IPM programs. Prior to this study, efforts to manage GWSS in citrus were primarily to suppress populations to limit the spread of *Xylella fastidiosa* in areawide management programs.

OBJECTIVES

This research was initiated to:

1. Address the impact of GWSS on fruit yield, and distribution of fruit size when GWSS are controlled compared to untreated blocks of Valencia oranges, 'Washington' navel oranges, and grapefruit.
2. Evaluate the effects of high GWSS populations have on fruit quality (sugar/acid ratios, peel thickness, sugar/acid ratio, juice quality, peel texture and firmness, susceptibility to post-harvest disorders) in Valencia and Navel oranges.
3. Evaluate the effects of large GWSS populations have on water stress, nutrient loss (Ca etc.) metabolite loss (amino acids, xylem translocated PGRs) due to xylem feeding and fruit drop and fruit quality, and fruit drop
4. Determine if Admire enhances fruit size, tree health and vigor in the absence of GWSS.

RESULTS AND CONCLUSIONS

Objective 1

The Navel orange experiment was initiated on 21 Aug 2001 for 'Washington' Navel oranges. A site was established in Mentone with a completely random design with 5 replications with high and low GWSS populations. Each population level has three rows of 43 trees (2 guard rows and 1 central harvest row). The low populations (as close to '0' as possible) were established by applying 32 oz of Admire 2F via drip irrigation 21 August 2001, 7 April 2002, and 6 May 2003. Insects were monitored weekly by trapping, and visually counting adults, nymphs & egg masses. Efforts to establish differential populations were successful. On 3 July 2003, visual searches revealed 139.6 adults/3 min search/tree (± 3.7 SEM) in the high population trees verses 3.0/3 min search/tree (± 0.5 SEM) in the low population trees (Figure 1). The adult peak for 2002 occurred on 25 June with 104.6 GWSS/3 min count (± 6.5 SEM). The high and low population trees had 2.7 (± 0.6 SEM) and 0.9 (± 0.2 SEM) egg masses/25 leaf turns respectively. One tree from a guard row was tented and fumigated for absolute counts on 27 August 2002. The absolute counts ranged from 1149-4999 GWSS/tree in the high population trees and 10-21GWSS/tree in the low population trees. The size distribution for the Valencia Experiment was statistically significant for the high population and low population trees. Significantly more oranges cartons of sizes 72, 88, 113, 138 were packed from the low GWSS population trees than the high population trees (Figure 2). When taking into account oranges rejected to the juice line, the overall yield by weight was also higher for the low population trees (Figure 3).

Objective 2

Navel oranges were harvested from 37 trees within the harvest rows 21 January 2003 and sent to the Blue Banner Packing House in Riverside for packout and evaluation. Two cartons from 2 sizes (88 and 113) and two grades (Choice and Export) from each replication (total of 96 cartons) were selected. Trans-Pacific shipment was simulated by storing the 96 cartons from at the packinghouse for 21 days at 37° F after which time the fruit was sent to Kearney Agricultural Center (KAC) for

storage at 68° F for 4 days followed by 55° F for 5 days. For post-harvest evaluation at harvest, initial measurements of general appearance, pitting, puff and crease, peel firmness, thickness, color, TA, TSS, and % juice were taken from a 20 fruit sub-sample. Fruit was evaluated for general appearance, rind pitting, and decay following simulated shipment.

The rind pitting is seemingly a postharvest disorder and is not caused by direct damage of the GWSS. Pitting was clearly a problem in the May 2003harvested Newhall Valencia's but there were no significant differences in the treated (i.e. low population trees) and the untreated (high population trees) ($F= 0.361$, $P=0.550$). The low population trees had 34.4% pitting (± 1.23 SEM) while the high trees had 36.5% pitting (± 1.2 SEM) following simulated trans-Pacific shipping as described above. Navel pitting on the Jan 2003 harvest following simulated trans-Pacific shipment was 3.9% (±0.3 SEM) for the untreated trees (high populations) and 4.1% (±0.5 SEM) on the treated (low or 0 population trees). Pre-shipment simulation pitting on the navels was 0.03% and 0.01% respectively. The preliminary information suggests a postharvest physiological problem that's not result of GWSS xylem feeding behavior. However, this xylem feeding behavior may be contributing to significant stress factors summarized below.

Objective 3

The results of year one provide significant evidence that (1) the peel nutrient status of navel and oranges is reduced in a manner related to GWSS population density and (2) peel metabolite concentrations indicative of stress also change in a manner related to GWSS population density.

The peel nutrient status of navel orange fruit collected from the high and low GWSS treatments at Mentone during a period of low population density in July 2002 were not significantly different. At this site the GWSS population density increased on approximately August 8 and remained high through the end of August-early September. Peels from navel oranges collected at the time of intensive GWSS feeding on shoots in the high GWSS treatment had significantly lower concentrations of the Ca, Mg and NO3 than peels of fruit collected from the low GWSS treatment. The results are consistent with reports in the literature that high concentrations of Ca and Mg are found in GWSS excretions. In addition, peel samples of fruit collected from trees with high populations of GWSS tended to have a 10% and 12% less Mn and Mo, respectively. However, these differences were not statistically significant. For fruit samples collected from the high GWSS treatment on 11 August 2002 just 3 days after the GWSS populations began to increase, the two contrasting population densities had no significant effect on peel arginine concentration. However, for navel orange fruit collected during the period of high GWSS populations (20 August 2002), peel arginine concentration was significantly greater for fruit from the high GWSS treatment than the low GWSS treatment. It is worth noting that arginine tends to accumulate in parallel with the build up of the GWSS population over time. Elevated arginine concentrations are indicative of biotic and abiotic stress conditions. The accumulation of arginine in navel orange peels in response to increasing GWSS population density also paralleled the increase in peel putrescine concentration. Arginine is the immediate precursor for the biosynthesis of putrescine, which is also known to accumulate under stress conditions. Depending on the regulation found in different plant species, concentrations of spermidine increase, decrease or remain the same during stress. Spermine, which is typically found in lower concentrations than putrescine and spermidine, is usually unaffected by stress. These results strongly suggest that high population of GWSS cause significant stress. Proline concentrations decrease under stress when carbohydrates become limiting. Whereas peel proline concentrations tended to be lower for fruit collected from the high GWSS treatment, there was no significant difference in peel proline concentration between the two GWSS population densities. For both GWSS populations, the proline concentration decreased with time.

Yield (kg/tree) for the individual navel orange trees sampled for fruit peel analyses was similar to the whole row harvest (37 trees/row) data obtained at Mentone. Trees in the high GWSS populations tended to have fewer commercially valuable large size fruit.

Objective 4: Woodlake Experiment – Valencia

One half of a Valencia orchard in Woodlake CA was treated 27 June 2002 and June 2003 with 32 oz of Admire per acre through the irrigation system. The treated and untreated areas were 5 rows wide by 79 trees long and replicated 3 times each. Twenty fruit of size 56 were randomly selected from each of the plots for analysis at the LREC fruit quality lab. Ten fruit were used to determine average length, width, and rind thickness. Ten additional fruit were processed whole through the Sunkist juicer to determine % juice, sugar/acid ratio, and percent soluble solids. These measurements were done on 27 May 2002 and on 14 May 2003 before the first and second treatments were applied.

There were no significant differences in any parameter before treatments were applied. In the post treatment samples conducted on 27 May 2002, approximately 1 year after treatment, very little difference in fruit quality, size, or quantity was found between treated and untreated areas. There was a significant difference only in the in sugar/acid ratio being slightly higher in the Admire treated areas.

LREC Experiment – Navels

The ground around 45 individual trees of block 23 at the Lindcove Field Station were treated by backpack sprayer with the equivalent of 32 oz of Admire per acre on 30 Apr 2002 and 20 Jun 2003. An equal number of trees were left untreated. Before treatment, the 90 trees were chosen based on similar yields (1 March 2002). After the first treatment (11 February 2003), fruit were harvested and evaluated for number, size and quality. There were no significant differences in any of the parameters tested for the treated and untreated trees.

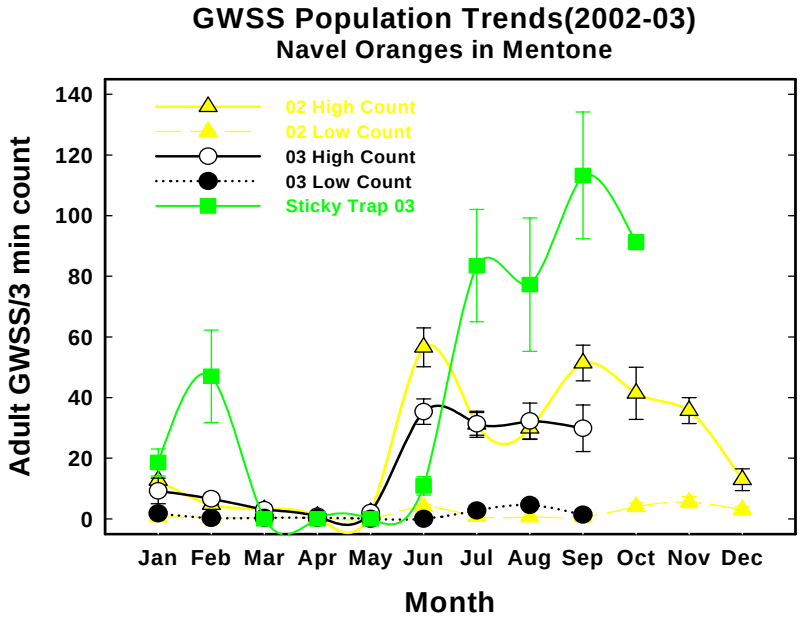

Figure 1. Means are based on nondestructive 3 min counts from 6 trees/replication/week. Each replication had 1 Seabright yellow panel trap. N = 5 ± SEM.

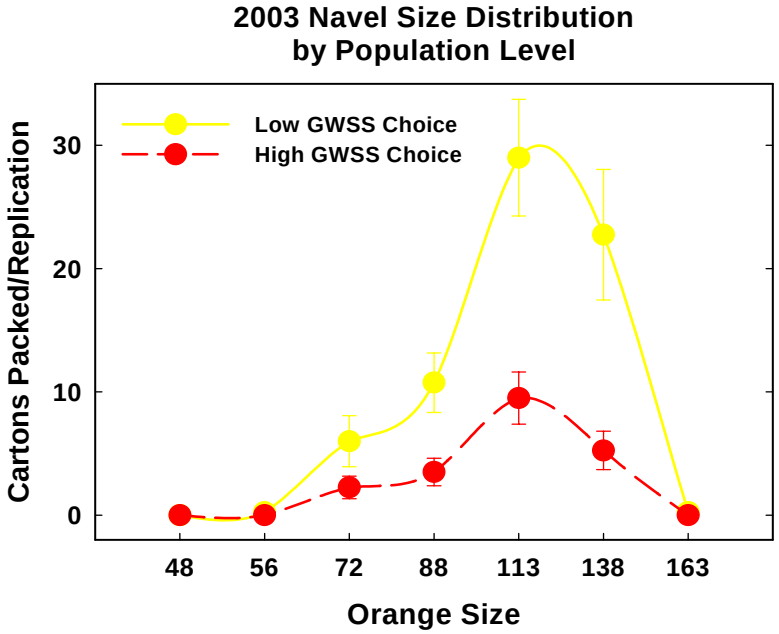

Figure 2. Mean numbers of export and choice cartons packed fresh between the high and low GWSS populations for the Jan 2003 Navels. Low population (virtually 0 GWSS) trees were treated on 7 April 2002. 5 reps (Each rep = 37 trees) ± SEM.

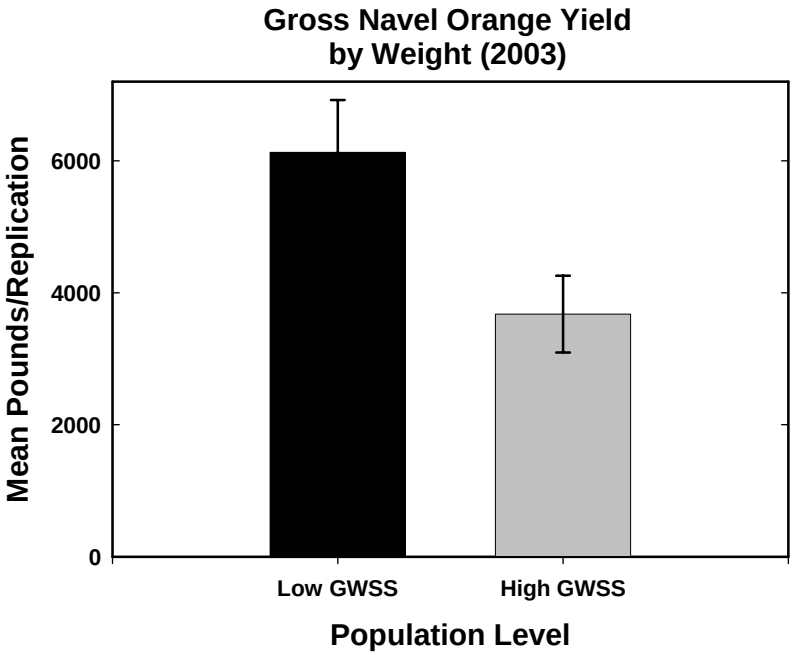

Figure 3. The low population trees produced more gross weight of navels than the high population trees. N = 5 ± SEM. 1 replication = 37 trees.

FUNDING AGENCIES
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COLD STORAGE OF PARASITIZED AND UNPARASITIZED EGGS OF THE GLASSY-WINGED SHARPSHOOTER, *HOMALODISCA COAGULATA*

Project Leader:
Roger A. Leopold
USDA-ARS
Biosciences Research Laboratory
Fargo, ND 58105

Cooperators:

Wenlong Chen
Department of Entomology
ND State University
Fargo, ND 58105

David J. Morgan
CDFA
Mt. Rubidoux Field Station
Riverside, CA 92521

George Yocum
USDA-ARS
Biosciences Research Laboratory
Fargo, ND 58105

Reporting Period: The results reported here are from work conducted from November 1, 2002 to October 1, 2003.

ABSTRACT

Excised leaves and cuttings of the *Euonymus japonica* plant were tested for cold tolerance over time at 2, 5, and 10° C in the dark for purposes of developing a protocol that would be favorable to cold storage of parasitized and unparasitized GWSS egg masses oviposited on the leaves of this plant. Cuttings generally survived longer than individual leaves at all temperatures tested, some as long as 165 days. The two lower temperatures were best for storage of the cuttings while 5°C was best for the individual leaves. Successful parasitism of GWSS eggs stored on euonymus cuttings for 20days at 13°C for both *Gonatocerus ashmeadi* and *G. triguttatus* was similar to control levels while parasitism steadily declined with longer storage periods. The threshold temperature for development of GWSS embryos appears to be approximately 12°C and, depending upon the age of the embryos when placed at 13°C, hatching can be as high as 36% after 40 days storage. We previously observed that *G. ashmeadi* within GWSS eggs stored for 7 days at 2°C were able to survive, however, our recent tests showed that neither *Gonatocerus* species were able to survive 20 days at this temperature.

INTRODUCTION

The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say), has become a serious economic problem in California as a vector of the xylem-inhabiting bacterium, *Xylella fastidosa* Wells (Sorensen and Gill 1996). Classical biological control typically relies on techniques of mass production of natural enemies to amass large quantities of individuals for eventual release and establishment of field populations (Miller 1995). Parasitoids in the families Mymaridae and Trichogramattidae are the most common natural enemies of GWSS in its native range (Turner and Pollard 1959) and in California (Phillips 1998). Three egg parasitoids, *Gonatocerus ashmeadi* Griault, *G. triguttatus* Girault, and *G. morrilli* Howard, are among the more important species being studied as agents for control of GWSS. Cold storage of insects during the rearing process of insects has proved to be a valuable aid to bio-control when implementing an IPM program. Low temperature storage of immature parasites within host eggs and storage of host eggs for later use have been extensively investigated (Leopold 1998). During the process of rearing and maintaining the GWSS and parasitoid colonies, it would be desirable to be able to store the GWSS egg masses and parasitized eggs for use at a later date.

In the absence of an effective artificial diet and ovipositional rearing system for *H. coagulata*, current rearing methods all require a constant supply of living plant materials. The GWSS females mostly deposit eggs into the lower epidermis of host plant leaves (Blua et al. 1999). Thus, the physiological status of these egg-bearing leaves has a direct impact on cold storage of GWSS eggs. Some host plants preferred for oviposition by the GWSS do not survive when placed under low temperature non-freezing conditions and subsequently, the developing insects also die (Leopold et al. 2002). While some of the intact host plant species survive cold storage relatively well, the space required for storing whole plants, each bearing a few egg masses, would be impractical when rearing large numbers of insects. Thus, many of the parameters that ensure vigor of the host plant leaves or cuttings during cold storage are yet to be defined.

Previous studies on short-term cold storage of GWSS eggs showed that the GWSS eggs could hatch in part or all after storage at 10º C for 1-6 days, but no eggs hatched after cold storage for more then 8 days at 10º C (Leopold et al. 2002). Another preliminary experiment conducted by Andress at al. (2000) indicated 67%, 16 % and 1% survived after 1, 2, 3, days respectively at 1ºC. Also, it has been suggested that GWSS eggs collected from different seasons may have different cold tolerance capabilities (Andress at al. 2000). However, data regarding seasonal changes and the effect of long-term cold storage on the development of the GWSS still remain unknown.

Although cold storage has been studied in many insects, there is still much to be learned about the effects of low temperatures on insect parasitoids (River et al. 2000). Two parasitoid wasps, *G. ashmeadi* and *G. triguttatus*, oviposit into GWSS eggs and their wasp progeny emerge within 15-20 days at 22º C. In the absence of practical techniques for propagation the wasps via artificial methods, the parasitoid wasps are mainly dependent on the provision of GWSS eggs collected in laboratory.

Apart from the internal factors controlling development of the parasitoids, other conditions including the developmental stage or age of the host, storage temperature, and humidity and lighting regimes may affect their development and/or survival. Preliminary experiments were conducted at 2 and 5ºC in fully dark environment in 2002 using a relatively low number of parasitized GWSS eggs (Leopold et al. 2002). This report presents current progress on investigations determining the cold tolerance of an ovipositional host plant and of parasitized and unparasitized eggs.

OBJECTIVES

1. Determine the effect of low temperatures on excised leaves and plant cuttings of host plants preferred by the GWSS for oviposition.
2. Determine the cold tolerance of GWSS eggs over time and parasitism by wasps on eggs that have experienced storage.
3. Determine effect of cold storage and age of GWSS egg on the emergence rate of egg parasitoids.

RESULTS AND CONCLUSIONS

Objective 1. Effect of low temperatures on excised leaves and cuttings of host plants

Excised leaves and cuttings of euonymus plants (*Euonymus* spp.*)* were examined for cold tolerance in constant temperature incubators set at 2, 5 and 10º C in the absence of light. Leaves were placed into slits cut into a water-soaked sponge, while all cuttings were placed vertically into plastic containers filled with water. About 50% of the excised leaves stored at 5º C survived for over 90 days, significantly longer than those stored at 2 or 10º C (Table 1). After 2 months, >42% of the leaves appeared chlorotic when stored at 2 ºC, 25% at 5º C and 75% at 10º C. Further, many excised leaves stored at 10º C died from the fungal or microbial infections. Euonymus cuttings generally lived longer than excised leaves. The LT_{50} for the cuttings ranged from 81 days stored at 10º C to nearly 100 days when stored at 5º C. Some even survived for more than 160 days. Cuttings stored at 10º C started to shed leaves after 7 weeks and were infected by bacteria or fungi after four weeks in storage. When stored at 13 and 14º C, the leaves of euonymus cuttings containing GWSS eggs were mostly covered by mildew after 30-35 days and were subsequently shed. Currently we are testing the effects of low level light administered to cold-stored cuttings and leaves using a short day photoperiod. We are also testing mildew retardants on cuttings placed in cold storage.

Table 1. Survival time of Euonymus leaves and cuttings exposed to low temperatures.

Temp.	Excised leaves				Cuttings			
	N	LT_{50} (days)*	Survival time (Mean ± SE)**	Range (days)	N	LT_{50} (days)*	Survival time (Mean ± SE)**	Range (days)
2 ºC	26	61.2	76.9 ± 3.0 a	64~112	20	84.5	96.3 ± 10.4 a	36~147
5 ºC	19	89.5	94.7 ± 6.1 b	64~134	15	99.1	104. 8 ± 9.9 a	40~165
10 ºC	16	60.0	74.4 ± 5.1 a	40~115	22	81.2	87.4 ± 4.4 b	48~110

* LT_{50} represented the storage time caused to kill 50% of the leaves or cuttings at a given temperature.

**Values followed by different letters in each column were significantly different ($P < 0.05$)

Objective 2. Cold tolerance of GWSS eggs over time and parasitism by wasps

To determine the effect of low temperature on the GWSS hatching and parasitism by the two parasitoids, GWSS egg masses deposited on euonymus cuttings were stored at 12, 13 or 14ºC for varying lengths of time at 1-2 days post oviposition. After being taken out of incubators, the egg masses were then exposed to either of the two wasps, *G. triguttatus* or *G. ashmeadi*, for 2 days at room temperature (22 ºC) and under a L 10: D 14 photoperiod. Before statistical analysis, all data were square-root transformed to correct non-normality because the number of eggs/mass size was not constant. Of the GWSS eggs stored at 13º C for 20, 30, 35 or 40 days, eggs stored for 20 days had approximately 85% successful parasitism (Successful parasitism is defined as the number of wasp offspring ×100 / the number of the GWSS eggs) (Figure 1). The parasitism of the eggs stored for 30 and 35 days was significantly lower than that of the control and those stored for 20 days. Only 23% GWSS eggs stored for 40 days were parasitized by *G. triguttatus*. The percentage parasitism was negatively correlated to the storage times of the GWSS eggs at 13º C (Figure 2). The wasp, *G. triguttatus*, successfully parasitized nearly 42% and 39% of GWSS eggs stored at 12 ºC for 30 and 40 days, respectively and only 2% for those eggs stored for 80 days (Figure 3). After storage at 14 ºC for 20 days, up to 89% GWSS eggs was parasitzed by *G. triguttatus*, significantly higher than those eggs for 40 days (29%) (Figure 4).

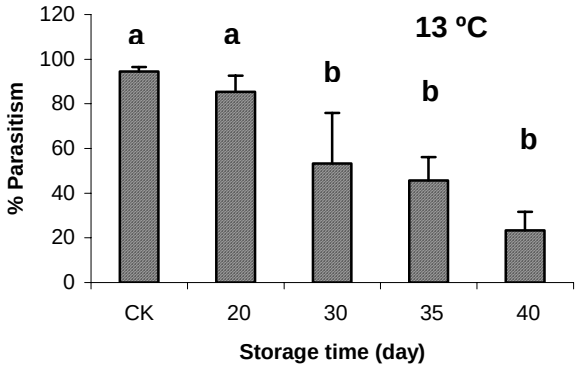

Figure 1. Parasitism of *G. triguttatus* on GWSS eggs submitted to different storage times at 13 ºC. Bars marked by different letters represent significant difference ($P < 0.05$)

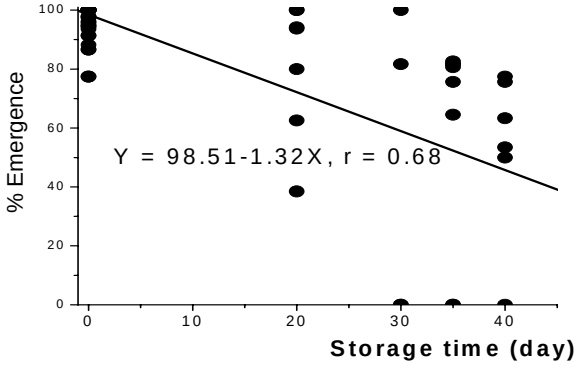

Figure 2. Relationship of the per cent emergence of *G. tiguttatus* to storage time of the GWSS eggs at 13ºC (F=61.1; df = 1, 70; $P < 0.001$)

Approximately 65% and 44% of the GWSS eggs stored at 14º C for 20 and 40 days, respectively, were parasitized by *G. ashmeadi* (Figure 5) as compared to 92% for the control. However, no wasp offspring emerged from those GWSS eggs stored at 14º C for 55 days. After storage for 30 days, the parasitism of the GWSS eggs at 12º C was nearly 49%, and 29% for 13 ºC (Figure 6).

During the process of stockpiling the GWSS eggs, we found that the eggs start to hatch in the incubator set at 14 ºC after 25 days. Within 30 days, approximately 65% eggs hatched (N= 169), and 84% (N = 74) after 40 days. Nearly 36% of the 5-8 day-old eggs when placed in storage (N = 128) hatch within 40 days at 13º C, but only 2% of the 1-3 day-old eggs hatch after storage for 50 days. Since GWSS hatching occurs while in storage at 13º C and the parasitism rate and/or wasp progeny emergence is low when GWSS eggs are held at 12º C, periods of 20 to 30 days may be reaching the limit for storage of viable unparasitized eggs because of certain factors that are not evident at this time.

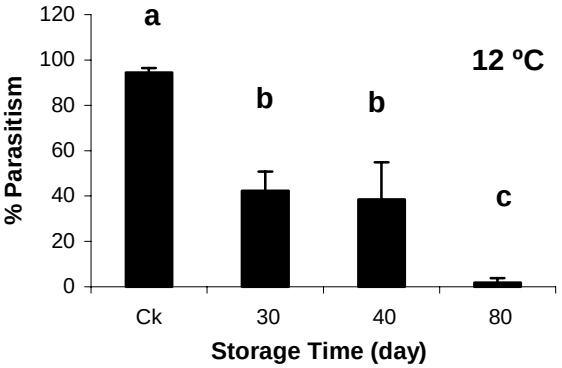

Figure 3. Parasitism by *G. triguttatus* on GWSS eggs submitted to different storage times at 12 ºC. Bars marked by different letters represent significant difference ($P < 0.05$)

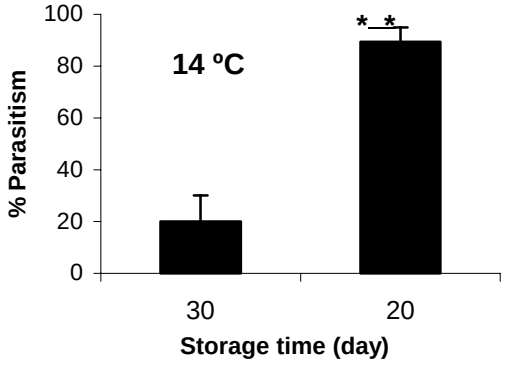

Figure 4. Parasitism by *G. triguttatus* on GWSS eggs submitted to different storage times at 14 ºC. Bar marked by an asterisk represents significant difference ($P < 0.05$)

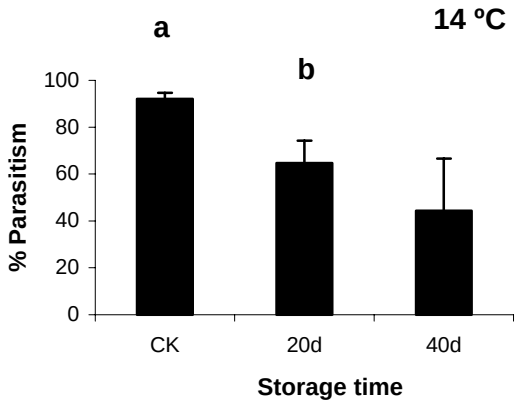

Figure 5. Parasitism by *G. ashmeadi* on GWSS eggs submitted to different storage times at 14 ºC. Bars marked by different letters represent significant difference ($P < 0.05$)

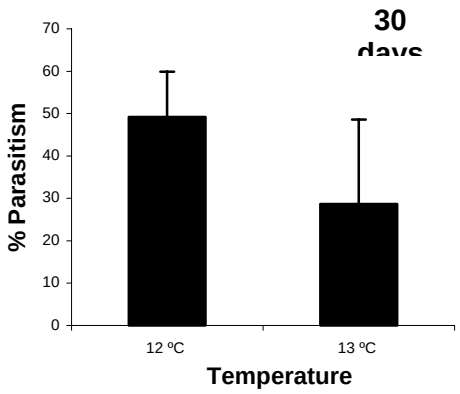

Figure 6. Parasitism by *G. ashmeadi* on GWSS eggs stored at 12 and 13 ºC for 30 days.

Objective 3. Cold tolerance of GWSS eggs over time and parasitism by wasps

Based on the results of short-term storage (< 6 days) at 2 ºC conducted last year, we attempted to lengthen storage time of parasitized eggs. After storage for 20 days (N = 63) and 30 days (N = 299) at 2º C, the eggs parasitized by *G. ashmeadi* did not emerge. Further, the eggs (N = 302) parasitized by *G. triguttatus* also did not emerge after storage for 30 days at 2º C. Additional studies will be focused on testing temperatures between 4 and 6º C for storage of the parasitized eggs.

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A PRELIMINARY STUDY ON PARASITISM OF *HOMALODISCA COAGULATA* EGG MASSES BY *GONATOCERUS ASHMEADI* AND *G. TRIGUTTATUS*

Project Leader:
Roger A. Leopold
USDA-ARS
Biosciences Research Laboratory
Fargo, ND 58105

Cooperators:

Wenlong Chen	David J. Morgan
Department of Entomology	CDFA
ND State University	Mt. Rubidoux Field Station
Fargo, ND 58105	Riverside, CA 92521

Reporting Period: The results reported here are from work conducted from April 1, 2002 to October 1, 2003.

INTRODUCTION

Parasitoid wasps in the families Mymaridae and Trichogramattidae are the most common natural enemies of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say), in its native range (Turner and Pollard 1959) and in California (Phillips 1998). Among its natural enemies, three egg parasitoids, *Gonatocerus ashmeadi, G. triguttatus,* and *G. morrilli* are important species being widely studied. The egg parasitoid, *G. ashmeadi*, is a mymarid wasp that accounts for 80-95% of the observed parasitism on the sharpshooter eggs in California (Phillips 2000). In Texas, GWSS eggs were highly parasitized by *G. triguttatus* throughout the year, but *G. ashmeadi* and *G. morrilli* were present in much lower numbers (Jones 2002). Classical biological control typically relies on propagation of natural enemies via mass-rearing to accumulate large quantities of beneficials for eventual release and establishment of field populations (Miller 1995). In the absence of practical techniques for propagation of the wasps via artificial methods, mass-rearing of these parasitoids mainly depends on a supply of host eggs also produced by a mass-rearing system.

When mass-rearing and releasing parasitoids, such as *G. triguttatus and G. ashmeadi*, it is very important to understand the fundamental biological characteristics of the insects including reproduction, development, and host-wasp interaction. An investigation by Jones (2002) showed that egg parasitism by *Gonatocerus* ssp. on GWSS eggs varied with respect to type of plant the sharpshooters used as their ovipositional host in the field. Furthermore, microscopic particles called brochosomes are placed by the female GWSS around its egg masses Hix (2001), Rakitov (2002). Jones (2002) observed that these brochosomes clearly slowed down the time that *G. triguttatus* spends in completing its oviposition on GWSS egg masses. Irvin and Hoddle (2001) investigated egg age preference of single *G. triguttatus and G. ashmeadi* females to GWSS eggs for a period of 2 hours. The interspecific competition for oviposition between these two wasps was also observed in laboratory by Hoddle and Irvin (2002). Other than these studies, little is known about the reproductive behavior of these species, the development of these two wasps within the GWSS eggs parasitized at different ages, and effects of host plants and brochosomes on wasp ovipositional behavior. Further investigation on the behavior of these two wasps will be valuable in maximizing their production for release as bio-control agents.

OBJECTIVES

1. Determine host-age preference of two parasitic wasps, *G. triguttatus* and *G. ashmeadi* for the GWSS eggs and to investigate developmental time of two wasps within host eggs of different ages.
2. Determine the effects of host plants and brochosomes on the oviposition behavior of the two wasps.
3. Determine daily and lifetime fecundity of *G. triguttatus* and *G. ashmeadi*.

RESULTS AND CONCLUSIONS

Objective 1. Host-age preference of two parasitic wasps

Multiple choice tests were employed to determine whether there is a host-age preference of *G. triguttatus* and *G. ashmeadi* when given an array of GWSS egg masses from 0 to 5 days of age. Euonymus cuttings bearing the GWSS eggs were collected daily and placed at room temperature (24 ºC) before the experiments began. The egg masses representing the different age groups were exposed to the caged (BioQuip-Bug Dorm) 2-5 day-old wasp colonies (230-250 individuals, sex ratio unknown) for 48 hours at 24 ºC. Our preliminary data on parasitism and wasp progeny emergence (Defined here as: the number of parasitoids emerged from the GWSS eggs × 100/ the number of the GWSS eggs) shows that the two wasp species had no host-age preference and that all ages of GWSS supported development of the wasp progeny (Table 1). More than 91% the eggs of all ages, except for the 3-day old (72%), were parasitized by *G. ashmeadi* and produced wasp progeny. For *G. triguttatus*, the wasps parasitized >93% of 2-and 3-day-old eggs, and approximately 68% and 55% of 4-and 5-day-old eggs, respectively. Although the successful parasitism of the older eggs was lower, the extensive variation within a limited number of egg masses showed no significant differences. The data from a separate choice test further showed that *G. triguttatus* and *G. ashmeadi* had no parasitism preference for the GWSS eggs of 6 or 7 days of age (Table 2). More than 98%

6-and 7-day-old eggs were parasitized by *G. triguttatus* and produced progeny. Further, *G. ashmeadi* parasitzed approximately 90% of 6-day-old and 72% of 7-day-old eggs. That the two wasps successfully parasitized even 6-and 7-day old GWSS eggs indicated the species had relatively wide range of host ages under laboratory conditions where the wasps out number the available egg masses. Interestingly, Table 3 shows that the developmental time of both species of wasps within the GWSS eggs was significantly influenced by the age of GWSS egg at the time it used for oviposition by the wasp female. Both wasp species took a significantly longer time to develop from egg to adult in young GWSS eggs (0 and 1day of age) than within the older ones (4 and 5 days of age). The results suggest that certain physiological and/or nutritional factors within the older GWSS embryos may facilitate wasp development.

Objective 2. Effects of host plants and brochosomes on the oviposition behavior of wasps

Although the white powdery-appearing brochosomes clearly slowed down the time of *G. triguttatus* to complete oviposition of GWSS egg masses (Jones 2002), our experiments found *G. triguttatus* didn't show a clear choice when the brochosomes were present or when they were physically removed from the GWSS eggs and then simultaneously exposed to ca. 230 wasps for 48 hours (T-test, $P = 0.772$) (Figure 1). Approximately 94% eggs without brochosomes and 90% of the brochosome-covered eggs were successfully parasitized. Like *G. triguttatus*, *G. ashmeadi* also had no ovipostional preference for non-brochosome- vs. brochosome-covered the GWSS egg masses within a colony of ca. 130 wasps ($P = 0.624$). Further, the ovipositional host plants of the GWSS also had no impact on parasitism by the two wasps on the GWSS eggs (Figure 2). There were no significant differences for preference in parasitism of eggs by *G. triguttatus* ($P = 0.713$) or by *G. ashmeadi* ($P = 0.598$) when they were offered the choice of egg masses on either euonymus or chrysanthemum leaves.

Objective 3. Daily and lifetime fecundity of G. triguttatus and G. ashmeadi

To determine the daily and lifetime fecundity of *G. triguttatus* females, we provided fresh GWSS egg masses on euonymus leaves for wasp parasitism every day. We found total life span of an ovipositing female was 25.0 ± 0.9 days (N=6), ranging from 22 to 27 days. It was observed that the female wasps essentially deposited eggs throughout their lifetimes (Figure 3). The number of eggs produced by a female during her lifetime was 99.3 ± 5.6 (N = 6) and ranged from 83 to 120. In the first and second day, the each species of females laid nearly 15 and 14 eggs, respectively. From the third day on, the number of eggs/day sharply plummeted, ranging from 2 to 6. The parasitism of the GWSS eggs within 3 days was higher than the other days (Fig. 4). Approximately 82% of the available GWSS eggs (n =110) were parasitized in the first day, 76% of eggs (n = 102) in the second day, and 47% of eggs (n = 66) in the third day. Parasitism during the rest of the days fluctuated between 1.4% and 29.4%.

Table 1. Parasitism by the two wasps, *G. ashmeadi* and *G. triguttatus*, on GWSS egg masses of different ages (Mean ± SE)

	G. ashmeadi				***G. triguttatus***			
Age (day)	No. of egg masses	No. of eggs	% Parasitism*	Range	No. of egg masses	No. of eggs	% Parasitism *	Range
0 d	8	77	92.5 ± 3.0	77.5-100	5	49	71.7 ± 19.2	0.0-100
1 d	9	107	90.8 ± 5.4	51.6-100	7	92	84.1 ± 9.6	40.0-100
2 d	8	88	98.1 ± 1.3	91.3-100	3	57	94.4 ± 3.7	87.5-100
3 d	9	95	71.5 ± 14.2	0.0-100	6	113	93.2 ± 5.4	66.7-100
4 d	3	42	96.1 ± 4.0	88.2-100	5	52	68.3 ± 19.4	0.0-100
5 d	3	24	93.2 ± 3.5	88.9-100	4	67	55.0 ± 17.8	23.8-91.3

*Means were analyzed using one-way analysis of variance (ANOVA). Data were square-root transformed before analysis. No difference was detected at the level of 0.05.

Table2. Parasitism by the two wasps, *G. ashmeadi* and *G. triguttatus*, on 6 day- and 7 day-old GWSS eggs (Mean ± SE)

	G. ashmeadi				***G. triguttatus***			
Age (day)	No. of egg masses	No. of eggs	% Parasitism *	Range	No. of egg masses	No. of eggs	% Parasitism *	Range
6d	5	67	90.4 ± 4.6	76.9-100	13	155	98.9 ± 7.7	90.9-100
7d	10	73	72.0 ±11.8	0.0-100	5	46	100 ± 0.0	-

*Means were analyzed using independent sample T test. Data were square-root transformed before analysis. No difference was detected at the significant level of 0.05.

Table 3. Developmental time of *G. ashmeadi* and *G. triguttatus* within GWSS eggs of different ages (Mean ± SE)

Age (day)	*G. triguttatus* n	*G. triguttatus* Developmental time (day)	*G. triguttatus* Range	*G. ashmeadi* n	*G. ashmeadi* Developmental time (day)	*G. ashmeadi* Range
0d	205	18.0 ± 0.1 a	16 - 22	138	17.9 ± 0.2 a	13 - 21
1 d	327	17.7 ± 0.1 a	15 - 22	121	17.5 ± 1.3 ab	16 - 22
2 d	127	16.7 ± 0.1 bc	15 - 21	206	17.8 ± 0.1 abc	16- 23
3 d	108	16.6 ± 0.1 bc	16 - 19	205	17.2 ± 0.1 bc	14 - 21
4 d	34	16.7 ± 0.2 b	16 - 20	40	17.1 ± 0.1 c	15 - 19
5 d	40	16.4 ± 0.1 c	16 - 20	19	16.3 ± 0.1 d	15 - 17
		F = 36.01			F = 6.69	
		df=5, 835			df =5, 723	
		P < 0.0001			P < 0.001	

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

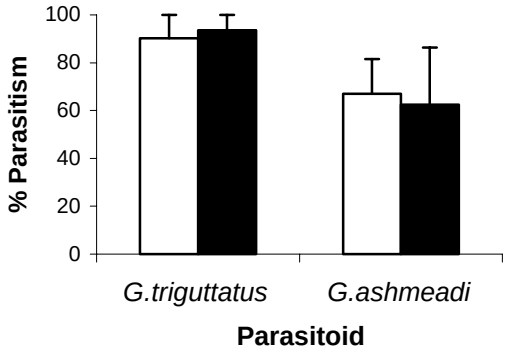

Figure 1. Parasitism of *G. triguttatus* and *G. ashmeadi* on GWSS eggs covered with brochosomes (blank) and no brochosomes (black).

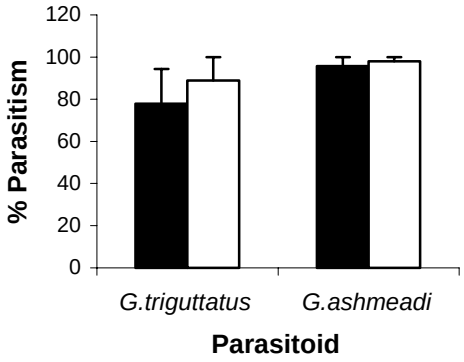

Figure 2. Parasitism of *G. triguttatus* and *G. ashmeadi* on GWSS eggs on euonymus (black) vs. chrysanthemum leaves (blank).

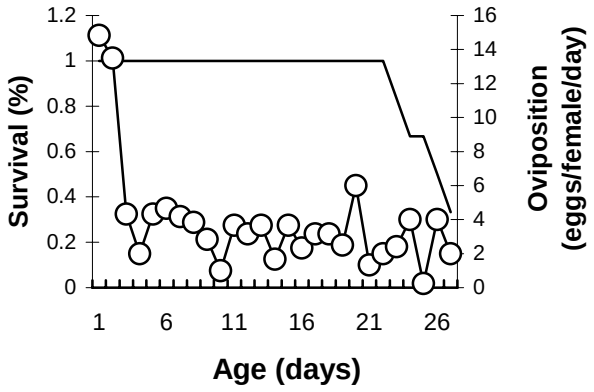

Figure 3. Oviposition (circles) and survivorship (solid line) of ***G. triguttatus*.**

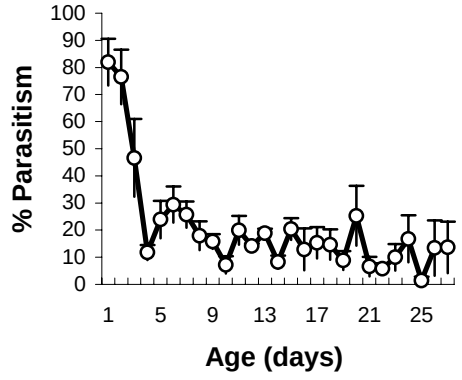

Figure 4. Successful parasitism by *G. triguttatus* females on the GWSS eggs over their lifetimes.

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FUNDING AGENCIES

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DEVELOPING A STABLE CLASSIFICATION OF THE GLASSY-WINGED SHARPSHOOTER GENUS *HOMALODISCA*

Project Leader:
Stuart H. McKamey
Systematic Entomology Laboratory
PSI, Agricultural Research Service
U. S. Department of Agriculture
c/o National Museum of Natural History
Smithsonian Institution
Washington, DC 20560-0168

Cooperators:
Roman Rakitov
Center for Biodiversity
Illinois Natural History Survey
Champaign, IL 61820

Andrew Hicks
University of Colorado Museum
Boulder, CO 80309-0218

Gustavo Moya Raygoza
Universidad de Guadalajara
Guadalajara, Mexico

Daniela Takiya
Center for Biodiversity
Illinois Natural History Survey
607 E. Peabody Dr.
Champaign, IL 61820

Carolina Godoy
Instituto Nacional de Biodiversidad
Santo Domingo de Heredia
Heredia, Costa Rica

Marco Gaiani
Facultad de Agrícola
Universidad Central de Venezuela
Maracay, Venezuela

Reporting Period: The results reported here are from work conducted from December 20, 2002 to October 15, 2003.

INTRODUCTION

The glassy-winged sharpshooter (GWSS), the leafhopper principally responsible for the spread of Pierce's disease on grape in California, is the species *Homalodisca coagulata*. This special capacity relates to the tissue upon which all sharpshooters (leafhopper subfamily Cicadellinae) feed: xylem, and the invasive status of the GWSS in California. It is noteworthy that of the 19 species in the genus, only one other species occurs in California and 18 species occur outside the USA (6 of these also occur in the USA). The genus is common in Mexico and also occurs southward through Central America, northern South America, and southeastern Brazil and Paraguay. That is, most species of *Homalodisca*, were they to reach California, have a destructive potential equal to the GWSS regarding the grape industry. The genus *Homalodisca* contains two other species that are already known to vector phytopathogens and it is practically expected that all species in the genus have the capacity to be, or become, serious vectors. Clearly, in a situation like this, we need to be clear about which species we are studying. The genus has never been revised.

Words are the tools of efficient communication and taxonomy is the vocabulary of species. By linking information to genus and species names, a classification of species becomes at once a very efficient system for storage and retrieval of information, and hence for meaningful communication, and a predictive tool, provided that classification is sound. Linking that information to species names that may be based on misidentifications, or belong to entirely different genera, will only add confusion to vector studies. In order to communicate effectively about the GWSS and its congeners, it is essential that everybody use the same names for the same species.

Access to all information on any group of organisms, including *Homalodisca*, is severely impeded by arbitrary generic limits, multiple names for some species and no name for others, or the absence of authoritative identification tools, or all three factors. The status of *Homalodisca* in this regard is below acceptable levels for a group of such economic importance.

OBJECTIVES

Broadly, the objective of the proposed research is to stabilize the classification of the genus *Homalodisca* so that all other information gathered (host plants, ecology, physiology, genomics, etc., which are all identified as priorities in the PD research program) can be linked to the correct names for meaningful communication. This will be accomplished through three major objectives:

1. Establish the limits of the genus *Homalodisca* though comparison to closely related genera, and the limits of all species in the genus, determine their valid names, and describe new species as necessary.
2. Characterization of brochosome structure and related behavior to allow identification of egg masses and females for most species.
3. Provide authoritative and electronically accessible identification aids and distribution data for all species, in addition to a hardcopy publication of the *Homalodisca* revision.

Also important for a revision is determining the relationship of *Homalodisca* to closely related genera. This is presently being addressed by Ph.D. student and proposal cooperator Daniela Takiya, with outside funding for four years and is consequently not a major objective of this project.

RESULTS AND CONCLUSIONS

Objective 1. About 1,000 specimens of *Homalodisca* have been examined in detail and locality data has been extracted and converted to decimal degree geographic coordinates for about 1,500 specimens. Morphological comparison among populations and species of *Homalodisca* has yielded a preliminary data matrix that will, when completed, be used to estimate relationships among species. At present, the species *H. insolita* appears to warrant its own genus, and the description of the type species of *Homalodisca* appears to refer to the genus *Propetes*, and not congeneric with other species currently in *Homalodisca*. As international rules of nomenclature require that a genus name must remain linked to its type species, there is a possibility that a new generic name may be needed to refer to the glassy-winged sharpshooter and its congeners. In June 2002, the first expedition of the project was conducted, in Costa Rica. It was an enormous success, yielding two species of *Homalodisca* with host and distribution data, oviposition behavior (recorded on film), and samples of egg masses for both.

This has enabled accurate illustration of color patterns (Figures 1, 2) which darken over time in preserved specimens, fresh material for molecular analyses of *Homalodisca*, and more progress in Objective 2.

Figure 1. *Homalodisca icthyocephala*, colors based on freshly captured specimen in Costa Rica.

Additionally, fresh specimens, hosts, oviposition behavior, egg samples, and two likely parasitoids were obtained for several close relatives of *Homalodisca*. These additional collections will facilitate development of a more predictive classification and improve quarantine efforts.

Figure 2. *Homalodisca insolita*, colors based on freshly captured specimen in Costa Rica.

Objective 2. In *Homalodisca* and a few related genera, females coat egg masses with brochosomes, minute, hydrophobic secretions of the Malphigian tubules that are only found in leafhoppers. These egg-brochosomes differ markedly from cuticular brochosomes and vary in structure among species. Postdoctoral fellow Roman Rakitov has now characterized the brochosomes and brochosome-related behavior of seven species of *Homalodisca* and several of its close relatives, particularly as a result of the team expedition to Costa Rica.

Objective 3. The identification aids and published revision of *Homalodisca* necessarily follow completion of Objectives 1 and 2. An on-line, image-driven key will be produced and placed on the USDA/ARS Systematic Entomology Laboratory server to maximize access and utility. A traditional key to species will accompany the hardcopy generic revision.

Delimitation of the genus and its relationship to other genera presently is being conducted by Ph.D. student Daniela Takiya. Because her analysis is based on molecules and morphology, obtaining fresh specimens of many sharpshooter genera is crucial for this facet of understanding *Homalodisca*. The fieldwork in Costa Rica has provided many specimens for her study, including fresh material of *Homalodisca* and several close relatives, even new species and one new genus in the process of being described.

FUNDING AGENCIES

Funding for this project was provided by the USDA Agricultural Research Service, Systematic Entomology Laboratory, and the University of California Pierce's Disease Grant Program.

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

Project Leaders:

Alexander Purcell
ESPM - Division of Insect Biology
201 Wellman Hall
University of California
Berkeley, CA 94720-3112

Steve Lindow
Plant and Molecular Biology
111 Koshland Hall
University of California
Berkeley, CA 94720-3102

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

ABSTRACT

This study will examine the effect of host plant tolerance on the epidemiology of Pierce's disease (PD), specifically on the ability of the insect vectors to acquire *Xylella fastidiosa* (*Xf)* from plants considered tolerant to PD. The examination of *Xf* population and distribution in tolerant and susceptible plants, and its relationship to xylem anatomy, symptom development, and bacterial acquisition by sharpshooters, may reveal what traits of a plant are important in the overall spread of PD. Since host plant resistance is an important component in the long-term goal of curing PD, it will be important to know how this resistance affects the spread of PD in areas permanently infested with sharpshooter vectors. This research will also assist with the short-term goal of controlling the spread of PD by enabling growers to evaluate grape cultivars on their ability to provide inoculum for vine-to-vine spread of Pierce's disease. We will examine host plant resistance and its effect on the ability of insects to be vectors of Pierce's disease with two tools, GFP-expressing *Xf* and insect transmission of the pathogen. Due to the short time available for experimentation, there are no substantive results available yet.

INTRODUCTION

The GFP-*Xf* retains typical virulence in grape, continually glows green when illuminated with blue light (Figure 1) and allows examination of plant tissues without the extensive fixation required with electron microscopy (8). Alternate hosts of *Xf* selected for their different patterns of *Xf* colonization following vector inoculation, lack of stem lignification, and absence of green autofluorescence under blue light (Figure 2) are annual morning glory (*Ipomoea purpurea* 'Grandpa Ott'), mugwort *(Artemisia douglasiana)*, sunflower (*Helianthus annuus)* and annual bur-sage (*Ambrosia acanthicarpa*) (6, 15). Grape cultivars with varying tolerance to PD selected for evaluation are tolerant 'Sylvaner', moderately susceptible 'Cabernet Sauvignon' and highly susceptible 'Chardonnay' cultivars of *Vitis vinifera* (11, 12). Both highly efficient blue green sharpshooters (BG) and glassy-winged sharpshooters (GW) will be used to infect plants and assess the efficiency of insect acquisition of *Xf* (1, 5, 10).

Anatomical comparisons between alternate hosts and grape cultivars will include measurements of vessel length and number, and vascular bundle number and distribution based on the techniques of Tyson *et al.* (14), utilizing confocal rather than electron microscopy, and Ewers and Fischer, modified to infuse the pigment via 100kPa pressure applied to the proximal end of the cutting (4, 9). We will evaluate 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 will inoculate a 3-cm stem section, and the plants will be 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, 6).

We will evaluate GFP-*Xf* acquisition by sharpshooters from the alternate hosts and grape cultivars after completion of anatomical characterization. Insects will be caged on GFP-*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 or 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 from remaining plant material of the same site, and symptom development and severity will be assessed. Data analysis will include comparisons between species for the number, length and distribution of xylem elements. Since acquisition efficiency has been related to bacterial populations (7), 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 in a confocal microscopy session 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. Based on an average of 20.3 bundles per stem (in annual bur-sage), we will be able to detect at least a 20% difference in *Xf* colonization with $\alpha = 0.05$ and $\beta = 0.10$ (13).

OBJECTIVES

1. 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.
2. 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.

RESULTS

We have just started work on the project, but have obtained the plants and equipment required for anatomical comparisons of the various grape cultivars and alternate hosts. Preliminary examinations of vascular tissue from mugwort and annual bur-sage revealed typical primary vascular bundle anatomy in with wide interfasicular regions (3). Initial observations showed annual bur-sage (average 20.3, n = 4, SE = 1.1) and mugwort (average 17.8, n = 4, SE = 1.5) to have the approximately the same number of vascular bundles, but different numbers of large elements within those bundles. Bur sage had an average of 21.4 vessels per bundle (n = 32, SE = 2.2) and mugwort had an average of 12.2 vessels per bundle (n = 20, SE = 1.2). 'Cabernet Sauvignon' grape and annual morning glory had continuous vascular cambium typical of dicotyledonous vines (3). Annual morning glory had large vessels distributed evenly along the cambium and grapes had rays dividing the cambium, an average of 48.4 rays per stem cross section (n = 4, SE = 1.2). There were an average 5.9 large vessels per ray (n = 29, SE = 0.6).

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. Using GFP-*Xf*, this research will systematically examine 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. Electron and confocal microscopy with *in situ* DNA hybridization has shown *Xf* densely packed in individual vessels, with adjacent vessels empty. Alternate hosts or tolerant grape cultivars with low overall populations may have just a few vessels completely colonized with bacteria, so acquisition would be highly variable and dependant upon sharpshooters encountering the few colonized vessels while feeding. Research by Hill and Purcell (7) indicated that *Xf* acquisition by sharpshooters increased along with bacterial populations in infected grapes. If the proportion if colonized vessels has a similar positive relationship to insect acquisition of *Xylella*, a relationship as in Figure 3 is expected.

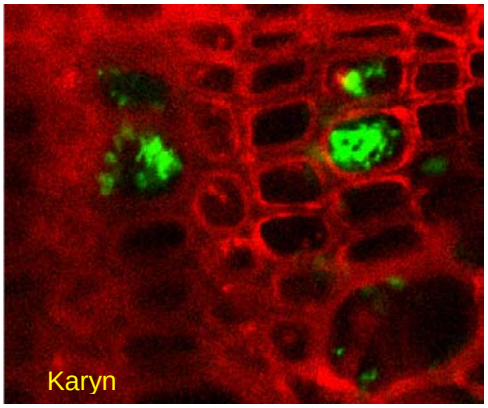

Figure 1. GFP *Xylella fastidiosa* (green) inside Inside vessels (red) of a grape petiole.

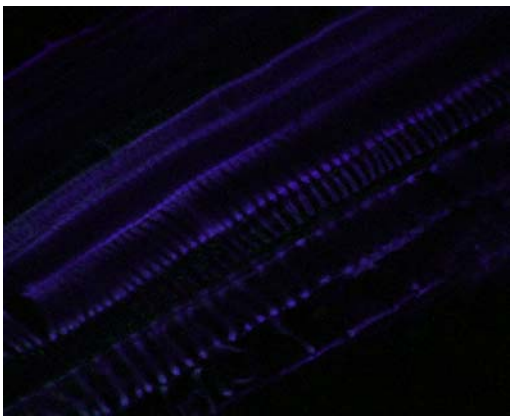

Figure 2. Longitudinal quinoa petiole section showing xylem structure with UV illumination.

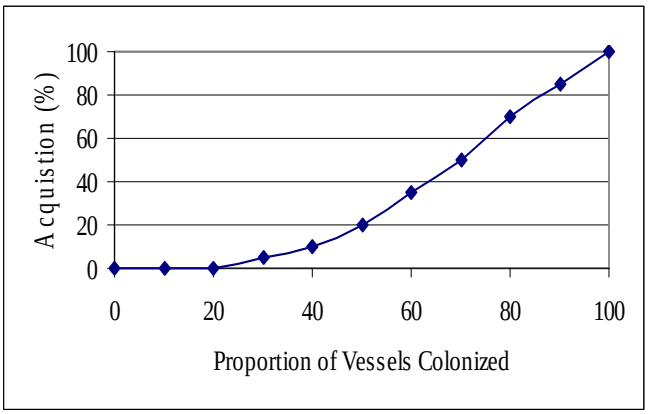

Figure 3: Proposed relationship between colonized vessels and *Xf* acquisition.

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FUNDING AGENCIES

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

PLANT-GLASSY-WINGED SHARPSHOOTER INTERACTIONS: BIOCHEMICAL MECHANISMS INVOLVED IN HOST PLANT SELECTION BETWEEN YOUNG AND OLD ORANGE TREES

Project Leaders:
Nick Toscano
Jian Bi
Frank Byrne
Department of Entomology
University of California
Riverside, CA 92521

Steven Castle
USDA-ARS
Western Cotton Research Lab.
Phoenix, AZ

Reporting Period: The results reported here are from work conducted from September 2002 to September 2003.

ABSTRACT
Glassy-winged sharpshooter (GWSS) population dynamics on young (5 years old) and old (20 years old) orange trees and related biochemical mechanisms were investigated in a field experiment. The numbers of GWSS were much higher on the young trees compared to those on the old trees. Levels of xylem asparagine, glutamine, threonine, valine, phenylalanine, tyrosine, isoleucine, leucine, lysine, methionine and histidine were also higher in the young trees in comparison with those in the old trees. These results suggest that particular amino acids play critical roles in GWSS host selection.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) *Homalodisca coagulata* is an exotic insect pest in California and is an important vector of *Xylella fastidiosa* that causes Pierce's disease (PD) in grapes. In California, citrus is an important host of GWSS and the proximity of citrus groves to vineyards influences the incidence and severity of PD in grapes. It is imperative that effective control strategies be implemented to curb the spread of the vector – vital to this would be to establish the host plant range of the GWSS and to determine the physiological and biochemical mechanisms for host selection. We previously reported GWSS host selection between lemons and oranges and related plant xylem chemistry. Here we report GWSS host selection between young and old orange trees and the potential role of amino acids in the host xylem fluid in host selection.

OBJECTIVES
1. Investigate the seasonal population dynamics of GWSS on young and old orange trees.
2. Determine amino acid levels of the xylem fluid upon which the insects feed.

RESULTS
An orange grove at Newhall Ranch, Piru, Ventura county was used for the experiment. Young and old orange trees (cv. Valencia grafted on Carrizo) in neighboring blocks were used. The young trees were planted in 1997 while the old trees were planted in 1973. Twenty trees were randomly selected from each block to monitor GWSS population dynamics and fifteen of them were used to extract xylem fluid. A bucket-sampling device was used to sample both immature and adult GWSS. Population dynamics of both adult and immature GWSS was monitored on a bi-weekly basis throughout the season. Xylem fluid in one-year old stems from each of the trees was collected bi-weekly to determine levels of free amino acids.

Adult GWSS numbers on young oranges were highest during October 2002 and a smaller peak was observed from mid-November 2002 to mid-February 2003 (Figure 1). The numbers were near zero after March 2003. The adult numbers on old oranges peaked from early-September to late October 2002. The adult numbers were up to 6.53-fold higher during peak population period on young trees in comparison with those on old trees. Peak immature GWSS counts on young trees occurred in early September 2002 and dropped to zero after late-October 2002, whereas the immature numbers on old trees were very low during this same interval (Figure 1). Throughout the experimental season, levels of xylem asparagine, glutamine, threonine, valine, phenylalanine, tyrosine, isoleucine, leucine, lysine, methionine and histidine were consistently higher in the young trees. In contrast, the levels of other protein amino acids such as proline, glycine, serine, arginine, alanine, cysteine, glutamate and aspartate did not show a similar consistent difference in the young and old trees (Figures 2a and 2b). These results are consistent with our previous findings of GWSS host selection between lemons and oranges.

CONCLUSIONS
In an orange grove in Ventura county with both young and old trees growing together in adjacent blocks, more GWSS adults and immatures occurred on young trees than on old trees. Levels of the amino acid asparagine, glutamine, threonine, valine, phenylalanine, tyrosine, isoleucine, leucine, lysine, methionine and histidine were consistently higher in the xylem fluid of young trees and possibly play important roles in GWSS host selection.

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

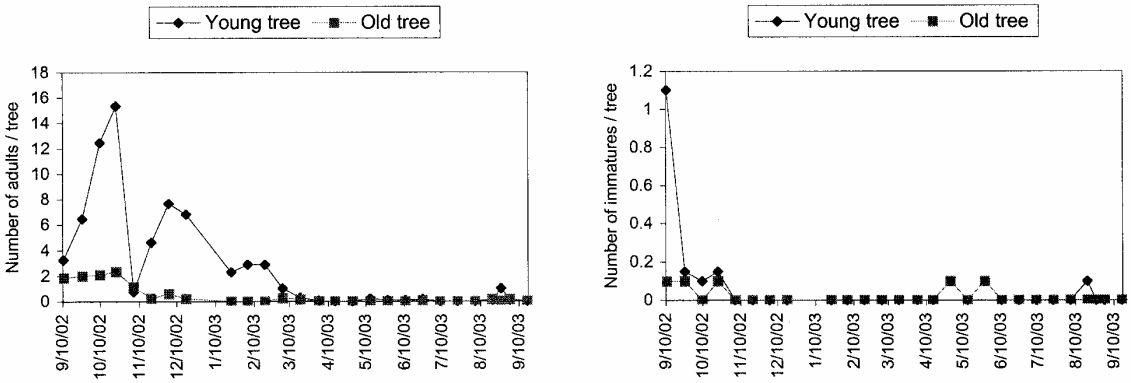

Fig.1 Seasonal population dynamics of GWSS on young and old orange trees

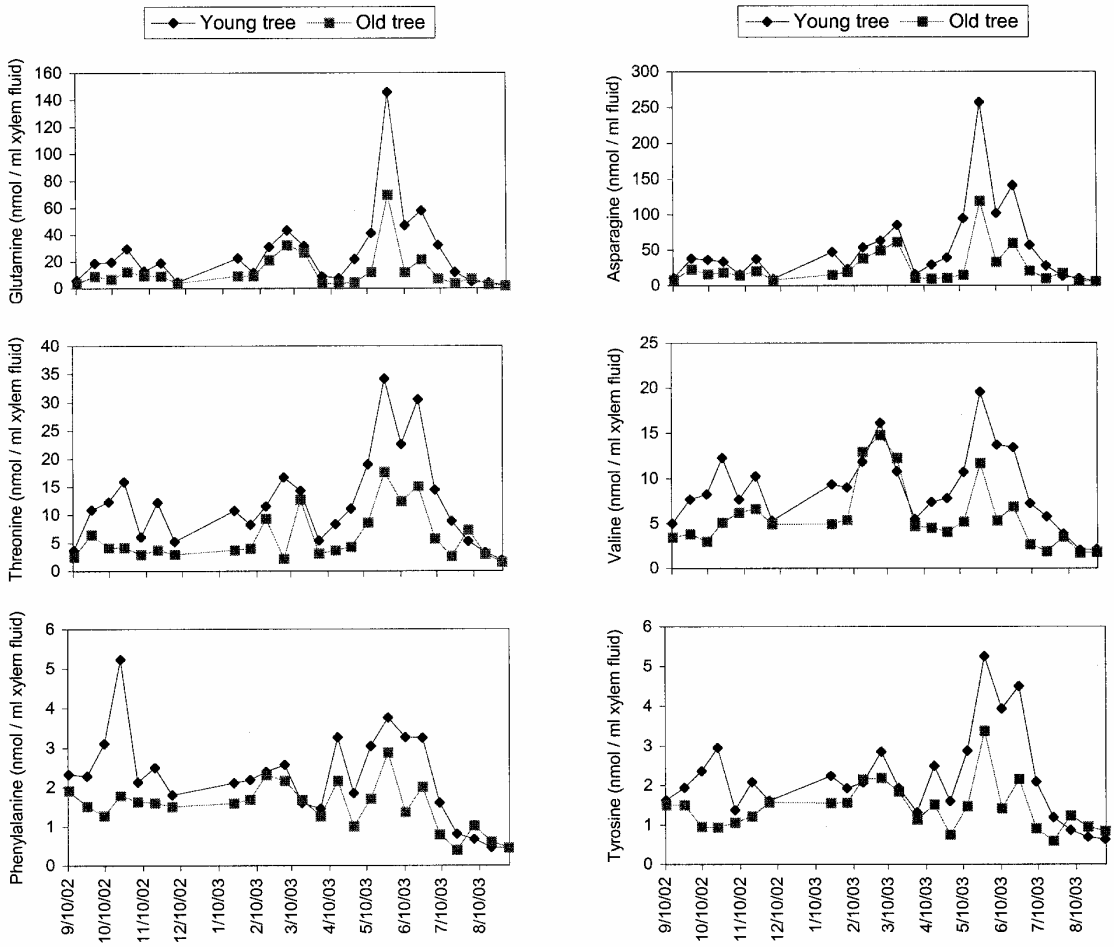

Fig. 2a Seasonal variation of some amino acid levels in xylem fluid of young and old orange trees

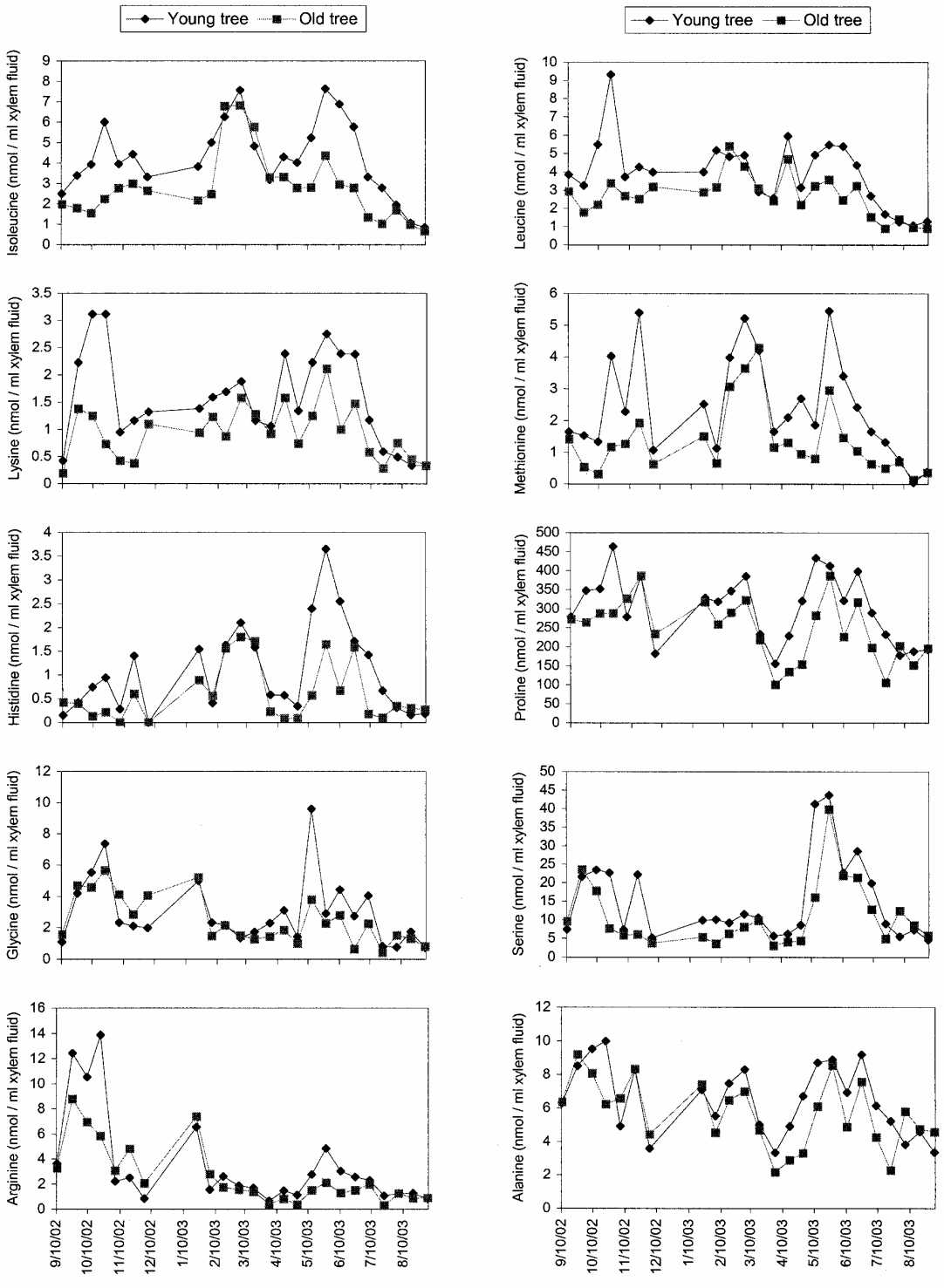

Fig. 2b Seasonal variation of some amino acid levels in xylem fluid of young and old orange trees

REPRODUCTIVE BIOLOGY AND PHYSIOLOGY OF FEMALE *HOMALODISCA COAGULATA*

Project Leaders:
Frank G. Zalom
Department of Entomology
University of California
Davis, CA 95616

Christine Y.S. Peng
Department of Entomology
University of California
Davis, CA 95616

Cooperators:
Nick C. Toscano
Department of Entomology
University of California
Riverside, CA 92521

Natalie A. Hummel
Department of Entomology
University of California
Davis, CA 95616

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

ABSTRACT
Female and male GWSS have been collected from July 2001 to October 2003, at monthly or bimonthly intervals, from citrus hosts at UC Riverside Ag Operations. A sub-sample of 10 females per month was dissected to determine ovary rank of the specimens collected. Dissections of these female specimens revealed repeated patterns related to the proportion of previtellogenic females in the field. These patterns indicated 2 distinct generations each year with a possible third generation late in the season. The results are currently being compared to patterns observed in samples from Tulare County, California. A host plant study, completed in the summer 2002 in which adult male and female *Homalodisca coagulata* were caged on grape, citrus, and oleander, has suggested differences in female fecundity and offspring survival. SEM and TEM studies are in progress in order to describe both sensory structures on the ovipositor and the process of vitellogenesis.

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

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

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

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

RESULTS AND CONCLUSIONS
Oögenesis Study
Female and male GWSS have been collected from July 2001 to October 2003. Samples were taken on monthly or bimonthly intervals. Dissections of female specimens collected from citrus hosts at UCR Agricultural Operations have revealed repeated patterns related to the proportion of previtellogenic females in the field (Figure 1). Dissected specimens were ranked according to ovary development state (stages 0-8) and then these categories were grouped to determine the proportion of previtellogenic, vitellogenic, and postvitellogenic females in the field. Patterns in the proportion of previtellogenic females indicate two distinct generations each year with a possible third generation late in the season. In 2001, an upward trend in the proportion of previtellogenic females was observed to begin in September with a peak in the proportion of

previtellogenic females occurring in October. In 2002, peaks in the number of previtellogenic females were observed in June and October with a slight peak appearing in December. In 2003, we have observed one distinct peak in the proportion of previtellogenic adults in June and are currently observing an increase in the proportion of previtellogenic adults in our September 2003 samples. Currently, 10 specimens have been dissected for each sampling date, and we are dissecting 10 additional specimens from each sampling date to expand our sample size to a total of 20 specimens per sampling date (Figure 2). Histological studies of the specimens from the female oögenesis study are being investigated to verify the data collected from the dissections.

Effect of Location on Number of Generations Per Year

Specimens were collected from October 2002 to May 2003 in Piru (Ventura County), California. The insect population unexpectedly disappeared in June 2003, and has since failed to reappear in our weekly sampling. Samples are now being taken at an alternate field site in Porterville (Tulare County), California, with sampling beginning in October, 2003.

Host Plant Study

The preliminary data of our host plant study in summer, 2002 suggested a potential difference in female fecundity on the different plant species. For this study, adult female and male GWSS were caged on citrus, grape, or oleander, and allowed to mate and oviposit on the plants. We were successful in promoting oviposition and in rearing GWSS from egg to adult stage on all three host plant types. A preliminary analysis of these data indicated a significant difference in the number of egg masses produced on each of these host plants, with citrus hosting the most oviposition, followed by grapes and then oleanders (Figure 3). The total number of offspring successfully maturing to the adult stage was greatest on citrus and lowest on grape (Figure 4). The sex ratio of the offspring that survived to become adults (Figure 4) differed by plant type. The proportion of male and female offspring was significantly different on oleander, while remaining nearly 50:50 on citrus and grapes. This experiment is currently being repeated on the late summer, overwintering generation of GWSS in citrus. This experiment will also be repeated in the spring using first generation adults. We hope to determine if oviposition preferences change, and if production of male versus female offspring is variable in the different generations.

Electron Microscopy Studies

A TEM study will be performed using insects reared at a site to be determined. This study will be initiated in February of 2004. Specimens will be reared on two different host plants (citrus and basil), and subsamples will be taken daily for the purpose of TEM study. This will result in a detailed study of the vitellogenesis cycle of the female GWSS.

SEM study of the ovipositor has been carried out since September 2003. The initial SEM sessions have revealed sensory structures on the first, second, and third valvulae of the ovipositor. A number of sensory hairs are also located on the pygofer of the female. TEM is necessary to determine the exact type of sensilla present. The external morphology suggests that these structures include various types of mechanoreceptors and chemoreceptors.

In conclusion, our study of the oögenesis cycle will provide better definition of the timing and number of generations of *H. coagulata* in California. Together with our study on the effect of host plant on female fecundity and sex ratio of offspring, resulting data will contribute to the available knowledge base on environmental and host plant influences on female development and reproductive success. Ultimately, this will lead to a greater understanding of factors important to successful control of *H. coagulata* populations by chemical and biological means.

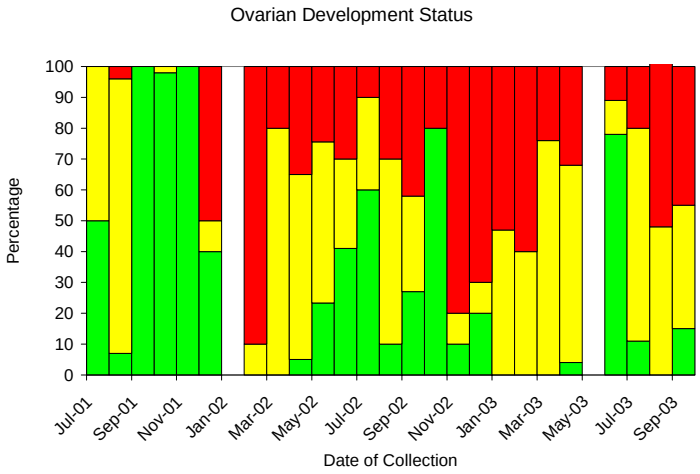

Figure 1. Proportion of previtellogenic (green), vitellogenic (yellow), and postvitellogenic (red) adult female *H. coagulata* per month, according to dissections (July 2001 to September 2003), collected from citrus plants located at the University of California, Riverside, Agricultural Operations.

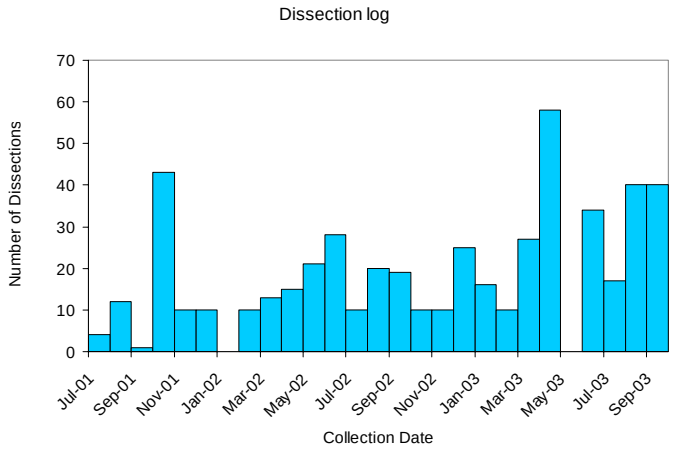

Figure 2. Number of dissections completed per month (July 2001 to September 2003) of female *H. coagulata* collected from citrus plants at the University of California, Riverside, Agricultural Operations.

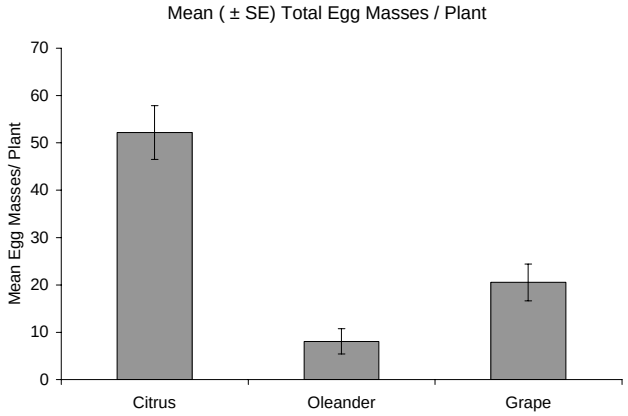

Figure 3. Mean number of egg masses per plant, obtained from female *H. coagulata* collected from citrus at UCR Agricultural Operations and reared in the greenhouse on grape, oleander, or citrus (3 July 2002 to 7 November 2002).

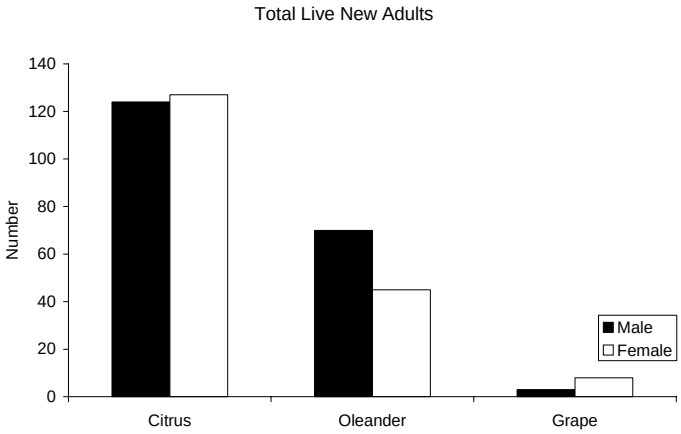

Figure 4. Total number of adults per each host plant type (totaled over all replicates), hatched from egg masses of female *H. coagulata* collected from citrus at UCR Agricultural Operations and reared in the greenhouse on grape, citrus, and oleander (3 July 2002 to 7 November 2002).

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FUNDING AGENCIES

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

IDENTIFYING GENETIC TARGETS FOR GLASSY-WINGED SHARPSHOOTER CONTROL: AVAILABLE GENETIC DATASETS FOR THE GLASSY-WINGED SHARPSHOOTER

Project Leaders:
W.B. Hunter and P.M. Dang
U.S. Horticultural Research Lab
Ft. Pierce, FL 34945

ABSTRACT
Genes identified from the glassy-winged sharpshooter, GWSS, (*Homalodisca coagulata*) are available on NCBI, ESTdb. Posted by Dr. Wayne Hunter at the U.S. Horticultural Research Laboratory, Ft. Pierce, FL. More genes are scheduled to be released by March 2004. Examples of the data construction, protocols, and BLAST results are presented. One set of genes identified was "Heat Shock Proteins". Eight unique genes for hsp20.8, hsp70, hsp90, hsp83, and others had a significant identity to heat shock proteins described from Drosophila, and other insects. The assembled sequences averaged just over 1,100 base pairs, with the longest being 1,361 bases. The identification of genes being expressed in adult GWSS and their putative functions in relation to stress, adverse environmental conditions, biology and development were categorized using the Gene Ontology classification resulting in genes for Cell communication, cell adhesion, cell signaling, cell death, cell differentiation, cell growth/maintenance. Developmental genes- Aging, cell differentiation, larval development, morphogenesis, Organs- eye, gut, heart, imaginal disc development, muscle, nerves, and tracheal system. Reproduction. Carbohydrate utilization and metabolism, transport, enzymatic pathways, cuticle biosynthesis and others. These cDNA libraries provide important information on thousands of potential genetic targets which may be used to develop methods aimed at reducing GWSS populations, and will increase our understanding of sharpshooter biology.

EXPANDING THE GENETIC TARGETS FOR SHARPSHOOTER CONTROL: GENETIC DATASETS FOR SHARPSHOOTERS THAT TRANSMIT PIERCE'S DISEASE

Project Leaders:
W.B. Hunter
U.S. Horticultural Research Lab
Ft. Pierce, FL 34945

P.M. Dang
U.S. Horticultural Research Lab
Ft. Pierce, FL 34945

C. Tipping
U.S. Horticultural Research Lab
Ft. Pierce, FL, 34945

R. Mizell
University of Florida
North Florida Research and Education Center
Quincy, FL 32351

ABSTRACT
Identification of genes and proteins isolated from the sharpshooter, *Oncometopia nigricans* are aiding our understanding of important genetic variations between leafhopper species which spread Pierce's disease. Presently 13,124 expressed sequences, culminating into the identification of ~4,649 unigenes for sharpshooters, *O. nigricans* and GWSS. These were represented in 1,229 assembled gene clusters, with 659 gene clusters being made up of sequences from both species leaving ~53% displaying specie uniqueness. Of the remaining 3,420 singlets. ~67% of the sequences belonged to the GWSS and ~33 % to *O. nigricans*. Gene frequency comparisons are now being completed and are being used to examine genetic similarities and differences between sharpshooters. Through gene frequency analysis it may be possible to determine important differences in the biology of sharpshooters to better understand their developmental biology, feeding and host plant utilization, as well as specific pathogen interactions. With this broad approach to sharpshooter management and understanding, new and more specific methods of control may be developed and implemented against these important economic insect pests to reduce the impact of Pierce's disease.

UNDERSTANDING GLASSY-WINGED SHARPSHOOTER FEEDING: GENE EXPRESSION IN GLASSY-WINGED SHARPSHOOTER SALIVARY GLANDS AND MIDGUTS

Project Leaders:

W.B. Hunter
U.S. Horticultural Research Lab
Ft. Pierce, FL 34945

P.M. Dang
U.S. Horticultural Research Lab
Ft. Pierce, FL 34945

E. Backus
USDA-ARS-PWA
SJV Agriculture Science Center
Parlier, CA 93648

J. Habibi
Department of Entomology
University of Missouri-Columbia
Columbia, MO 65211

D. Morgan
Calif. Dept. of Food and Agriculture
Riverside, CA 92501

ABSTRACT

To better understand the dynamics of sharpshooter feeding, a gene expression library was made from specific tissues isolated from adult glassy-winged sharpshooter, GWSS, (*Homalodisca coagulata*) salivary glands and midguts. The putative proteins identified from these tissues are listed in the following table:'GWSS Salivary Gland library.'

A list of some of the "Matches" Significant identity by BlastX search:

Carbonic Anhydrase	CDC-like Kinase	ADP/ATP translocase
Ferritin	ATPase	Apolipoprotein
Stretchin	Insecticyanin Form A, B	Aliphatic lipid binding protein
Gluthione transferase	Lysosomal thiol reductase	Laccase 1
Catalase	FingerProteins	Serine Protease
Biliverdin (Lipocalin)	Chitinase	
Protein Kinase	Initiation factor	Fibromodulan
Phosphopyruvate (enolase)	Histone	Defensin A, C
Aldose 1-epimerase	Proline oxidase (dehydrogenase)	Polyubiquitin Protein
Lysine-deficient Kinase	Cytochrome P450	Inositol polyphosphate phosphatase
Lipase	NEFA interacting Nuclear Protein	Zuotin related Factor 1
α-2 platein precursor	NADH-dehyerogenase	Troponin-C
Transposase	Glutaredoxin	Amino Acid Transporter
Proprotein Convertase	Juvenile hormone esterase	Splicing Factor, Sfrs7
Deoxyribonuclease	Synaptic Nuclear Envelope 1	Cytochrome oxidase

Some of the identified genes and their putative protein functions are discussed.

Section 3B: Glassy-winged Sharpshooter Population Ecology and Biological Control

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

Project Leaders:
Carlos E. Coviella
Robert F. Luck
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:

Mark Hoddle	Peter Andersen
Department of Entomology,	Southern Region Pest Management Center
University of California	University of Florida
Riverside, CA 92521	Quincy, FL 32351

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

ABSTRACT
Unlike other insect pests, the glassy-winged sharpshooter (GWSS) threat is associated with its ability to vector a variety of *Xylella fastidiosa* strains, one of which causes PD in grapes. That GWSS feeds on xylem fluids is probably the reason why adult GWSS are able to feed on a remarkable range of host plant species: they avoid secondary plant compounds, and develop dense populations, particularly on several Citrus species. The insect's high mobility contributes to the efficiency in vectoring *X. fastidiosa*. The insect is likely to establish generally in the State and transmit several forms of *X. fastidiosa* that affect other important perennial crops, e.g., almonds, olives, and stone fruits. These characteristics imply that to disrupt *X. fastidiosa* transmission and prevent *X. fastidiosa* epidemics, GWSS population densities need to be driven to be exceedingly scarce (Redak, 2001). Commonly accepted control measures, albeit useful in slowing the spread of such epidemics, are unlikely to drive GWSS densities low enough to completely disrupt transmission. This study will provide the information to determine whether it is possible to devise a control tactic and strategy along with its temporal and spatial scale that, when applied at the scarcest density of GWSS population i.e., its weakest point in the annual cycle, has the potential to eradicate local GWSS populations.

OBJECTIVES
This project seeks to provide information about GWSS' spatial and temporal dynamics involving its annual population cycle on its dominant host species. We seek to identify periods in this cycle during which selective control measures appropriately applied might drive the GWSS population below its critical density, thus leading to its local extinction. To fulfill this goal, we propose the following objectives:

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

RESULTS AND CONCLUSIONS
Our current work suggests that a period exists during GWSS' annual cycle where its populations are likely to be close to their minimum viable population size (MVP). In particular, on citrus and in California, GWSS appears to have a single (effective) generation per year. A second generation, albeit present at high densities during the egg stage (Figure 1), contributes very few nymphs Figure 2) and adults to the population. Approximately 1.3% of the total annual adult production arises from these eggs (Figure 3). Although the source of this egg and nymphal loss still needs to be explored, we have measured egg parasitism ranging from 78% to 92% of the eggs during the second half of the year. It is at this point that the population's annual cycle is vulnerable to a selective control measure tailored to drive the GWSS population below its MVP, and possibly leading to its' local eradication. We are in the first stages of field data collection and results are not available to be presented at the time of this report.

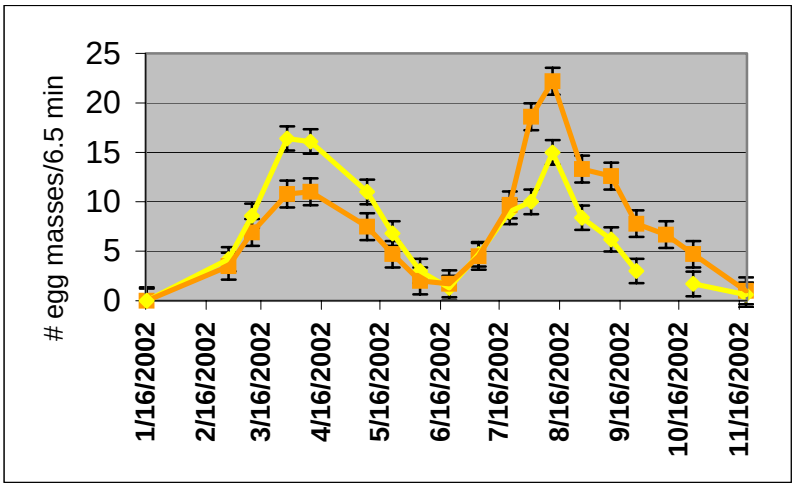

Figure 1: Egg densities per tree on orange trees (orange) and lemon trees (yellow). Data from Ali Al-Wahaibi and Joseph Morse.

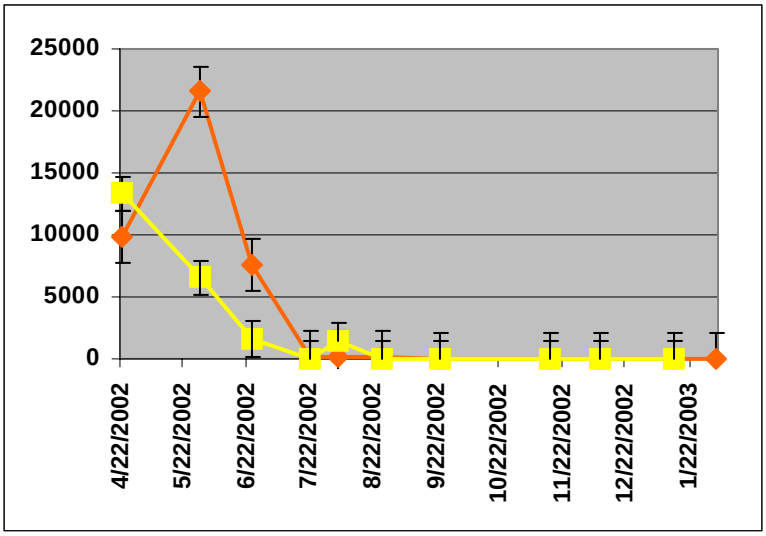

Figure 2: Nymphal production per tree (all stages) on orange trees (orange) and lemon trees (yellow).

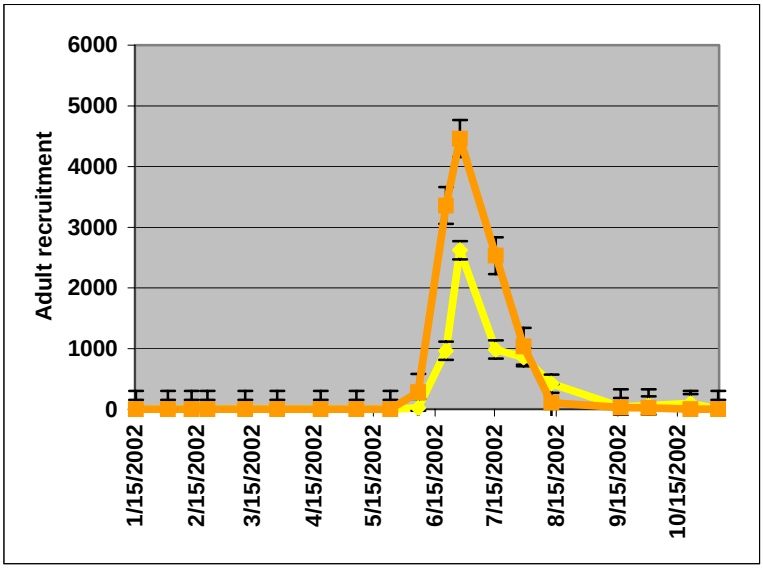

Figure 3: Adult production per tree on orange trees (orange) and lemon trees (yellow).

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FUNDING AGENCIES

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

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

Project Leaders:

Kent M. Daane
Division of Insect Biology,
Department of Environmental
Science, Policy and Management
University of California
Berkeley, CA 94720

Marshall W. Johnson
Department of Entomology
University of California, Riverside
Kearney Agricultural Center
Parlier, CA 936348

Cooperators:

Tarcisio Ruiz
Division of Insect Biology
University of California
Berkeley, CA

Jennifer Hashim
UC Cooperative Extension
Kern County
Bakersfield, CA

Alexander Purcell
Division of Insect Biology
University of California
Berkeley, CA

Elaine Shapland
Division of Insect Biology
University of California
Berkeley, CA

James Hagler
USDA-ARS
Western Cotton Research Lab
Phoenix, AZ

Robert Luck
Department of Entomology
University of California
Riverside, CA

Russell Groves
USDA – ARS
San Joaquin Valley ASC
Parlier, CA

Carlos Coviello
Department of Entomology
University of California
Riverside, CA

Reporting Period*:* The results reported here are from work conducted from November 1, 2002 to November 1, 2003.

INTRODUCTION

The primary focus of this research is the description of glassy-winged sharpshooter (GWSS) preference, egg deposition, age structure, population dynamics and levels of natural regulation on different host plants in urban / agricultural interface in the SJV where untreated populations serve as an inoculum source for nearby vineyards and citrus. We will also test sampled GWSS, from selected host plants and ecosystems, for the presence of *Xylella fastidiosa*. This work will aid researchers currently mapping out PD and *X. fastidiosa* sources in the San Joaquin Valley.

A description of GWSS biology and ecology on host plants in urban areas of the SJV will help understand GWSS seasonal movement and infestation foci. For example, information on the abundance, host plant use, and seasonal dispersal patterns of resident sharpshooters (e.g., blue-green sharpshooter) (Goodwin & Purcell 1992, Perring et al. 2001). The same critical information for GWSS is lacking for the SJV. This work will provide a needed baseline on resident natural enemies of GWSS in the SJV and their contribution to GWSS mortality. Information on GWSS movement and host plant succession in the SJV may also be useful for modification of surrounding vegetation or traps crops can potentially suppress GWSS movement into a vineyard.

OBJECTIVES

1. Determine glassy-winged sharpshooter (GWSS) biology and ecology throughout the season, particularly its age structure on and utilization of the different host plants that represent common breeding or dispersion refuges for GWSS in the San Joaquin Valley.
2. Determine the presence of *Xyella fastidiosa* in GWSS collected from different host plant species and in selected ecosystems in the San Joaquin Valley.
3. Begin to evaluate predator release as an additional suppression tactic.

RESULTS AND CONCLUSIONS

We categorized GWSS age structure, ecology, and resident natural enemies (particularly predators) on different host plants common in the SJV from spring through winter 2003. Our methodologies included a controlled experiment using potted host plants to test the preference of resident GWSS and natural enemy populations to different host species, and field surveys for GWSS of different host plants in untreated urban regions in Kern County. Potted (6.6 L) plants were used to provide a replicated array of similarly-conditioned (e.g., age, size, irrigation) GWSS host species. These preference studies were

conducted in an unsprayed, GWSS infested citrus orchard, and two unsprayed residential areas in Bakersfield, California. Perennial species included ivy, photinia, citrus, gardenia, privet, euonymus, hibiscus, agapanthus (lily of the Nile), grapevine, crape myrtle, eucalyptus, and oleander. Annual (or weed) species included prickly lettuce, little mallow, annual sowthistle, coast fiddleneck, common groundsel, London rocket, fox tail brome, lambsquarters, blue grass, and shepperd purse. Both perennial and annual species were set in a randomized block design (3 blocks each in the citrus orchard and residential area), with data analysis separate within each category. GWSS eggs, nymphs and adults and GWSS predators and parasitoids were counted weekly from late-March through October 2003.

Results show GWSS seasonal-long densities were influenced by host plant species, with a significant difference (ANOVA, $P < 0.001$) among host plants, for both perennial and annual categories, in the numbers of GWSS adults, egg masses, nymphs and natural enemies. Results are provided for perennial host plants in the citrus orchard, which had the highest GWSS densities and provided the best data on host plant preference. Figure 1 shows a 20-fold difference in the number of GWSS on ivy, the least preferred host planted tested, and grape, the most preferred. The data show a similar pattern for GWSS adults (Figure 2) and nymphs (Figure 4). These data represent seasonal averages, and, therefore, the host plant densities are biased towards those host species that were preferred in June and July, when GWSS densities were the highest in this plot. Interestingly, GWSS egg mass density (Figure 3) was not related to adult or nymph density (P=0.25, r^2=0.03; P=0.35, r^2=0.01, respectively). We conclude that GWSS adults have oviposition preferences that may be different from the nymph feeding preference. For example, GWSS nymphs were significantly more common on oleander, which had few GWSS egg masses, than citrus, which had the most GWSS egg masses. We believe this difference is a result of both GWSS adults and nymphs switching among host plants for better food resources throughout the season (e.g., Luck et al. 2001), and to a disparate level of predator and parasitoid activity.

Within each array of potted host plant species, plants were close enough to each other that GWSS adults could fly and nymphs hop between adjacent host plants. Citrus was the more preferred overwintering host plant, however, during the summer months there was a clear preference for crape myrtle for egg deposition and feeding. The most obvious differences between egg deposition and nymph abundance were with oleander, which had few eggs masses (Figure 3) and many nymphs (Figure 4), and euonymus or Photinia, which had the opposite abundance pattern.

Another possibility is disparate egg and nymph mortality among treatments. Support is found in the significantly positive relationship between the number of spiders found, the most common predator group, and the number of GWSS egg masses ($P < 0.001$, r^2=0.28); in contrast, there was no relationship between the density of egg masses and either GWSS nymphs (P = 0.20, r^2=0.014) or adults (P = 0.78, r^2=0.001). These results suggest that natural enemies of eggs (Triapitsyn et al. 1998) and small nymphs significantly reduced the GWSS population between the egg to the nymphal stage. On the potted host plants, we found a season-long “egg mass” parasitism rate of 44%; when an egg mass was attacked most of the eggs were parasitized (there were an average 13.8 eggs per egg mass). A subsample of emerged parasitoids shows *Gonatocerus ashmeadi* and *G. triguttatus* present. Results also suggest that parasitoid and predator densities tract GWSS density. Abiotic and biotic mortality factors accounts for a reduction of ca. 3.9 eggs to 0.22 large GWSS nymphs per plant.

Similar data were collected for the described replicated blocks of perennial and annual host plants at three locations. These data have not yet been analyzed; however, GWSS densities at these sites were low and it is unlikely that clear separation of GWSS host plant preferences will be found.

Surveys of three urban areas in Bakersfield, CA were made to determine GWSS and natural enemy host plant relationships. Data are still being processed. Initial observations show GWSS host plant preference in urban settings followed a similar pattern as in our controlled experiment, with GWSS preference for carpe myrtle, euonymus, photinia, crab apple and Xylosma. GWSS nymphs were commonly found on all these species, as well as oleander. During the GWSS surveys, egg masses are collected to determine parasitoid species composition and activity. Similarly, predator species and density are recorded. During the urban survey, more than 800 predators were collected, identified to family or genus, and stored at -80ºC. These specimens are currently being processed by Dr. James Hagler with immunologically-based assays that employ pest-specific monoclonal antibodies (MAbs) that can be used in an ELISA to identify the key predators of GWSS (Hagler et al. 2001).

During the urban surveys, we collected plant material (e.g., potential vector host plants) and potential insect vectors to determine the incidence of *X. fastidiosa* in different geographic regions. This material is being stored at either -80ºC (plant material) or in acetone (insects). Before we begin to process this material, we have practiced extraction techniques on infected almond and grape plant material and sharpshooters that were exposed for 2-3 days to infected plant material. These “known” positives were analyzed for *X. fastidiosa* presence using immunocapture DNA extraction procedures, developed by the Purcell (UC Berkeley), Kirkpatrick (UC Davis), and Blua (UC Riverside) laboratories, and their help is gratefully acknowledged. Initial results show nearly 100% of positive plant material was found to be positive for *X. fastidiosa* DNA; >50% of the positive insect material was found to be positive for *X. fastidiosa* presence. Results suggest that our initial proposal of identifying levels of infected GWSS from different host plant species may require 1000s of insect samples. For

this reason, we will change methodologies in 2003 to determine first *X. fastidiosa* presence in resident host plants, and then cage and collect GWSS on these plants.

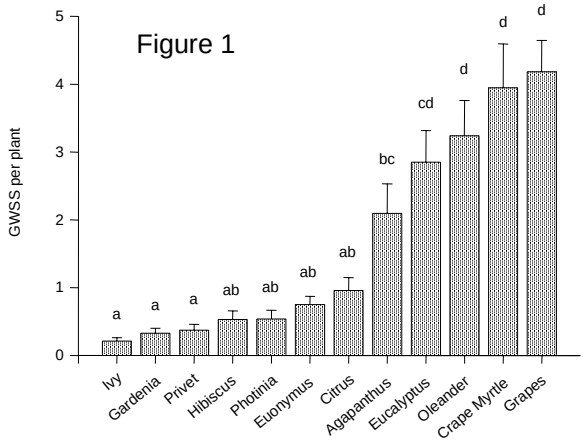

Figure 1. Average densities (± SEM) of GWSS (nymphs and adults) were significantly different among perennial host plants, Tukey's HSD at $P < 0.05$.

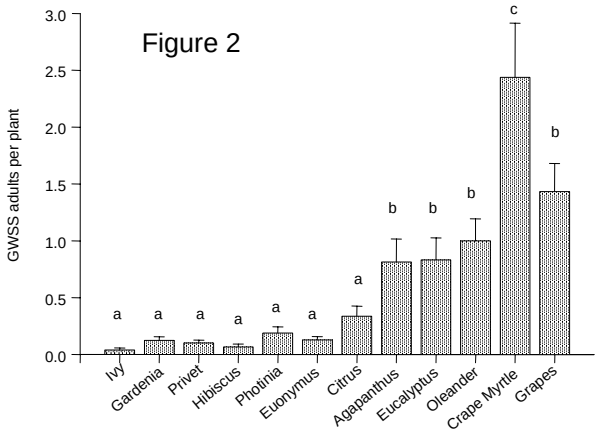

Figure 2. Average densities (± SEM) of GWSS adults were significantly different among perennial host plants, Tukey's HSD at $P < 0.05$.

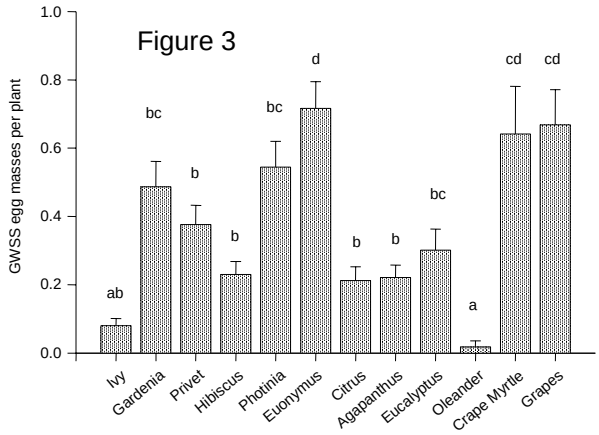

Figure 3. Average densities (± SEM) of GWSS egg masses were significantly different among perennial host plants, Tukey's HSD at $P < 0.05$.

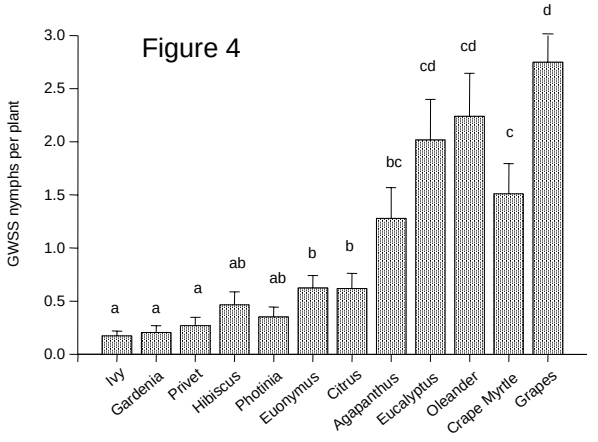

Figure 4. Average densities (± SEM) of GWSS nymphs were significantly different among perennial host plants, Tukey's HSD at $P < 0.05$.

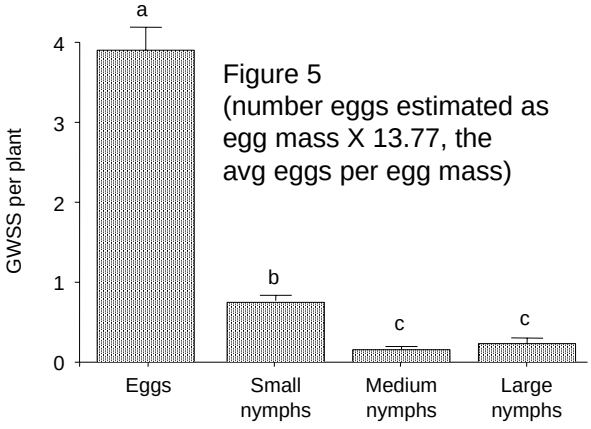

Figure 5. Average densities (± SEM) of GWSS separated by development stages, Tukey's HSD at $P < 0.05$.

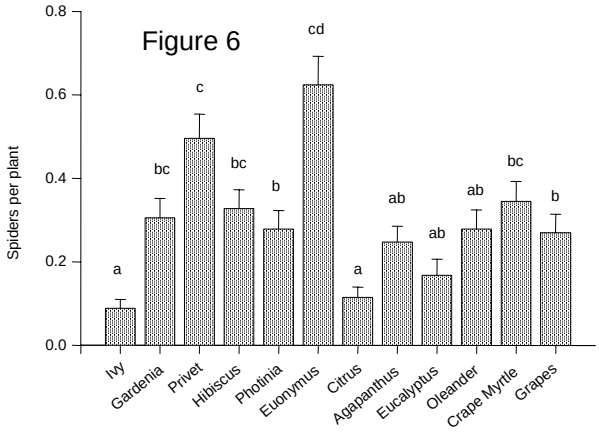

Figure 6. Average densities (± SEM) of spiders were significantly different among perennial host plants, Tukey's HSD at $P < 0.05$.

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INTERSPECIFIC COMPETITION BETWEEN *GONATOCERUS ASHMEADI, G. TRIGUTTATUS,* AND *G. FASCIATUS* FOR GLASSY-WINGED SHARPSHOOTER EGG MASSES

Project Leader:
Mark Hoddle
Department of Entomology
University of California
Riverside, CA 92521

Cooperator:
Nic Irvin
Department of Entomology
University of California
Riverside, CA 92521

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

ABSTRACT
Interspecific competition between *G. ashmeadi*, *G. triguttatus* and *G. fasciatus* for GWSS egg masses was investigated in the laboratory using three experimental designs. Overall parasitism by *G. ashmeadi* was consistently higher (up to 76.0%) compared with *G. triguttatus* and *G. fasciatus*, for all three experimental designs. Exposing females to individual egg ages (1, 3 or 5 days) showed that *G. ashmeadi* parasitized a significantly higher (48.1%) proportion of GWSS eggs compared with *G. triguttatus*, whereas when females were exposed to all egg ages simultaneously, parasitism by *G. ashmeadi* and *G. triguttatus* was equivalent. Results from visual observations showed that *G. triguttatus* allocated a significantly higher (up to 36.4% and 22.9%, respectively) proportion of time to resting/grooming, compared with *G. ashmeadi* and *G. fasciatus*. *Gonatocerus fasciatus* spent a greater (19.6% and 9.6%, respectively) proportion of time off leaves with GWSS egg masses compared with *G. ashmeadi* and *G. triguttatus*. *Gonatocerus triguttatus* parasitized a significantly higher proportion (25%) of GWSS eggs compared with *G. fasciatus*, whereas time allocated to oviposition by each species was equivalent. *Gonatocerus ashmeadi* and *G. triguttatus* demonstrated aggressive behavior towards congeneric competitors for GWSS egg masses, whereas no aggressive behaviors were observed for *G. fasciatus*. The implications of these results for interspecific competition and biological control of GWSS by these three mymarid parasitoids are discussed.

INTRODUCTION
Gonatocerus ashmeadi has been resident in California since 1978 and genetic studies indicate it is native to the southeast U.S.A. and probably accompanied *H. coagulata* from its home range in the southeast U.S.A. *Gonatocerus triguttatus* has been imported from eastern Texas and released in California since 2001, while *G. fasciatus* was liberated in 2002 (CDFA, 2003). All three *Gonatocerus* species are mymarid parasitoids that attack GWSS eggs. The introduction of more than a single natural enemy to control a target pest as part of a classical biological control program may induce interspecific competition and result in either competitive exclusion or coexistence (Zwolfer, 1971; Myers et al., 1989). Coexistence of natural enemies can potentially lead to the disruption of biological control because interspecific competition can result in lower densities of the more efficient parasitoid which causes pest populations to be higher than what would occur with the more efficient species acting alone on the target population (Briggs, 1993; Rosenheim et al, 1995). A better understanding of the competitive interactions between *G. ashmeadi*, *G. triguttatus* and *G. fasciatus* for GWSS egg masses may provide insight into predicting and interpreting field outcomes following the establishment and proliferation of *G. triguttatus* and *G. fasciatus* in California, as they compete with the already well established and widely distributed *G. ashmeadi*. Irvin and Hoddle (2002) demonstrated that competition outcomes from laboratory studies can be contradictory and results greatly varied depending on the experimental designs used and the questions being addressed. Therefore, the following investigations were conducted to investigate which *Gonatocerus* parasitoid species is most competitive using three different experimental designs that varied GWSS egg age and egg density available for attack.

OBJECTIVES
Investigate interspecific competition between *G. ashmeadi*, *G. triguttatus* and *G. fasciatus* for GWSS egg masses of varying age and density.

RESULTS
Three experimental designs were used: the first involved exposing approximately 30 GWSS eggs of known age to one mated female *G. ashmeadi*, *G. triguttatus* and *G. fasciatus* (~24 hrs of age) for 24 hours in a 3 inch ventilated vial cage at 25^{o}C. This was repeated for eggs one, three and five days of age. The second experiment involved the above procedure, but exposed approximately 15 GWSS eggs of each age category (total of ~ 45 eggs), simultaneously to one mated female of each species. The third experiment involved exposing one egg mass (3-6 eggs and 24-48 hours of age) simultaneously to one mated female of each species searching together for one hour in a single 2 inch Petri-dish lined with moist filter paper. Visual observations for aggressive behavior were made every 5 minutes during the third experiment and behaviors at the

observation point were recorded for each female. All experiments were replicated 20 times. The proportion of GWSS eggs parasitized by *G. ashmeadi*, *G. triguttatus* and *G. fasciatus* was calculated for each experiment and compared between species using Friedman's Chi-Square.

Overall parasitism by *G. ashmeadi* was consistently higher (up to 76.0%) compared with *G. triguttatus* and *G. fasciatus* for all three experimental designs (Figures 1-4). These results suggest that female *G. ashmeadi* may be more 'aggressive' than *G. triguttatus* and *G. fasciatus*, and therefore show greater potential as a biological control agent of GWSS. Results from laboratory studies presented here indicate that *G. ashmeadi* could out compete *G. triguttatus* and *G. fasciatus* in the field and hinder their successful establishment and impact in California. Previous studies have shown that *G. ashmeadi*, *G. triguttatus* and *G. fasciatus* demonstrate strong preferences for GWSS eggs aged two, three and four days, respectively (Irvin and Hoddle, in preparation), suggesting that field interspecific competition for egg masses may be less prevalent, compared with laboratory no choice situations when populations of GWSS of varying age are naturally co-occurring in field situations. Competitive advantages between parasitoid species are most likely to become important when host densities are low. It is possible that as GWSS biological control progresses in California, and GWSS populations diminish and egg masses become increasingly scarce, there may be a shift in parasitoid species dominance to the more competitively superior species at low egg mass densities.

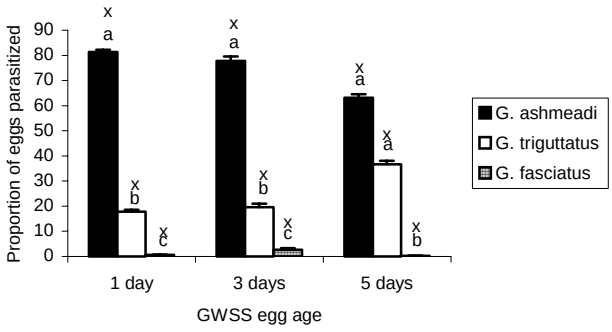

Figure 1. Proportion of GWSS eggs parasitized by *G. ashmeadi*, *G. triguttatus* and *G fasciatus* for eggs one, three and five days of age (different letters (x, y, z) indicate significant ($p < 0.05$) differences within species, different letters (a, b, c) indicate significant ($p < 0.05$) differences between species).

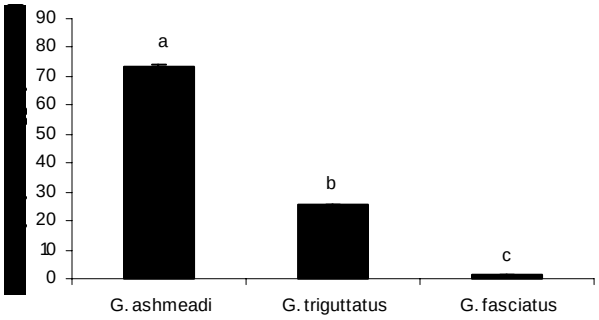

Figure 2. Over all proportion of GWSS eggs (combining one, three and five days of age) parasitized by *G. ashmeadi*, *G. triguttatus* and *G fasciatus* (different letters indicate significant ($p < 0.05$) differences between species).

When female *G. ashmeadi*, *G. triguttatus* and *G. fasciatus* were exposed to eggs one, three or five days of age concurrently in individual vials (experiment 1) the proportion of eggs parasitized by *G. triguttatus* increased from 17.8% for eggs one day of age, to 36.6% for eggs five days of age (Figure 1). This result was significant ($F = 3.14$, $df = 2$, $p = 0.053$) and supports previous research that showed under no choice conditions *G. triguttatus* demonstrated a preference for older hosts, by parasitizing a higher proportion of eggs three-six days of age (Irvin and Hoddle, in preparation). In the current study, parasitism by *G. ashmeadi* was 63.6% and 58.2% higher than *G. triguttatus*, when females were exposed to eggs one and three days of age, respectively. Whereas for eggs five days of age, there was no significant difference in parasitism between these two species. This indicates that *G. triguttatus* larvae, resulting from eggs deposited in GWSS eggs five days of age, were more able to compete with *G. ashmeadi* larvae because this more preferred GWSS egg age may have provided a highly favorable environment for *G. triguttatus* development. Alternatively, *G. triguttatus* females aggressively defended eggs from *G. ashmeadi* and prevented multiparasitism from occurring. In contrast, the proportion of eggs parasitized did not significantly vary between egg ages for *G. ashmeadi* ($F = 2.62$, $df = 2$, $p = 0.08$) and *G. fasciatus* ($\chi^2 = 1.93$, $df = 2$, $p = 0.38$) (Figure 1), whereas previous studies have shown that *G. ashmeadi* and *G. fasciatus* demonstrate strong preferences for eggs one and three days of age, respectively (Irvin and Hoddle, in preparation). Parasitism by *G. fasciatus* may not have been significant between egg ages in the current study, because interspecific competition and potential mutual interference caused extremely low parasitism rates (0.3-2.7%) (Figure 1). *Gonatocerus ashmeadi* may not have demonstrated a preference for GWSS eggs three days of age in the current study because exposure time was higher for the current study (24 hours compared with 2 hours in the previous study), perhaps causing higher rates of parasitism at less preferred egg ages, or the presence of competitors in the current study may have initiated *G. ashmeadi* aggressiveness and oviposition.

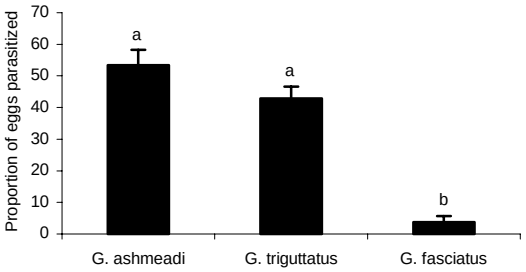

Figure 3. Percentage of GWSS eggs parasitized by *G. ashmeadi*, *G. triguttatus*,and *G fasciatus* when eggs one three and five days of age are presented simultaneously to one female of each species (see Figure 2).

Irvin and Hoddle (2002) showed that competition outcomes between *G. ashmeadi* and *G. triguttatus* varied greatly depending on the experimental design and GWSS egg density used. In the current research, pooling results from exposing females to individual egg ages showed that *G. ashmeadi* parasitized a significantly higher (48.1%) proportion of GWSS eggs compared with *G. triguttatus* (Figure 2), whereas when females were exposed to all egg ages simultaneously, parasitism by *G. ashmeadi* and *G. triguttatus* was equivalent (Figure 3). This may be due to *G. ashmeadi* out competing *G. triguttatus* when females were only provided eggs one or three days of age, since these ages are more favorable for *G. ashmeadi* development, and *G. triguttatus* prefers older hosts (Irvin and Hoddle, in preparation). We speculate that when *G. ashmeadi* and *G. triguttatus* were exposed simultaneously to eggs one, three and five days of age, females parasitized egg ages most preferred by each species, thereby decreasing interspecific competition and resulting in equivalent parasitism rates between species. These results may have important implications for the field environment, where a range of host ages are present at one time, and may suggest that *G. ashmeadi* and *G. triguttatus* can coexist in California without significant interference.

Parasitism by *G. fasciatus* was consistently significantly lower (17.4-76.0% lower) than both *G. ashmeadi* and *G. triguttatus* for all three experimental studies (Figures 1-4). *Gonatocerus fasciatus* may have performed poorly because exposure to GWSS eggs was restricted to one or 24 hours in these studies, and *G. fasciatus* being smaller may require a longer period of time for each individual host handling, and lay fewer eggs at each oviposition event compared with *G. ashmeadi* and *G. triguttatus.* Lifetime fecundity, longevity or offspring production was not investigated in the current studies so it is unknown whether *G. fasciatus* may perform more effectively in the field, over many generations. However, female *G. fasciatus* are capable of producing two or more offspring per *H. coagulata* egg and gregarious reproduction may be advantageous early in the spring when GWSS egg masses are relatively rare and parasitism levels are low (Triapitsyn et al., 2003).

There are many factors that influence the competitive ability of female Hymenoptera. Extrinsic competition refers to the exploitation of the host population (Zwolfer, 1971) and involves factors associated with host finding efficiency, host discrimination between parasitized and unparasitized hosts, aggressive behavior between adult parasitoids, reproductive capacity of female parasitoids, host developmental stage attacked, sex ratio, and phenological synchronization with the host (Lewis et al., 1990; Tumlinson et al., 1993). When multiparasitism does occur, intrinsic competition refers to the interaction between immature parasitoids and involves larval combat or physiological suppression (Chow and Mackauer, 1984; Lawrence, 1988; DeMoraes et al., 1999). The competitive inferiority of *G. fasciatus* shown in these studies may be attributed to poor competitive ability of *G. fasciatus* larvae. Firstly, eggs oviposited by female *G. fasciatus* are conceivably smaller than *G. ashmeadi* and *G. triguttatus*, due to comparative differences in female size between species (Triapitsyn et al., 2003) . Larvae that emerge from larger eggs may potentially give rise to larger parasitoid larvae, which may tend to be better competitors, especially if they hatch earlier (Collier and Hunter, 2001). Secondly, *G. fasciatus* larvae probably do not participate in larval combat, since gregarious larvae often frequently contact one another under normal development (Salt, 1961), and therefore such species may be at an intrinsic disadvantage against congeneric species cohabiting a resource.

Alternatively, the competitive inferiority of *G. fasciatus* may simply be due to subordinate aggressiveness when competing for egg masses with congenerics. For example, in one hour egg age choice experiments where oviposition was required, Irvin and Hoddle (in preparation) showed that 115% and 65% additional female *G. fasciatus* were required to obtain sufficient replication when compared with *G. ashmeadi* and *G. triguttatus*, respectively. Results from the observation study (experiment 3) presented here showed that *G. fasciatus* allocated a significantly higher proportion (31.5%) of time to resting/grooming, compared with oviposition, whereas female *G. ashmeadi* allocated equal time to both activities (Figure 5). Results from both studies may suggest that female *G. fasciatus* are less aggressive than *G. ashmeadi* and *G. triguttatus*, or under the experimental conditions used oviposition motivation was diminished. Furthermore, 39.6% of time allocated by female *G. fasciatus* was spent off leaves with GWSS egg masses (Figure 5), and it was observed that *G. ashmeadi* and *G. triguttatus* often aggressively protected the GWSS egg mass, sometimes excluding access by *G. fasciatus*. Also, female *G. fasciatus* are smaller than *G. ashmeadi* and *G. triguttatus* (Triapitsyn et al., 2003). Together these observations may explain

why *G. fasciatus* spent significantly (9.6-19.6%) more time off the egg mass compared with both *G. ashmeadi* and *G. triguttatus* (Figure 5).

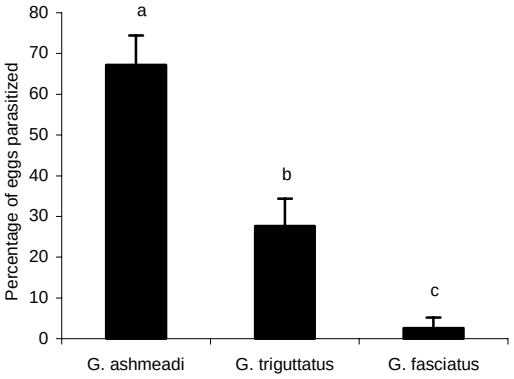

Figure 4. Percentage of eggs parasitized by *G. ashmeadi*, *G. triguttatus* and *G fasciatus* when one GWSS egg mass is presented simultaneously to one female of each species (See Figure 2).

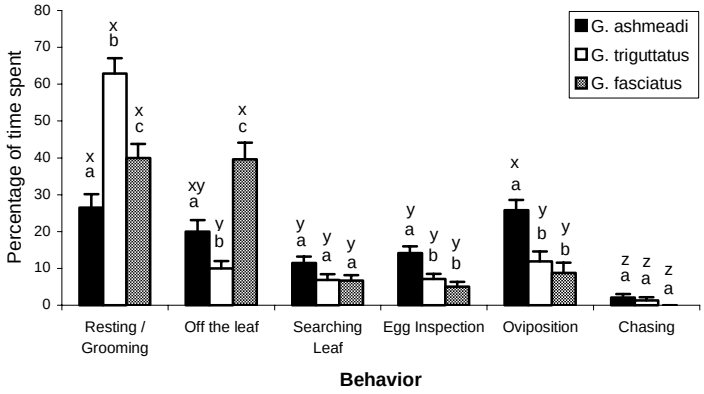

Figure 5. Proportion of time spent in each quantified behavior for female *G. ashmeadi*, *G. triguttatus* and *G fasciatus*, when one GWSS egg mass is presented simultaneously to one female of each species (See Figure 1)

In the Petri-dish observation trial (experiment 3), parasitism by *G. triguttatus* was significantly (39.6%) lower compared with *G. ashmeadi* (Figure 4). This contradicts a similar study that showed that parasitism by *G. triguttatus* was 53% higher compared to *G. ashmeadi* (Irvin and Hoddle, 2002) and iterates the question raised by Irvin and Hoddle (2002), of which experimental designs should be used to decide which parasitoid species shows the most potential as a classical biological control agent for GWSS. *Gonatocerus triguttatus* may have performed differently in the current study because an additional species (*G. fasciatus*) was incorporated into the design. Data from the current study showed that 62.9% of behavioral observations for *G. triguttatus* were resting/grooming, which was significantly higher (36.4% and 58.9%, respectively), when compared with *G. ashmeadi* and *G. fasciatus* (Figure 5). This suggests that female *G. triguttatus* may have a longer pre-oviposition period and require more time resting before ovipositing, or that when three parasitoid species are present concurrently, the assertiveness of female *G. triguttatus* declines.

In Experiment 3 (visual observations in Peri dishes), *G. triguttatus* parasitized a significantly higher proportion (25%) of GWSS eggs compared with *G. fasciatus* (Figure4), whereas time allocated to oviposition by each species was equivalent (Figure 5). This may indicate that *G. triguttatus* is more competitive when ovipositing or its larvae are more aggressive towards congeneric species. Scanning electron microscopy of *G. triguttatus* larvae four days of age has revealed that *G. triguttatus* larvae possess anterior tusk-like appendages, that could possibly be used in larval combat for host procurement (Irvin and Hoddle, in preparation). Irvin and Hoddle (in preparation) conducted a study that involved exposing one GWSS egg mass to one female *G. triguttatus* for one hour, and one hour following the removal of *G. triguttatus*, the same egg mass was exposed to one female *G. ashmeadi* for one hour. Parasitoid emergence data from egg masses in which ovipositor insertion of both species was observed were analyzed. Results showed that the proportion of *G. triguttatus* offspring emergence was 88%, compared with 12% for *G. ashmeadi* when *G. triguttatus* oviposited first (Irvin andHoddle, in preparation). However, when the sequence of species introduction was reversed, the proportion of *G. ashmeadi* emergence was 49%, compared with 51% for *G. triguttatus*. With a four-hour intermission between introducing *G. triguttatus* to the previously parasitized egg mass the proportion of *G. ashmeadi* offspring were not significantly higher than *G. triguttatus*. Only with a 24-hour intermission between female introductions, did *G. ashmeadi* produce significantly more offspring (64%) than *G. triguttatus* (36%) (Irvin and Hoddle, in preparation). These results support the theory that *G. triguttatus* larvae may be superior competitors when multiparasitism of GWSS egg masses occurs.

Zwolfer (1971) proposed that due to life history trade-offs, parasitoid species that are intrinsically inferior should win in extrinsic competition, and vice versa. Therefore it is possible, that while *G. fasciatus* performed poorly in the current studies, females may be extrinsically superior in the field due to potentially higher reproduction rates (gregarious reproduction), oviposition preference for young GWSS egg masses, and greater host finding efficiency at low host densities. A previous study investigating percentage parasitism and offspring emergence of each species showed that parasitism by *G. fasciatus* was up to 15.9% lower than *G. ashmeadi* and *G. triguttatus*. But because of the gregarious reproduction habit, *G. fasciatus* offspring emergence was 77.2% and 82.9% higher compared with *G. ashmeadi* and *G. triguttatus*, respectively (Irvin and Hoddle, in preparation).

CONCLUSIONS

- Overall parasitism by *G. ashmeadi* was consistently higher (up to 76.0%) compared with *G. triguttatus* and *G. fasciatus* for all three experimental designs suggesting that *G. ashmeadi* may show greater potential as a biological control agent of GWSS and could out compete *G. triguttatus* and *G. fasciatus* in the field.
- The aggressive behavior demonstrated by *G. ashmeadi* and *G. triguttatus*, towards other females, may give these species a competitive advantage over *G. fasciatus* which does not display similar aggressive tendencies.
- Parasitism by *G. fasciatus* was consistently significantly lower (17.4-76.0%) than both *G. ashmeadi* and *G. triguttatus* for all three experimental studies, suggesting that this species may be an inferior competitor under laboratory conditions. However *G. fasciatus* may be superior in the field, due to potentially higher reproduction rates, younger host age attacked and greater host finding efficiency.

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FUNDING AGENCIES

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INVESTIGATING THE BEHAVIOR AND BIOLOGY OF EXOTIC MYMARID PARASITOIDS RELEASED FOR GLASSY-WINGED SHARPSHOOTER CONTROL, AND EVALUATING TECHNIQUES FOR ENHANCEMENT IN THE FIELD

Project Leader:
Mark S. Hoddle
Department of Entomology
University of California
Riverside, CA, 92521

Cooperators:
Serguei Triapitsyn
Robert Luck
Dept.of Entomology
University of California
Riverside, CA 92521

Reporting Period: The study is scheduled to commence in February 2004.

INTRODUCTION

Glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Say) (Hemiptera: Cicadellidae), is an exotic insect pest in California that vectors a phytopathogenic xylem-dwelling bacterium, *Xylella fastidiosa*. This bacterium causes serious scorch-like maladies in a variety of economically important plants including grapes {Pierce's disease (PD), almonds (almond leaf scorch), and oleanders (oleander leaf scorch)}. Pesticide use has increased dramatically in citrus orchards (major crop reservoir of GWSS) adjacent to vine yards as sprays are applied to suppress populations of migratory GWSS into grapes in spring. Increased use of broad-spectrum insecticides to control GWSS has severely disrupted citrus IPM in southern California, in particular, Temecula in Riverside County. A similar situation of preemptive spraying in citrus has developed in the Central Valley, especially in Tulare County.to mitigate GWSS migration into grapes. Consequently, pesticide usage has increased substantially in vineyards to kill invading GWSS that survived citrus sprays or migrated in from untreated wilderness and residential areas. GWSS is the subject of a major classical biological control program in California. Mymarid egg parasitoids from the home range of GWSS in southeastern USA are being imported and released in California in an attempt to control inordinate pest densities.

Two new parasitoid species previously lacking in California have been imported over the last two years – *Gonatocerus triguttatus* from Texas and *G. fasciatus* from Louisiana. It is likely that *G. triguttatus* has established in southern California, as this parasitoid has been recovered from different release sites several times. It is unknown whether *G. fasciatus*, which was released last year, has established. These two parasitoids join the resident *G. ashmeadi*, a parasitoid with a geographic range that includes Florida through eastern Texas. *G. ashmeadi* is most likely not native to California and was probably introduced with GWSS in the early 1990's and may have established on eggs of *H. liturata*, a native California sharpshooter.

The research proposed in this grant is essential for the long-term management of the GWSS-PD epidemic because imported biological control agents are expected to play a fundamental role in the permanent suppression of GWSS populations that vector PD. Consequently, management decisions for GWSS that are reliant on combined components of biological, cultural, or chemical control strategies as part of an IPM program will require information on the basic biology, ecology, and behavior of imported *Gonatocerus* spp. Maximal exploitation of imported natural enemies, effective management, conservation, and mass rearing of mymarid parasitoids for GWSS suppression will require information that can only be attained if comprehensive studies of the biology, behavior, and impact of the three *Gonatocerus* spp. alone and in combination are made. Therefore we aim to achieve as quickly as possible a thorough understanding of what we consider to be the most important aspects of *Gonatocerus* spp. ecology, biology and behavior to maximize the role of biological control for GWSS suppression. Our research objectives have been designed to achieve this rapid understanding.

OBJECTIVES

1. Address competition between the three mymarid species under conditions of high and low host densities, and simple and complex environments. These studies are very important as they provide the necessary mechanistic understanding to explain the observed dominance of particular species under varying GWSS densities and habitats and may provide insights into which species could be the most effective parasitoid thereby focusing mass rearing and establishment efforts on this species.
2. Investigate factors affecting sex allocation and subsequent ratios of males to females. This objective is fundamental to our understanding of factors influencing the production of females, especially in mass rearing situations where males tend to dominate and this reduces the rate at which colonies can grow and produce wasps for liberation. *G. fasciatus* is a gregarious species (this was unknown until we reared this parasitoid) unlike the other two *Gonatocerus* spp. that are

solitary parasitoids. Consequently, we know nothing about factors influencing sex allocation of *G. fasciatus* and factors affecting its subsequent population growth.

3. The most fundamental aspects of the developmental and reproductive biology of the three mymarid parasitoids have not been studied and this is a serious impediment to our understanding of these natural enemies. We know nothing about pre-oviposition periods, fecundity, developmental times, longevity, and survivorship rates at different temperatures. Attaining this basic knowledge will greatly improve decisions on colony management, interpretation of field results, and will allow construction of degree-day models to predict potential parasitoid impact through the range GWSS occupies.
4. Laboratory assays have clearly demonstrated that floral resources can enhance parasitoid vigor. Field evaluation in citrus orchards of understorey plantings is necessary to determine if survivorship of over wintering parasitoids can be enhanced through resource provisioning. This cultural control strategy may significantly enhance mortality of the spring GWSS generation (a major weakness of *G. ashmeadi* is the low level of attack on the spring generation of GWSS eggs) because of increased parasitoid survivorship. We envision the following short-term benefits from this research:
    a. Improved understanding of biological and behavioral factors affecting mass rearing of mymarid parasitoids resulting in more cost effective management protocols and enhanced production.
    b. Mechanistic understandings of factors affecting parasitoid establishment, impact, and spread under varying sharpshooter densities, habitats, and climatic zones.
    c. Empirical assessment of the feasibility and magnitude of parasitoid impact on spring and summer GWSS populations resulting from habitat manipulations designed to provision parasitoids with resources that enhance their vigor.

RESULTS AND CONCLUSIONS

This work has not been commenced. We are currently recruiting a new postgraduate researcher to fill this position

FUNDING AGENCIES

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

IS THE GLASSY-WINGED SHARPSHOOTER PARASITOID *GONATOCERUS ASHMEADI* (HYMENOPTERA: MYMARIDAE) ONE SPECIES OR A COMPLEX OF MORPHOLOGICALLY INDISTINGUISHABLE SIBLING SPECIES?

Project Leaders:
Mark S. Hoddle
Richard Stouthamer
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:
Danel Vickerman
Serguei Triapitsyn
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted from September 2002 to September 2003.

ABSTRACT
Gonatocerus ashmeadi Girault (Hymenoptera: Mymaridae) is a common and seemingly widespread egg parasitoid of glassy-winged sharpshooter (GWSS). Location records for *G. ashmeadi* indicate its natural range to be the southeastern USA and northeastern Mexico (which coincides with the presumed native range of GWSS), and possibly southern and central California (the adventive range of GWSS). *G. ashmeadi* was collected in California from eggs of the native smoke-tree sharpshooter, *Homalodisca literata* Ball, as well as from GWSS eggs before *G. ashmeadi* releases began as part of an organized biological control program and was therefore thought to be native to California. However, the earliest *G. ashmeadi* specimen from California that we found after a search of the Entomology Museums at UC Davis, Berkley and Riverside, the San Diego Natural History Museum, California Department of Food and Agriculture, USNM and the California Academy of Sciences sources, is from Riverside County in 1978 (first identified by Huber 1988). The purpose of our work is to determine whether *G. ashmeadi* in the USA and northeastern Mexico is one species or a complex of cryptic species that can't be separated on the basis of currently employed morphological characters. We used three approaches to determine the species identity of different *G. ashmeadi* populations: (1) reassessment of key morphological features using scanning electron microscopy (SEM) to determine if subtle morphological differences exist between *G. ashmeadi* populations which could indicate species differences; (2) mating compatibility studies to determine if different populations of *G. ashmeadi* are reproductively isolated, or if mating occurs, whether offspring are viable thereby defining species groups on the basis of successful interbreeding; (3) to determine if molecular differences exist between *G. ashmeadi* populations collected from different regions by comparing mitochondrial and ribosomal DNA sequences. Results from these three areas (morphology, behavior, and DNA sequences) have been evaluated together, leading us to the conclusion that *G. ashmeadi* as it is currently viewed is a valid species and not an aggregate of morphologically indistinguishable cryptic species.

INTRODUCTION
A classical biological control program is currently underway for glassy-winged sharpshooter (GWSS), which is an exotic pest in California. The native range of GWSS is the southeastern USA and northeastern Mexico (Triapitsyn and Phillips, 2000). GWSS is thought to have invaded California around 1990 as egg masses that were accidentally imported on ornamental plants from Florida. Species of GWSS egg parasitoids, not present in California, are currently being prospected for in the native range of GWSS. Promising candidate natural enemy species that attack eggs are being imported and released in CA for GWSS control (Triapitsyn et al., 1998; Triapitsyn and Hoddle, 2001). Interestingly, one species of egg parasitoid associated naturally with GWSS in California, *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae), is also widely distributed in the home range of GWSS, but at the time of its initial discovery in California, *G. ashmeadi* had not been intentionally released here and was thought to be native to California. However, it is a possibility that *G. ashmeadi* is not native to California and was unintentionally introduced to southern California and became established. A potential host for *G. ashmeadi* in California prior to the arrival of GWSS could have been the native *Homalodisca liturata* which has had unidentified *Gonatocerus* spp. reared from its egg masses collected in the San Diego area (Powers, 1973). Later, Huber (1988) identified several species of *Gonatocerus*, including *G. ashmeadi*, from eggs of *H. liturata* in Riverside Co. in California collected in 1978 and later. After a search of the Entomology Museums at UC Davis, Berkley and Riverside, the San Diego Natural History Museum, California Department of Food and Agriculture, USNM and the California Academy of Sciences sources, this 1978 specimen is the earliest we have found.

G. ashmeadi is the key natural enemy of GWSS egg masses in California (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). The success and failure of a number of

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

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

OBJECTIVES

1. Reassessment of key morphological features using scanning electron microscopy (SEM) to determine if subtle morphological differences exist between *G. ashmeadi* populations which could possibly indicate species differences.
2. Mating compatibility studies to determine if different populations of *G. ashmeadi* are reproductively isolated, or if mating occurs, whether offspring are viable thereby defining species groups on the basis of successful interbreeding.
3. Studies to determine if molecular differences exist between *G. ashmeadi* populations collected from different regions by comparing mitochondrial and ribosomal DNA sequences. Molecular dissimilarities of key regions could potentially indicate the existence of different species.

RESULTS

Objective 1. Reassessment of key morphological features

Morphological studies entailed using scanning electron micrographs (SEM), as well as point-mounted and slide-mounted specimens. Examination of key morphological characters including head, antennal and mesosomal morphology (Figure 1) did not result in a differentiation between insects from several populations. However, specimens from San Luis Potosí, Mexico (not shown here) will be re-evaluated for possible morphological distinctions.

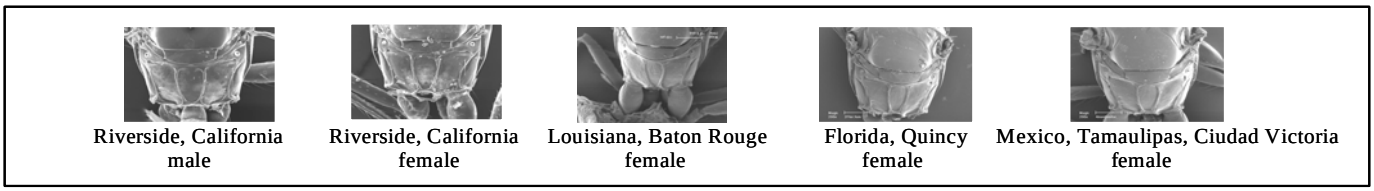

Figure 1. SEM photos showing the propodeum of *G. ashmeadi* from several locations.

Objective 2. Mating compatibility studies

Live insects were obtained from collaborators in Texas, Louisiana and Florida. The California insects were collected in areas where other biotypes had not been released. We were unable to obtain live insects from Mexico for use in mating crosses. We carefully orchestrated paired mating crosses of isofemale lines from within each geographical area in order to establish the regional compatibility of these lines. A single isofemale line (regional standard) was then used to cross the California isofemale line with Texas, Louisiana, and Florida. The mating protocol of 10 replicates each was as follows: 1) female line A and male line B; 2) female line B and male line A; 3) female line A and male line A (control cross); 4) female line B and male line B (control cross); 5) virgin females of line A; 6) virgin females of line B. The virgin females controls were done to assure that the females used in the crosses were 1) indeed virgins and 2) do not produce daughters through parthenogenetic reproduction. Offspring were reared out and sexed. Production of female offspring indicated sexual compatibility between individuals of the same species and production of males only indicated sexual incompatibility between different populations of *G. ashmeadi*. This experimental set up allowed us to estimate the relative compatibility by comparing offspring sex ratios of all crosses with each other (females/ females + males). No significant differences were found between crosses (Figure 2; Kruskal-Wallis, $H = 4.343$, $df = 9$, $P = 0.888$).

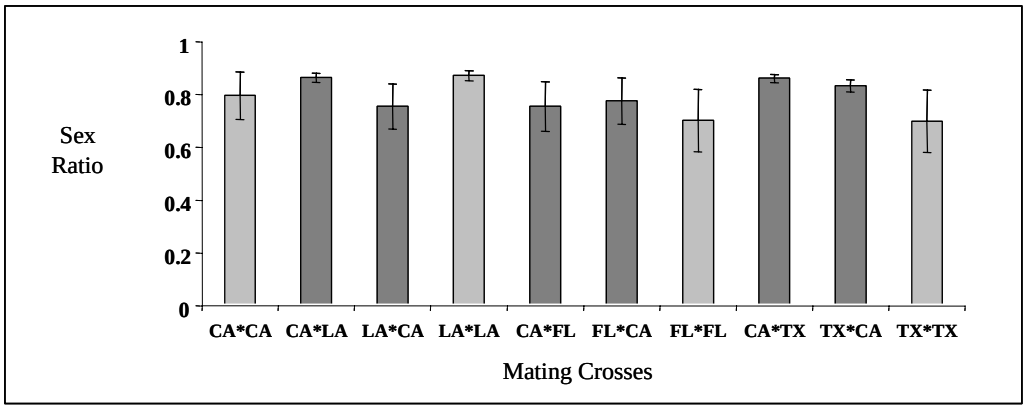

Figure 2. Mating compatibility of isofemale line crosses within and between populations, shown here as the sex ratio (females/ females + males).

Objective 3. Studies to determine if molecular differences

Using the polymerase chain reaction (PCR) we have amplified and sequenced the mitochondrial genes COI and COII for 7 populations (Tamaulipas Mexico, Louisiana, Texas, Florida, Georgia, South Carolina and California). In addition, the ribosomal genes ITS1 and ITS2 have been amplified for all 7 populations using PCR. Since ribosomal genes are known to occur as multiple copies within an individual, these gene regions have been cloned, and 4 clones from one individual per population have been sequenced for the ITS regions. Sequences of 4 additional *Gonatocerus* species have been used as a comparison of the variation between species. Molecular sequences of the COI, COII, ITS1 and ITS2 regions do not demonstrate sufficient variation between populations that could identify any one population as different from the others (Figure 3).

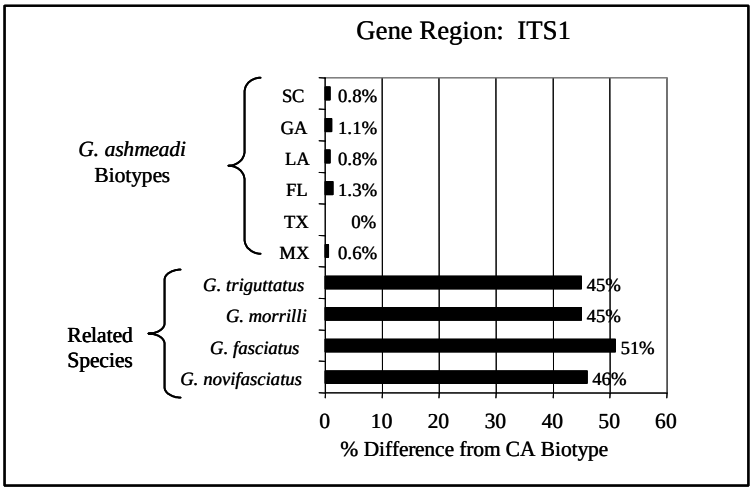

Figure 3. Percent difference from the CA biotype in basepairs of the ITS1 gene region for *G. ashmeadi* biotypes and related species. A similar trend was observed for other sequenced gene regions.

CONCLUSIONS

Results from these three areas (morphology, behavior, and DNA sequences) have been evaluated together, leading us to the conclusion that *G. ashmeadi* as it is currently viewed is a valid species and not an aggregate of morphologically indistinguishable cryptic species. Further investigations should determine if releasing different biotypes of this parasitoid may be beneficial if they fill niches not currently occupied by the biotype of *G. ashmeadi* already present in California.

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FUNDING AGENCIES

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

Project Leaders:
Mark S. Hoddle
Serguei V. Triapitsyn
Department of Entomology
University of California
Riverside, CA, 92521

Cooperators:
David J.W. Morgan
California Department of Food and Agriculture
Riverside, CA

Roman A. Rakitov
Illinois Natural History Survey
Champaign, IL

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

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 their native range in central and eastern United States justifies 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 Georgia, Alabama, Mississippi, Louisiana, and Texas. Prior research showed presence of the mymarid *Gonatocerus fasciatus* Girault there (Triapitsyn et al. 2003). In addition, a number of trichogrammatid genera and species have been recognized in central and eastern USA (Table 1).

Table 1. The following species of trichogrammatid egg parasitoids were identified from eggs of proconiine sharpshooters in central and eastern USA (Triapitsyn 2003):

Genus and species of egg parasitoid (family Trichogrammatidae)	Known records from: (state)	Host(s)
Oligosita ?americana	Georgia	*Homalodisca insolita*
Paracentrobia sp.	Florida, Georgia	*Cuerna costalis, Homalodisca insolita, ?H. coagulata*
Ufens niger	Georgia, Indiana, Maryland, Washington, D.C	*Cuerna costalis*
Ufens new species	Florida	*Homalodisca coagulata*
Zagella spirita	Florida, Georgia, Louisiana, Maryland, Mississippi	*Homalodisca coagulata, Oncometopia orbona*

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. (Arkansas, Illinois, Kentucky, Missouri, Oklahoma, northern Texas, Virginia, etc.) for introduction into California, establishment of cultures in UCR quarantine, and a following evaluation. Several short exploratory trips will be made during spring-summer 2004 to those states and parasitized egg masses of the sharpshooters will be collected there and sent to UCR quarantine facility under a permit. Where possible, local collaborators will be employed to collect adult sharpshooters to obtain and expose sentinel egg masses.
2. Curatorial work – It is expected that hundreds of collected voucher specimens of mymarid and trichogrammatid egg parasitoids will require appropriate curation as a result of the proposed exploratory work; these will need to be critically point-dried from ethanol, point- or card-mounted, labeled, and identified to genera and species. Then representatives of each species (of both sexes) will be selected, dissected, and slide-mounted. The specimens will be deposited in the collections of Entomology Research Museum, UC Riverside, as well as in the California State Arthropod Collection, California Department of Food and Agriculture, Sacramento, California.

RESULTS

One exploratory trip was made to Kentucky and Tennessee by S. Triapitsyn in July 2003. Adults of *Cuerna costalis* (Fabricius) were collected on Johnson grass in central Tennessee, but no egg masses were found. An old egg mass of *O. orbona* was also found on a holly leaf. The late availability of funding made it difficult for us to conduct any further exploratory work during 2003 as the collecting season was over, so we will concentrate our efforts during next spring and summer.

Our collaborator, Roman A. Rakitov, attempted to obtain egg parasitoids of *O. orbona* in Illinois using sentinel eggs, but no parasitoids emerged. Further attempts will be made next year.

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. During 2002 we searched for and collected egg parasitoids of GWSS in southeastern USA, mainly Louisiana, where the target species for importation into California was *Gonatocerus fasciatus* Girault (Triapitsyn et al. 2003) and also in northeastern Mexico. The result of our introductions, *Gonatocerus fasciatus* has been already mass-reared and released in California by the CDFA researchers.

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FUNDING AGENCIES

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ENTOMOPATHOGENIC FUNGI FOR BIOLOGICAL CONTROL OF THE GLASSY-WINGED SHARPSHOOTER, *HOMALODISCA COAGULATA*

Project Leader:
Harry K. Kaya
Department of Nematology
University of California
Davis, CA 95616

Cooperators:

Tony Bonner	Michael McGuire
Department of Nematology	USDA-ARS
University of California	Shafter Research and Extension Center
Davis, CA 95616	Shafter Research and Extension Center

Reporting Period: The results are from work conducted from July 1, 2003 to October 30, 2003

INTRODUCTION

The glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, is a threat to the grape industry because it is an efficient, mobile vector of the bacterium, *Xylella fastidiosa*, the causal agent of Pierce's disease. GWSS is native to the southeastern USA and has been established in southern California for more than a decade. As this insect moves and establishes in new areas, without its natural complement of natural enemies, control measures are needed. Although chemical insecticides are available, there are environmental and human health concerns with their use. Moreover, chemical insecticides can be disruptive in IPM programs where insect parasitoids and predators effectively control other pests. Thus, alternative control approaches are needed. One of these approaches is microbial control and we are examining the potential of utilizing entomopathogenic fungi to control GWSS. Entomopathogenic fungi may be effective against this insect with piercing-sucking mouthparts because fungi can infect directly through the integument whereas other pathogens require ingestion to cause infections.

Entomopathogenic fungi offer promise as control agents because they have been recovered from GWSS and its close relatives. Mizell and Boucias (2002) found mycosed (*Hirsutella* spp.) GWSS cadavers from Florida and Georgia. In addition, a number of entomopathogenic fungi have been reported from related leafhopper species. These fungi include *Erynia* spp. (Matsui et al. 1998; Sierotzki et al. 2000), *Torrubiella hemipterigena* (Hywel-Jones et al., 1997), *Zoophthora radicans* (McGuire et al. 1987 a, b; Galaini-Wraight et al. 1991, 1992; Magalhaes et al. 1991) and *Beauveria bassiana* (Lane et al. 1991). In California, McGuire et al (2001) and McGuire (2002) have isolated *B. bassiana* from the *Lygus* bug from alfalfa fields. In one field more than 65% of the *Lygus* bugs were infected with *B. bassiana*. These reports indicate that a number of fungal species infect plant bugs, leafhoppers and sharpshooters and could be used as biological control agents.

OBJECTIVES

1. Conduct an extensive survey for entomopathogenic fungi infecting GWSS in natural populations in California.
2. Culture the fungi and assay them against susceptible stages of the GWSS.
3. Evaluate California isolates of fungi from insects closely related to GWSS.
4. Conduct small-scale tests against overwintering adults in citrus groves.

RESULTS AND CONCLUSIONS

We have only recently initiated our project, and therefore, we have not accumulated much data. However, for objective 1, we have isolated a fungus tentatively identified as Beauveria sp. from an adult GWSS from a citrus grove in Riverside County. The Beauveria sp. was cultured on selective medium and tested against adult GWSS but preliminary results indicate that it was not highly pathogenic. Although a number of other dead individuals have been found, they have not yielded any entomopathogenic fungi. For objective 3, we are in the process of evaluating several entomopathogenic fungal isolates from Lygus bugs collected in California. Those that are pathogenic to GWSS will be tested further.

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FUNDING AGENCIES

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

EVALUATION OF OLFACTORY RESPONSES BY *GONATOCERUS ASHMEADI,* A PARASITOID OF GLASSY-WINGED SHARPSHOOTER EGG MASSES ON VARIOUS HOST PLANTS

Project Leaders:

Robert F. Luck	Carlos E. Coviella	David Morgan
Department of Entomology	Department of Entomology	CDFA Mount Rubidoux
University of California	University of California	Field Station
Riverside CA 92521	Riverside CA 92521	Riverside, CA 92501

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

ABSTRACT

This proposal seeks to improve the use of *Gonatocerus* spp. (Hymenoptera: Mymaridae) i.e., egg parasitoids, in urban and riparian habitats where it is a politically accepted tactic for suppressing glassy-winged sharpshooter, *Homalodisca coagulata* (Say) (Homoptera: Cicadellidae) (GWSS). Such releases are part of an area wide pest management strategy for suppression of GWSS. This proposal has two general objectives: 1) to determine the relative importance of volatile cues (host plant versus host egg-mass) for *Gonatocerus* spp. to locate and parasitize GWSS egg masses, and 2) to determine whether *Gonatocerus* spp released in an urban or riparian environment is equally successful in locating egg-masses in the variety of host plants utilized by GWSS as oviposition sites in these habitats. To fulfill these objectives, olfactometer techniques will be used to study the long-range host location behavior of *G. ashmeadi* to determine whether it a) is innately attracted via chemical cues to GWSS egg masses, b) learns these cues after attacking an egg mass in a particular host plant species or emerging from an egg-mass in a particular host plant species, or c) gains experience by attacking an egg-mass located in a particular host plant species that modifies its innate host location behavior for egg masses laid in other host plant species. Host location-host selection information will identify host plant species with GWSS egg-masses preferred by *Gonatocerus* spp for parasitization and identify those that are not recognized or readily recognized by these parasitoids. These results will then be used to compare the host finding behavior of the other *Gonatocerus* spp.

INTRODUCTION

Several of the important habitats in which GWSS populations thrive, i.e., the urban and riparian habitats, are not amenable to chemical suppression from a practically or politically perspective. It is these habitats to which the release of natural enemies is most suited and the single most important tactic available to reduce GWSS populations in these habitats. This proposal addresses optimizing the utilization of this tactic in these habitats (i.e., the release of biological control agents for the suppression of glassy winged sharpshooter egg-masses).

Currently, biological control agents are being released against GWSS and principally involve mymarid egg parasitoids belonging to the genus, *Gonatocerus*. Several of these species are being mass-reared and released by the California Department of Food and Agriculture. These parasitoids attack GWSS eggs and are the focus of this proposal. We propose to use olfactometer techniques to identify and assess the variation and relative importance of both host-plant and egg-mass associated cues that allow *Gonatocerus* species to locate GWSS egg-masses. Information on host-location selection will identify host plant species preferred by these parasitoids for oviposition and identify those that are not recognized or readily recognized by *Gonatocerus* species.

Successful parasitoid-host associations depend on a parasitoid's ability to locate its hosts in a complex and heterogeneous environment and to produce offspring from those hosts it locates and accepts (Vet and Dicke, 1992). Host location is frequently mediated by volatile infochemicals arising from the host, the host plant, and/or from an interaction between the two (Vinson 1976, Vet and Dicke 1992). Such infochemicals may also provide an estimate of the local environment's profitability (i.e., the number of hosts present and their quality) (Vet and Dicke 1992). Recently, Ives (1995) and Vet (1999, 2001) have argued that the sequence of steps describing the host parasitoid association also forms the link between parasitoid behavior, i.e., the variability in parasitism rates among host patches, and the coupled dynamics between the host and the parasitoid population. Since biological control, or the lack of it, is the parasitoid population's collective effect on the host's population dynamics (Vet 2001), the sequence of steps involved in host location and acceptance becomes a means of assessing a parasitoid's potential efficacy as a biological control agent (=natural enemy).

Furthermore, it has been shown that parasitoids can modify their searching behavior on the basis of prior experience (Vet and Dicke, 1992; Turlings et al, 1993; Vet et al, 1995). This associative learning can be exploited to prime the parasitoid (i.e., to imprint it with the cues of a particular host and/or host plant species, thus improving its ability to suppress GWSS by increasing its parasitization rate of GWSS egg-masses). Such a parasitoid is expected to locate its host more efficiently (see for e.g. Hare, 1996). Thus, this project will provide important information to improve mass rearing and release techniques for this parasitoid that can increase parasitization rates of GWSS egg-masses in urban and riparian environments.

OBJECTIVES

1. To determine whether chemical cues are used in long-range host finding by *G. ashmeadi*, and to determine the source(s) from which these chemical cues emanate (i.e., from an egg mass, a host plant in which the egg-mass is laid, or as an interaction between the two).
2. To assess whether the success of *G. ashmeadi* in locating its' host innately varies among the different host plant species in which GWSS lays its egg-masses.
3 To determine if egg-mass density affects the success of *G. ashmeadi* in locating GWSS egg-masses.
4. To assess whether emergence from or experience with an egg-mass on a specific host plant species affects the success with which *G. ashmeadi* subsequently finds additional egg-masses on this or other host plant species.

RESULTS AND CONCLUSIONS

Funding for this project was approved on August 2003. We are currently in the process of buying the necessary equipment. Mr. Kapranas, which will be in charge of the experimental part of the project is training with the software that will be used with the equipment. No data are yet available at the time of this report.

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FUNDING AGENCIES

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SPATIAL AND TEMPORAL RELATIONS BETWEEN GLASSY-WINGED SHARPSHOOTER SURVIVAL AND MOVEMENT, XYLEM FLUX PATTERNS, AND XYLEM CHEMISTRY IN DIFFERENT HOST PLANTS

Project Leaders:
Rober F. Luck
Mark Hoddle
Department of Entomology
University of California
Riverside, CA92521

Cooperators:

Carlos Coviella	Peter Andersen
Department of Entomology	North Florida Research Center
University of California	University of Florida
Riverside, CA	Monticello, FL

Reporting Period: This report covers the fieldwork performed from September 2002 through September 2003. However, we are including the whole dataset from October 2001 through September 2003 when reporting the results and analysis.

ABSTRACT
We use weekly whole tree samplings on commercial size citrus trees to quantify actual GWSS population densities and to follow its population dynamics. The dataset presented includes data taken from oranges and lemons, and we recently started the same sampling on tangerines and grapefruit. The results for the whole period from October 2001 through September 2001 show no significant difference between oranges and lemons. However there are significant differences at different times of the year. There is also a clear decline in population density from the averages detected in 2001/2 and the averages for 2003. We will continue sampling through at least 2004 to assess whether this trend continues. At the moment of this report, the results from the xylem chemistry 2002 dataset as well as the recruitment of nymphs and adults for 2003 are being processed, and will be shown at the meeting.

INTRODUCTION
As stated in our original proposal, we seek to identify those aspects in the GWSS-host plant interaction that explain GWSS' variation in reproductive performance and population dynamics. Following a species' population dynamics in the field requires a reliable method of estimating its densities. Currently, yellow sticky traps, net beatings and/or visual counts of GWSS are the principal methods used to estimate its field densities. We also continue to use a specially designed Schölander bomb to extract xylem fluid for chemical analyses. The physical and chemical properties of xylem collected from different host plants throughout the season will be correlated with changes in GWSS' densities and reproductive performance.

OBJECTIVES
1. Quantify xylem flux patterns and characterize xylem fluid chemistry to determine potential correlations with GWSS movement from surrounding alternate host plants into vineyards
2. Quantify egg production, nymphal survival, and adult production and movements in different host plants and correlate GWSS demographic statistics with xylem flux and chemistry.

RESULTS
During the third year of this project, we continue to use our insect sampling and xylem extraction methods we developed during the first year of this project. On average, we recover 89% of the GWSS in a commercial-sized citrus tree. We use this number as a correction factor for each tree. We currently use this to estimate GWSS densities in orange and lemon trees at a mixed orange/lemon grove, Agricultural Operations, UC Riverside. In 2003 we started sampling Tangerines and Grapefruit at the same location to get a better picture of the overall GWSS dynamics on its most important hosts. To follow GWSS' population dynamics, and to correlate its changes in densities over time in different host plants, we will characterize xylem flux and xylem chemistry over time obtained from the same sampled trees. We plan to continue the whole tree sampling and the sampling of xylem fluid during the third year of this grant. This will provide a 2.5-year database of GWSS dynamics and xylem chemistry profiles for two citrus varieties (Oranges and Lemons) and 1.5 years for two other varieties (Tangerines and Grapefruit). We expect that by the end of the third year, this database will enable us to explain some of the patterns involved in GWSS' host plant selection, oviposition, and overall changes in population densities, which is one of the critical aspects governing the dynamics of Pierce's disease epidemics in California.

Our results show that adult GWSS densities vary seasonally, depending on plant host. Three patterns appear to characterize these changes. First, adult GWSS densities on orange and lemon trees do not differ significantly during most of the winter and early spring, that is until they begin ovipositing in the spring. Secondly, they are significantly denser on oranges during late spring and most of the summer, and are significantly denser on lemon trees during late summer, fall and early winter. Thirdly, the results this year suggest a decline of these populations, both in lemons and oranges (Figure1)

Also our results for the year 2002 suggested that one (effective) generation per year occurred on citrus at UCR's Ag Ops even though a second period of egg laying occurred in late summer early fall. This second egg-laying period however contributed very few nymphs and adults to the total annual production of adult GWSS (approximately 1.3%; Figure 6). At the time of this report, we are still analyzing the results for 2003 to assess whether the same results occurred in late summer early fall 2003.

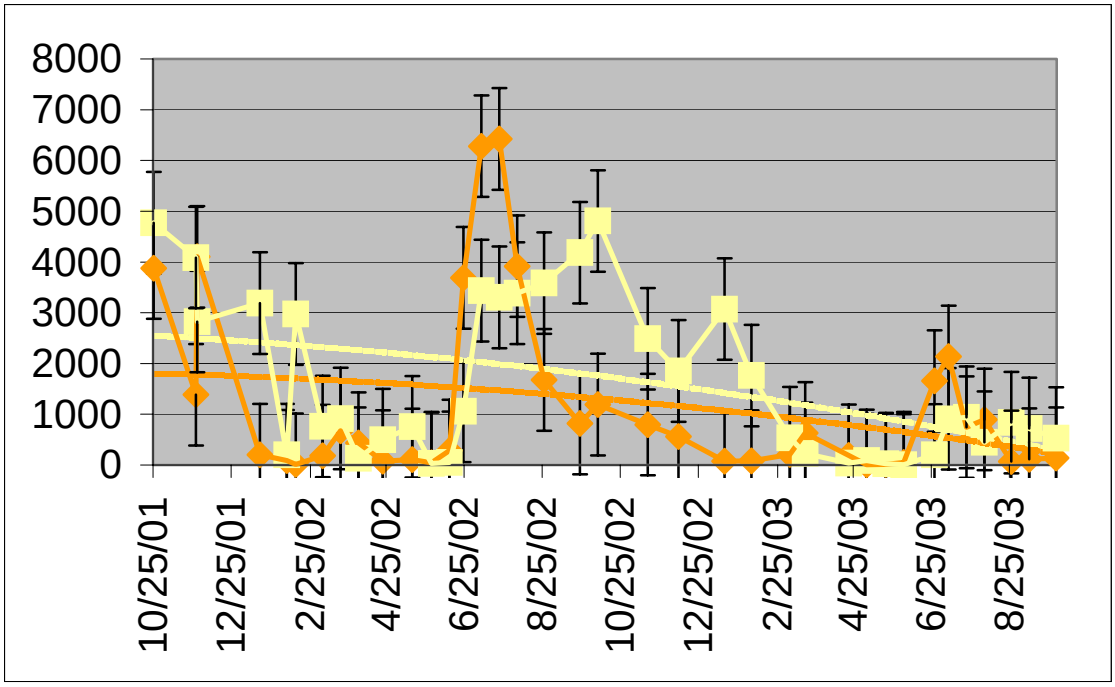

Figure 1: Total number of adult GWSS per sampling date on oranges (orange) and lemons (yellow). Standard error bars and trend-lines are shown.

Although the causes of egg mortality still need further exploration, egg parasitism is substantial and ranged from 78% to 92% during the second generation (eggs) in 2002. These results suggest, that after the period egg parasitism on citrus in late summer-early fall, GWSS populations may be vulnerable to a control measure that are tailored to drive the GWSS populations to densities below those necessary to maintain its local population.

We are using Turner and Pollard's estimations, as well as data gently provided by Dr. Isabel Lauziere on the duration of each nymphal instar to calculate adult GWSS recruitment from our field data. Newly emerged adults have a distinctive bright red coloration in their wings. This coloration fades over time but it is obvious for the first 15 days after emergence. We are using this coloration to calculate the proportion of newly molted adults comprising the adult GWSS on each sampling date and thus, to calculate GWSS adult recruitment on a monthly basis (Figure6).

CONCLUSIONS

The partial results presented here, continue to support last's year result that the total annual GWSS density on lemons and oranges do not differ significantly, whereas, significant differences in the GWSS densities on these to hosts do occur at different times during the year. The results also show a decline in GWSS densities during the two years. It is important to note that the sampling was performed on a citrus grove that is not being treated, (and that any decline in density would likely be due to the decline in GWSS populations elsewhere? I don't understand this point). The third field season next year will provide information on whether this decline is a significant trend, and whether GWSS populations in untreated areas are steadily declining.

FUNDING AGENCIES

BEHAVIORAL AND PHYSIOLOGICAL DETERMINANTS OF THE GEOGRAPHICAL RANGE OF THE GLASSY-WINGED SHARPSHOOTER

Project Leaders:
Russell F. Mizell, III
Peter C. Andersen
University of Florida, NFREC-Quincy
Quincy, FL 32351

Cooperators:
Carlos Coviella
Robert Luck
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted during 2002-2003.

ABSTRACT
This project concerns the important behavioral and physiological mechanisms of glassy-winged sharpshooter (GWSS) and its parasite, *Gonatocerus* spp. that affect overwintering survival and determine geographical range potential. An understanding of these mechanisms would enable researchers to predict the host range of GWSS during the dormant season, the potential for colonization by GWSS and *Gonatocerus* spp. of specific geographical locations in relation to ecological and climate and weather factors, and the potential survivorship of GWSS under specific weather conditions.

INTRODUCTION
Little is known about the overwintering and diapausing biology of GWSS. In north Florida, GWSS populations vary greatly from year to year; abundance may be greatly impacted by the success of the late-summer generation of GWSS that mature to adults in August of the previous year. Overall fecundity rates of GWSS begin to decline as early as July, but reproductive variability is great and we have noted some individuals ovipositing as late as November. We have documented that GWSS may overwinter as eggs as well as adults. Since GWSS are active in north Florida for a relatively short period (ca. 4 months), overwintering and diapause play a critical role in population dynamics of these insects. Understanding cues for diapause and overwintering is also essential for researchers attempting to rear these insects throughout the year.

We addressed the reproductive biology of this second generation of GWSS by detailed dissection and examination of fat body, ovariole and egg development. Typically, fat body development precedes ovariole development as vitogellins are synthesized in fat bodies and are required for ovariole and ova development. Thus, examining the sequence of fat body, ovariole and ova development allows us to characterize the reproductive state of leafhoppers. We note, however, that advanced fat body development does not necessarily infer ovariole development, in diapausing insects, nutrients from the fat bodies can remain stored or be utilized for non-reproductive functions.

OBJECTIVES
Determine the interactive effects of late season and dormant season host quality, temperature, and light regimes on GWSS physiology, behavior, and survivorship and the survivorship of its egg parasite.

RESULTS
We assessed the normal pattern of reproductive development of the late generation of GWSS and determined if host plant selection contributed to the noted variability in leafhopper reproductive development. Late summer populations of newly maturing adults on holly were collected at adult maturation in early August. Individuals from these populations were collected and dissected weekly through autumn. At two week-intervals, subsets of these insects were collected and confined to five different host species to determine the effects of host plants on reproductive development. Lastly, in greenhouse experiments, newly eclosed adults reared in the laboratory were confined on six different hosts for ten days to determine host effects on the reproductive biology of recently matured females. Quantification of reproductive development of the late-summer generation of GWSS collected on a weekly basis indicated that fat body development began slowly, increased substantially after several weeks and continued to increase at a moderate rate into autumn. Ovariole development increased within the first week, and increased again in September. Dissections in late September showed fully developed ovarioles in most insects that contained no eggs. The presence of eggs (ova development) was sporadic throughout August with ca. 25-30% of developing females having some ova development. In September egg production had almost ceased entirely; only 3 on the 40 females dissected had any sign of ova development.

The reproductive status of leafhoppers confined to specific hosts also showed strong effects of host species. This experiment was replicated three times. Initially, forty late instar nymphs were confined on each of five host species and allowed to

develop for two weeks. In the two subsequent experiments, twenty female and five to ten males were sleeved on each of the hosts (four replicates per host), and reproductive status was assessed by dissection after two weeks. For each of the experimental periods, host species significantly impacted fat body development. Ovariole development was significantly affected by host species in mid-August and September. Egg development was sporadic, although egg development for insects feeding on crape myrtle was significantly greater than on other host species in mid-August. Consumption rates and xylem chemistry were also determined on each host. As expected, consumption rates were in general correlated with fat body development (GWSS that ingested more nutrients appeared to store more nutrients). Ovariole development, however, appeared to be time or leafhopper age-dependent. In mid-August, the preferred feeding hosts crape myrtle and *Vitis* supported greater ovariole development, but development on these hosts ceased in September. In contrast, ovariole development on citrus and sycamore appeared slow initially (through mid-August) but development consistently increased and these hosts exhibited the highest ovariole development by September.

Lastly, newly eclosed females reared on soybean in our laboratory that were less than 24 hours old were caged for 10 days on five different host plants in August. Typically, during late June and early July, GWSS reared in our greenhouses on soybean will begin to oviposit 10-14 days after maturation. Results from this experiment showed that reproductive development had slowed immensely by August; no females had any signs of ova development. However, even within this ten day period, development of fat body and ovarioles varied as a function of host species. Females feeding on citrus, crape myrtle and soybean had significantly higher ovariole development than leafhoppers feeding on two common host species of holly, and fat body development on crape myrtle exceeded that on any other host.

CONCLUSIONS

Our data substantiate that fat body and ovariole development are significantly affected by host, but the relationship between development of these organelles merits further investigation. Host selection can have profound effects on reproductive development, even at the earliest stages of adult development. Ovariole development, appeared to be time or leafhopper age-dependent.

FUNDING AGENCIES

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KEYS TO MANAGEMENT OF THE GLASSY-WINGED SHARPSHOOTER: INTERACTIONS BETWEEN HOST PLANTS, MALNUTRITION, AND NATURAL ENEMIES

Project Leaders:
Russell F. Mizell, III
Peter C. Andersen
University of Florida, NFREC-Quincy
Quincy, FL 32351

Reporting Period: The results reported here are from work conducted during 2002-2003.

ABSTRACT
Our previous work has shown that glassy-winged sharpshooter (GWSS) eggs and nymphs face two primary mortality agents: egg parasitism and malnutrition. Female GWSS chose the host plants for oviposition and consequently the habitat that exposes the eggs to parasitism and also the potential host plant nutrition immediately available to the nymphs. We have developed a conceptual model of these behaviors, choices and tradeoffs facing female GWSS and are interested in the behaviors and cues used by GWSS in making these choices. The effects of host plant assemblages and host plant chemistry on the distribution, performance and behavior of GWSS and its natural enemies are keys to understanding the system. Categorizing host species as to their importance to GWSS life stages is necessary to direct any plant manipulations aimed at population suppression or manipulation. Research in this proposal addressed several aspects of these interactions

INTRODUCTION
Our research in North Florida has documented that different host species are utilized by GWSS for different purposes. Within our region, adult GWSS are most conspicuous on adult feeding hosts (i.e. Prunus in early summer, crape myrtle in mid-summer). Confining GWSS on specific hosts has shown that these same hosts may be poor ovipositional hosts or inadequate for the development of immature GWSS. Larger numbers of egg masses are often found on hosts where GWSS adults are only occasionally observed. We have also quantified the nutritional basis for adequacies as adult feeding hosts versus developmental hosts; our understanding of ovipositional preference is much less complete. Conflicting nutritional needs throughout the GWSS life cycle suggest that multiple host usage is required for optimal population growth of GWSS. Quantifying how the life stages of GWSS utilize each host species and elucidating the behaviors and important behavioral cues provides the background data necessary for formulating strategies to disrupt the life cycle of GWSS.

OBJECTIVES
1. Determine the relationship of host plant xylem chemistry on host selection, feeding and ovipositional behavior of GWSS and its parasites.
2. Assess host plant acceptance and subsequent feeding rate, host plant selection and acceptance for oviposition and the survival and performance of early and late instar nymphs as a function of host plant species.
3. Quantify the impact of these plant variables on the behavior and parasitism rate of eggs by *Gonatocerus ashmeadi.*

RESULTS
The survival and movement behavior under field and laboratory conditions of nymphs of GWSS and two other leafhopper species was determined. We investigated the visual acuity of the nymphal stages of three leafhopper species: *Homalodisca coagulata*, *H. insolita*, and *Oncometopia nigricans*. Under laboratory conditions, the nymphs of *H. coagulata* and *O. nigricans* could discriminate spectra and were highly attracted to hues of yellow with safety yellow being the most attractive. The nymphs of *H. insolita* were also attracted to yellow hues, but were more attracted to cream yellow. In the laboratory, maximum jumping distance of third instar *H. coagulata*, *H. insolita*, and *O. nigricans* was 68.0, 49.7, and 39.2 cm respectively, when provided with a target. The fifth instars of *H. coagulata*, *H. insolita*, and *O. nigricans* had a maximum jumping distance of 78.8, 29.2, and 45.5 cm, respectively. GWSS nymphs were able to walk at least 30 m in less than two hours indoors using a tile floor as a substrate,. Additionally, all nymphal stages of *H. coagulata* dispersed up to 10 meters after three days under field conditions when released into an outdoor grass-covered arena. The neonates of *H. coagulata*, *H. insolita*, and *O. nigricans* survived on average, 83.5, 70.5, and 83.0 hours without plant feeding, respectively.
We investigated the effects of soybean genotype on GWSS development, and if nutritional changes of xylem fluid affected developmental success. Three glabrous isolines (D90-9216, D88-5320 and D88-5328) and one pubescent genotype (Hagood) were examined. All three glabrous genotypes proved suitable developmental hosts, yielding sixty to two hundred successfully matured adults in cages originally loaded with 8 mating pairs of leafhoppers. Minor variations in life history characteristics (growth rates, success and time of development) suggest that the genotype D88-5328 was marginally superior as a developmental host. Chemical analyses of xylem fluid showed that this genotype provided higher dietary nitrogen in the form of amides and ureides as leafhoppers approached maturation. The pubescent genotype Hagood was a much less suitable host. Chemical analyses of xylem fluid showed that Hagood was not nutritionally deficient; thus, we conclude that trichomes of pubescent soybean were a deterrent to leafhopper feeding and development.

While all three glabrous isolines consistently produced matured GWSS, developmental times were extended in several instances. Chemical analyses of xylem fluid showed that under conditions of heavy leafhopper feeding, nutrients in xylem fluid began to decline over time which may have further contributed to slow leafhopper development. In these experiments only pre-plant slow release fertilizer was applied. Our results suggest that supplemental nitrogen may be necessary to consistently develop GWSS with minimal developmental periods.

We quantified the relationship between host selection, consumption rates, ovipositional preference and developmental suitability for several of the host species most utilized in California (Navel oranges, Spanish Pink Lemon and Chardonnay grapes). Experiments were repeated with several of the hosts most important in Florida (crape myrtle, oak leaf holly and the native yaupon holly) to determine if utilization patterns were the same for both sets of hosts. No-choice tests were run simultaneously on each host species (4 plants of only one host species per cage as opposed to one plant from each host species combined in each cage) to determine performance on individual hosts. Since GWSS is in a non-reproductive state the majority of the year, tests were run on both non-reproducing (autumn) and producing (summer) cohorts of insects. In the initial experiment of non-reproducing GWSS we also compared the response of field collected GWSS to those reared and maintained on soybean to examine if feeding history impacted insect behavior or performance.

Choice tests were conducted by releasing 12 male and 12 female GWSS into cages containing the three host species and glabrous soybean. Soybean was included for comparative purposes as it is one of the few hosts that we have found that is a consistent developmental host. Host selection was visually assessed 1 to 2 times daily for 10-22 days. At the termination of the experiment, surviving leafhoppers were counted and weighed. All leaves in each cage were examined and eggs were counted. Individual GWSS were then confined on each host plant in feeding assemblies for determination of consumption rates. Xylem fluid was collected from half of the plants of each species for subsequent chemical analyses. Thirty to forty neonates were placed on each of the remaining hosts and checked daily to determine developmental suitability of each host.

In the first choice test (Navel, Spanish Pink Lemon, Chardonnay and Soy), abundances of both reproducing and non-reproducing adults were highest on Navel. Subsequent tests of consumption rates and immature development showed Navel to be a very poor feeding and developmental host. It was, however, the preferred host for oviposition. One of the most surprising results was that GWSS remained on Navel when not reproductively active (experiments run in autumn), even though consumption rates were low. Tests for non-reproducing GWSS were run for over three weeks; those of reproducing GWSS had to be terminated after ten days so that eggs could be quantified. During the latter portion of the three week period of selection, abundances of GWSS eventually began to decline on Navel with very gradual shifts to the superior feeding hosts.

Trends in the second choice test (crape myrtle, yaupon holly, oak-leaf holly and soy were very different. For field collected GWSS in autumn (non-reproducing) crape myrtle and yaupon holly had the highest abundances. For reproducing adults, the vast majority of GWSS remained on crape myrtle, which was also the superior feeding host during this time period. Oviposition and development on crape myrtle were not significantly different than the two holly species.

Two other trends in the data are noteworthy. Soy reared GWSS were significantly different than field collected GWSS in terms of host selection and consumption rates. The data suggest that it is important to be aware of feeding history when assessing GWSS behavior. Secondly, soy was a very poorly selected host (both abundance and oviposition) despite being the superior rearing host in both sets of experiments, and an adequate feeding host. Our previous research has documented that GWSS does very well when confined on glabrous soybean. The inability of GWSS to recognize soybean (and perhaps other legumes) as a suitable host merits further investigation.

Chemical analysis of xylem fluid of all plants used in the experiments has recently been completed. Statistical analyses of how nutrients relate to GWSS utilization are currently being performed. Some trends are already obvious. In our selection experiments, Spanish Pink Lemon was rarely selected by GWSS. Chemical analyses showed that xylem of lemon used in these experiments was extremely deficient in nutrients, containing ca. 30% of the organic nitrogen of Navel. These results contrast xylem fluid of Navel and lemon that we have analyzed from California (in collaboration with Carlos Coviella), in which field grown lemon have organic nitrogen 30-50% higher than Navel and nearly ten-fold higher than our potted plant material. Both the chemical data from the field in California and data from our greenhouse show consistent relationships between xylem nutrients and host selection (higher amino acids resulting in higher selection). The failure of Spanish Pink Lemon to remain vigorous under greenhouse conditions highlights the need to assess GWSS host utilization in conjunction with chemical data and available field data.

CONCLUSIONS

Leafhopper nymphs are capable of surviving without a plant food source for in excess of three days and also are able to move relatively long distances from plant to plant and on the ground. This information is highly relative to the determination of potential female ovipositional strategies. If nymphs are highly mobile and capable of finding suitable host plants on their own, then female choice of host plants for oviposition may be less critical. This in turn may indicate that oviposition is affected by factors other than host plant chemistry relative to the special requirements of nymphs.

The nutritional experiments indicate how we can assess GWSS host utilization, and also highlight some of the biggest remaining questions in understanding the life cycle of GWSS. Results to date are consistent with what we know about how xylem nutrients impact adult consumption rates and the development of immature GWSS. The clearest gap in our knowledge is in our understanding of how GWSS selects hosts for oviposition, and how these same cues may even be impacting non-reproducing GWSS. To address this question we are currently examining and comparing different genotypes of Citrus and other preferred ovipositional hosts relevant to California. We have shown that glabrous genotypes of soybean are suitable developmental hosts, yielding sixty to two hundred successfully matured adults in cages originally loaded with 8 mating pairs of leafhoppers.

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FUNDING AGENCIES

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

Project Leaders:
Russell F. Mizell, III
University of Florida, NFREC-Quincy
Quincy, FL 32351

Drion G. Boucias
Department of Entomology and Nematology
University of Florida
Gainesville Fl.32611-0620

Reporting Period: The results reported here are from work conducted during 2002-2003.

ABSTRACT
To date, the fungi isolated from field-collected mycosed glassy-winged sharpshooter (GWSS) includes a combination of potential pathogens in the genera *Verticillium, Paecilomyces,* and *Hirsutella* and other saprobic fungi (*Fusarium, Apergillus, Acremonium,* etc). We have identified a primary pathogen, *Hirsutella* sp. developed a laboratory culturing method for it and identified a unique metabolite from the organism. We are developing a genetic profile of the pathogen and evaluating it for pathgenicity against several species of leafhoppers. The pathogen appears to be a good candidate for release into California as a classical biological control.

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

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

RESULTS
In the past field season we surveyed GWSS populations in the Southeast. The purpose of this survey was twofold: first, to piece together a better picture of the distribution of the glassy-winged sharpshooter in the area. Secondly, it gave us the opportunity to investigate the varieties and incidence of fungal pathogens associated with this host. The survey area encompassed three states. Relatively intensive sampling was done in Florida and Georgia, from the Orlando area north to Cairo, Georgia, and west to Defuniak Springs, Florida. This was coupled with a collecting trip through much of Georgia and South Carolina. In total, 41 sites were sampled, 34 of which harbored insect populations. After the first few weeks, it became apparent that crape myrtles were the preferred host plant for our sampling purposes. While we did not restrict our search to crape myrtle, it did comprise the bulk of what is included in the aforementioned sites. Crape myrtle is prevalent in both nurseries and landscaping in the South, and was therefore easily accessed. It is also possible to locate the insects through visual means, and to collect them with a gentle beating method that minimizes damage to the plant.

Pathogen Detection and Isolation
As mentioned above, concurrent with the insect populations, we also found a patchwork population of fungal pathogens. Of the 41 collection sites, six yielded fungi. Classical culturing methods met with limited success, although we were able to isolate and grow colonies of *Sporothrix* sp., *Acremonium* sp and *Pseudogibellula* sp. These were the less common fungi that were found. By far, the most prevalent pathogen found on dead GWSS was a *Hirsutella*-like organism. It proved to be extremely fastidious, and could not be grown on any media when taken directly from the mycosed insect samples. We also were not able to isolate it from any of the plant material or affiliated lichens.

We initiated a survey of microbes harbored by live GWSS from areas hosting epizootics of *Hirsutella*. Approximately one third of hemolymph samples examined from these insects contained hyphal bodies. Importantly, samples collected from the hemolymph grew in insect tissue culture. Parallel inoculations of these hemolymph samples onto various mycological substrates failed to produce colonies. These results demonstrate the fastidious nature of this pathogen. Significantly, all

cultures produced conidiophores that were identical to the *Hirsutella* species identified from cadavers collected over the past two seasons. It appears that the causal agent of the epizootics in the Southeast is due to this fastidious fungus. Five independent isolates of this pathogen are being maintained on tissue culture media.

Bioassay and Transmission of Isolated Fungi

Attempts to transmit or observe the disease life cycle in the laboratory have proven problematic. Spores harvested from mycosed insects and/or available cultures were applied topically to second instar nymphs from the greenhouse colonies with no effect. It should be noted that until recently we did not have the available *Hirsutella* cultures for bioassay. It should also be noted that this season hundreds of live insects were collected from an area with heavy pathogen populations and were monitored in the greenhouse over a seven-day period. None of these sharpshooters died of mycosis. In all of the sampling that we have done, covering much of the southeastern U.S. and thousands of individual host plants, the vast majority of mycosed insects were affiliated with crape myrtle. As mentioned above, the majority of our sampling focused on crape myrtle, but we did include other plants, often adjacent to infested stands of crape myrtle. Mycosed GWSS have been observed on a broad range of host plants and the number found on crape myrtle is indicative of the high numbers of GWSS that use crape myrtle for feeding.

CONCLUSIONS

We have identified and have in culture several isolates of a primary pathogen and potential GWSS biological control agent, *Hirsutella* sp. In addition we have identified an important metabolite of the fungus. Other potential primary pathogens exist and we are also working with them.

FUNDING AGENCIES

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

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

Project Leaders:
Russell F. Mizell, III
University of Florida, NFREC-Quincy
Quincy, FL 32351

Peter C. Andersen
University of Florida, NFREC-Quincy
Quincy, FL 32351

Pascal "Eve" Oevering
UC Cooperative Extension
Ventura, CA 93003

Phil Phillips
UC Cooperative Extension
Ventura, CA 93003

Cooperators:
Serguei Triapitsyn
Carlos Coviella
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted during 2003.

ABSTRACT
To fully support decision making and to supplement what is observed in the field, we need to develop a glassy-winged sharpshooter (GWSS) performance database on the host plant species that are identified as truly critical to GWSS survival (particularly for immature GWSS). There are no quantitative data available on the relative suitability of single or multiple hosts most relevant in California to GWSS growth and development. This proposal will not only provide this baseline information, but also identify host plant limitations at different life stages and identify key nutrients responsible for this phenomenon.

INTRODUCTION
The project aims to predict ecological events (including possible establishment of GWSS throughout much of the grape growing regions of CA) and to target control tactics (including potential manipulations of host plants and leafhoppers) for different regions of California. Understanding the seasonal occurrence of ecological events as they are affected by climatic variables is critical for management decision making. Also, there is tremendous utility in understanding the underlying mechanisms responsible for GWSS/Pierce's disease scenarios from both predictive and control perspectives.

OBJECTIVES
Determine the relative phenology (timing of biological events as influenced by the environment and intrinsic biological phenomena) of:

1. Host plants used by the glassy-winged sharpshooter;
2. Other leafhopper vectors and
3. Their natural enemies, as well as
4. *Xylella fastidiosa* in host plants and vectors in key California locations at three different latitudes: South Coast (Ventura), Central Valley (Kern County), and North (between Visalia and Sacramento).

RESULTS AND CONCLUSIONS
Personnel have been hired in California and they are currently setting up the experimental plots.

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

Section 4B: Chemical and Cultural Control

CONTROL OF IMMATURE AND ADULT GLASSY-WINGED SHARPSHOOTERS: EVALUATION OF BIORATIONAL INSECTICIDES FOR ORGANIC USE

Project Leaders:

David H. Akey
USDA-ARS-PWA-WCRL
Phoenix, AZ

Matthew J. Blua
Dept. of Entomology
University of California
Riverside, CA

Thomas J. Henneberry
USDA-ARS-WCRL
Phoenix, AZ

Cooperators:

Ed Civerolo
USDA-ARS-PWA-SJVASC
Parlier, CA

Nick C. Toscano
Dept. of Entomology
University of California
Riverside, CA

Reporting Period: The results reported here are from work conducted from September through October 2002, and June through September 2003.

ABSTRACT

We conducted a pyrethrum synergism study for organic control of GWSS that evaluated rosemary oil, or cinnamon oil (as cinnamic aldehyde) as piperonyl butoxide PIP (synergist) substitutes. Pyrethrum, alone or combined with PIP, was not consistent in efficacy against GWSS. Pyrethrum, and pyrethrum with PIP, gave effective GWSS control in a June 2003 trial but not in two September trials (2002, 2003). Efficacy of PIP alone was not significant from controls in any of the trials. No significant synergism occurred between pyrethrum and PIP. Neither Hexacide® nor Cinnacure® alone controlled GWSS in this study; nor did they synergize pyrethrum.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) is a newly introduced, now common, pest insect in southern and central California (Purcell et al., 1999; Blua et al. 2000). There, GWSS is a primary vector of *Xylella fastidiosa*, the causal agent of Pierce's disease (PD). PD is an important factor in grape production (wine and table) and nursery stock in California with the occurrence of serious grape and vine losses; and added inspection costs to the nursery and citrus industries (Blua et al. 1999). Earlier, we reported studies that identified effective conventional and biorational insecticides for control of GWSS in grapes and citrus (Akey et al., 2001a,b; 2002). Also, management methods are being developed for GWSS that are economically, ecologically, and socially acceptable (Akey et al, 2002; Blua, et al. 2000). But a concern is the limited number of resources available to organic growers for control of GWSS. This concern should not be restricted only to organic producers. Organic production may be (or perceived to be) "nurseries" for GWSS. Organically-approved pyrethrum insecticide is one agent that may be used. In conventional use, usually, this insecticide is combined with the synthetic synergist, piperonyl butoxide (PIP, aka PBO). PIP acts as a synergist by inhibiting the detoxifying p-450 cytochrome/mixed function oxidases and that allows the insecticide a longer presence in cell tissue. Here, we report a pyrethrum study for organic control of GWSS that tested rosemary oil (an octopamine inhibitor), or cinnamon oil (as cinnamic aldehyde) as PIP substitutes.

OBJECTIVES

Discover organically-approvable agents that can be substituted effectively for piperonyl butoxide (PIP).

RESULTS AND CONCLUSIONS

Experiments were conducted with naturally occurring GWSS populations on 6-7 foot tall orange trees during egg-to- nymph-to-adult development September through October, 2002, and June through September, 2003. The experimental design was a 3-replicate completely randomized block (four treatments including control, table 1) at University of California, Riverside, Agricultural Operations. Plots were 0.114 acre in size; 25 by 66 ft, 3 trees / plot with guard rows on each side. Pretreatment count of GWSS was made in all plots. Post-application counts were made 1-7 days (varied), (table 2, 3, and 4). Applications were made by windmill blast-type sprayer (John Bean Div., FMC) that delivered 200 psi at 300 gal/ac with 5 swivel-nozzle bodies (Tee Jet) on one side (10 nozzles, each had a core 23, disc 6, and slotted strainer). An "organically-certifiable" adjuvant, Nu-Film®-17 (Miller Chemical & Fertilizer Corp.) was used. Spray penetration was studied previously (Akey et al. 2001a, b). LSD mean separation tests were made if F values were significant by ANOVA. For analysis, data were transformed by $\sqrt{(x + ½)}$ to adjust zeros in data sets. Efficacies of materials evaluated for control are shown in tables 2, 3, and 4.

Pyrethrum, alone and combined with PIP, was not consistent in efficacy against GWSS. The pyrethrum (with PIP) trial in June of 2003 had suitable efficacy but not in the two September trials (2002, 2003) (see tables 2 and 4). Efficacy of PIP alone was not significant from controls in any of the trials (tables 2-4). No significant synergism occurred with pyrethrum and PIP (tables 2-4). Neither Hexacide® nor Cinnacure® alone controlled GWSS in this study; nor did they synergize pyrethrum combined in sprays (table 2). This area of study will require more work to determine under what conditions

pyrethrum may be effective (if any). Candidate insecticides for organic GWSS IPM programs need to be identified and evaluated. It is likely that such insecticides may be less effective than conventional insecticides but may have a place in organic IPM programs adjacent to or embedded within conventional area-wide programs.

Table 1. Trade names, chemistry classes, formulations and rates per acre of foliar biorational insecticides evaluated for glassy-winged sharpshooter control in citrus, Riverside, CA, 2002 and 2003.

	Name		Chemistry		Per Acre		
Year	**Trade**	**Generic**	**Class**	**Formulation**	**Product**	**lb AI**	**Company**
2002,03	Pyganic®	pyrethrins[1]	Cyclo-propane compound	EC 1.4	64.0 fl oz	0.06	McLaughlin, G.K. Com.
2002,	Pyronyl®62	pyrethrins	as above	EC 6	16.0 fl 0z	0.06	Prentiss, Inc.
2002,03	Incite C/A	Piperonyl butoxide PIP	Methylene dioxyphenyl	91.3	8.8 fl oz	0.53	Loveland Industries, Inc.
2003	Hexacide®	Rosemary oil	octopamine inhibitor	5	3.0 gal	0.42	Eco Smart Tech. Ind.
2003	Cinnacure®	Cinnamon oil	Cinnamic aldehyde	30 F	3.0 gal	7.56	ProGuard, Inc.

[1] Pyrethrum is a naturally-occurring group of insecticides from chrysanthemum flowers. Pyrethrum has six active insecticides; two are pyrethrins from which similar mode-of-action synthetics (pyrethroids) were developed.
[2] Pyronyl® 6% without PIP was prepared by Prentiss Inc.; commercial product is Prentox® Pyronyl® 60-6 EC

Table 2. Mean numbers (± SE) and percentage mortalities (beat sample data) following one application of chemical(s) for adult glassy-winged sharpshooter control in citrus at Riverside, CA, 2003.

Treatment	**Post application day**					
	1		**2**		**5 or 61**	
	$\bar{x}$ **2**	**%3**	$\bar{x}$	**%**	$\bar{x}$	**%**
Pyganic	1.7 ± 0.9	81 b[4]	2.0 ± 1.5	54 a	3.0 ± 1.0	85 b
Incite	8.0 ± 1.5	8 a	9.7 ± 0.9	---- a[5]	12.7 ± 3.3	37 a
Pyganic + Incite	1.0 ± 0.6	88 b	4.3 ± 4.3	---- a	4.7 ± 1.2	77 b
Control	8.7 ± 2.4	---- a	4.3 ± 2.4	---- a	20.0 ± 4.6	---- a
Pyganic	13.3 ± 13.3	----- a	9.3 ± 6.3	---- a	4.7 ± 1.2	---- a
Hexacide	20.0 ± 3.6	------a	9.3 ± 2.3	---- a	5.0 ± 1.5	---- a
Pyganic + Hexacide	6.0 ± 5.5	38 a	11.3 ± 6.9	---- a	3.3 ± 0.7	9 a
Control	9.7 ± 5.4	--- a	7.3 ± 3.2	---- a	3.7 ± 1.8	--- a
Pyganic	2.0 ± 1.0	74 a	6.3 ± 0.7	24a	5.0 ± 1.0	29 b
Cinnacure	5.3 ± 1.2	31 a	8.3 ± 1.8	---- a	10.3 ± 0.9	a
Pyganic + Cinnacure	3.0 ± 2.1	61 a	5.3 ± 3.0	36a	6.0 ± 1.0	14 b
Control	7.7 ± 0.9	---- a	8.3 ± 2.2	---- a	7.0 ± 0.6	---- ab

[1] Post-application sample day: Pyganic/Hexacide and Pyganic/Cinnacure – day 5; Pyganic/Incite – day 6.
[2] Means of 3 replicates of each treatment.
[3] % efficacy = percent reduction from control.
[4] Means in columns by group(s) with different letters, are significantly different by ANOVA and LSD at $P \leq 0.05$, analyses were based on transformed data, $\sqrt{(x + ½)}$ to adjust zeros in data sets.
[5] Numbers equal to or greater than control, i.e. no % reductions from control.

Table 3. Mean numbers (± se) and percentage mortalities (beat sample data) following one application of chemical(s) for glassy-winged sharpshooter nymph control in citrus at Riverside, CA, 2003.

Treatments	Post application day					
	1		2		5 or 6[1]	
Small nymphs[2]	$\bar{x}$ [3]	%[4]	$\bar{x}$	%	$\bar{x}$	%
Pyganic	2.0 ± 1.0	75 a[5]	2.3 ± 0.9	63 a	1.3 ± 0.9	84 a
Incite	2.7 ± 1.8	67 a	3.7 ± 1.5	42 a	3.3 ± 2.4	60 a
Pyganic + Incite	3.0± 0.6	63 a	1.7 ± 1.2	74 a	1.3 ± 0.3	84 a
Control	8.0 ± 3.2	---- a	6.3 ± 3.2	--- a	8.3 ± 3.8	---- a
Large nymphs						
Pyganic	0.7 ± 0.3	85 a	0.3 ± 0.3	80 a	0.0 ± 0.0	100 a
Incite	2.7 ± 0.9	38 a	1.0 ± 0.6	40 a	0.7 ± 0.7	33 a
Pyganic + Incite	2.3 ± 0.3	46 a	2.3 ± 1.3	--- a[6]	0.0 ± 0.0	100 a
Control	4.3 ± 2.4	---- a	1.7 ± 0.3	---a	1.0 ± 0.6	---- a

[1] Post-application sample day: Pyganic/Hexacide and Pyganic/Cinnacure – day 5; Pyganic/Incite – day 6.
[2] Nymphs were grouped by instars: small, instars 1 – 3; and large, instars 4 – 5.
[3] Means of 3 replicates of each treatment.
[4] % efficacy = percent reduction from control.
[5] Means in columns by group(s) with different letters, are significantly different by ANOVA and LSD at $P \leq 0.05$; there was no significance, analyses were based on transformed data, $\sqrt{(x + ½)}$ to adjust zeros in data sets.
[6] Numbers equal to or greater than control, i.e. no % reductions from control.

Table 4. Mean numbers (± se) and percentage mortalities (beat sample data) following one application of pyrethrum and/or piperonyl butoxide for adult glassy-winged sharpshooter control in citrus at Riverside, CA, 2002-2003.

Treatments	Trial			Post application day 6 or 7[1]	
	no.,	month	year	$\bar{x}$ [2]	%[3]
Pyronyl®6	1	Sept.	2002	15.7 ± 4.2	24 a[4]
Incite				16.0 ± 1.5	23 a
Pyronyl®6+ Incite				14.7± 2.4	29 a
Control				20.7 ±4.9	--- a
Pyganic	2	June	2003	3.0 ± 1.0	85 a
Incite				12.7 ± 3.3	37 a
Pyganic + Incite				4.7± 1.2	77 a
Control				20.0± 4.6	--- a
Pyganic	3	Sept.	2003	7.7 ± 2.2	36 a
Incite				6.0 ± 1.0	50 a
Pyganic + Incite				8.0 ± 3.1	33 a
Control				12.0 ± 2.5	--- a

1 Post-application sample day: June and Sept. 2003 –day 6; Sept. 2002 –day 7.
2 Means of 3 replicates of each treatment.
3 % efficacy = percent reduction from control.
4 Means in columns by group(s) with different letters, are significantly different by ANOVA and LSD at $P \leq 0.05$; there was no significance, analyses were based on transformed data, $\sqrt{(x + ½)}$ to adjust zeros in data sets.

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FUNDING AGENCIES

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IMPACT OF A SCREEN BARRIER ON THE INTROGRESSION OF THE GLASSY-WINGED SHARPSHOOTER INTO A NURSERY YARD

Project Leaders:

Matthew J. Blua
Department of Entomology
University of California
Riverside, CA 92521

Richard Redak
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:

Craig Hanes
Cal. Dept Food and Agriculture
Bell, CA 90210

David Morgan
Cal. Dept Food and Agriculture
Riverside, CA 92501

Brad Bowers & Robert Crudup
Valley Crest Tree Co.
Fillmore, CA 93015

Reporting Period: The results reported here are from work conducted from December 2002 to September 2003.

ABSTRACT

We are seeking unique tactics to curtail the movement of *Homalodisca coagulata*, the glassy-winged sharpshooter (GWSS), on nursery stock to non-infested areas of California. On the basis of our prior experiment that examined the height of GWSS flight out of citrus, we tested the potential of 5 m high screen barriers to reduce the influx of GWSS into a nursery yard from surrounding citrus and native vegetation. We also examined GWSS flight direction and behavior when placed near or on a barrier. We found two distinct periods of GWSS incursion into the nursery yard: the first of the year and in mid-summer. In sticky traps set a 1, 3, 5, and 7 m high, more than 99% of all GWSS were caught at 5 m or less. Although more GWSS were caught immediately inside areas where 5 m high barriers were constructed than areas without barriers, fewer GWSS were caught inside barriers than outside barriers. When released midway between the barrier and adjacent vegetation fewer GWSS flew in the direction of the barrier than away from the barrier.

INTRODUCTION

Strict regulations have been imposed on the nursery industry to curtail movement of *Homalodisca coagulata*, the glassy-winged sharpshooter (GWSS) via nursery stock transported to non-infested counties. These regulations require that potted plants destine for transport be thoroughly inspected for any stage of the GWSS, and treated with an insecticide if warranted. GWSS egg masses are particularly difficult to find and treat with insecticides. Inspections and treatments are labor-intensive and time consuming, resulting in additional costs to growers and county agricultural commissioners' offices. Some growers in GWSS infested areas have stopped shipping product to non-infested areas rather than risking a shipment of nursery stock being rejected after transit. This reveals the need for a means to reduce the GWSS population density in nursery yards.

The best GWSS management strategy we can bring to bear on this problem involves integrating two or more management tactics. The circumstances surrounding wholesale nurseries lends itself well to a management strategy that combines isolation from GWSS infestation, and the judicious use of insecticides to induce mortality and curtail oviposition.
Our experiments investigating GWSS flight height indicate a possibility that screen barriers could be constructed to reduce movement of GWSS from vegetation that supports reproduction and high population densities, into crop plants that require protection. In a prior experiment in which we monitored GWSS entering a vineyard from citrus or native vegetation at 1, 3, 5, and 7 m above the ground, we found that through the period of peak flight activity, 97% of all *H. coagulata* adults were trapped at an altitude of 5 m or lower (Blua and Morgan 2003).

OBJECTIVES

The primary objective of this study is to determine the impact of screen barriers on GWSS movement into a nursery yard, and their impact on GWSS flight behavior and movement.

RESULTS AND CONCLUSIONS

GWSS trap catches

Since our experiment was initiated on December 12 2002, we have examined weekly catches of GWSS on yellow sticky traps set at 1, 3, 5, and 7m above the ground. These traps were set inside and outside the border of a nursery yard where we placed 5 m high barriers, and at untreated control areas in a blocked experiment with three blocks at Valley Crest Tree Company (VCTC) in Fillmore, CA. Below we report GWSS catches from December 12 2002 to September 25 2003. Data are analyzed as weekly GWSS catches, and catches by treatment (barrier vs. no barrier) and trap height (1, 3, 5, and 7 m). All statistical analyses were carried out using chi-square after appropriate transformation of data.

We detected significant differences in the numbers of GWSS caught through time ($\chi^2 = 2399$, df=41, $P<10^{-4}$). Catches peaked in January and generally declined afterwards with another peak begging in July 2003, declining in August (Figure 1).

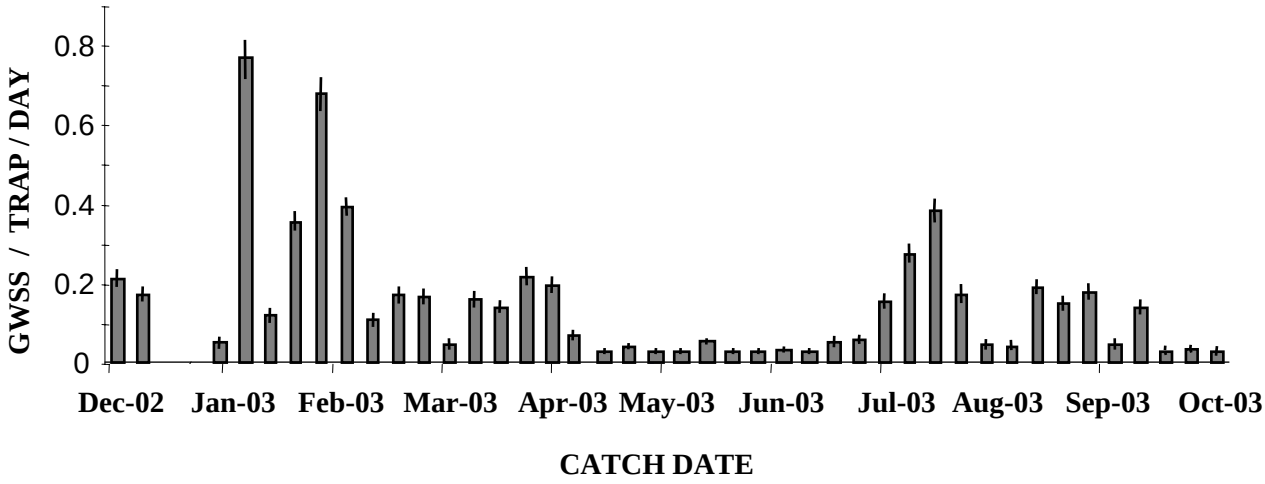

Figure 1. Glassy-winged sharpshooters trapped from December 12 2002 through August 25 2003 at Valley Crest Tree Co. Numbers represent Least Square Means $\pm$ SE across treatments and trap height.

We detected significant differences in the numbers of GWSS caught among trap heights (χ^2 =1643, df=3, $P<10^{-4}$). Over 99% of trapped GWSS were collected at 5 m (barrier height) or less, with the single greatest number caught at 3 m (45%), then 1 m (38%) and 5 m (17%) (Figure 2). Less than 1% of the GWSS were caught on traps set at 7 m above the ground. We caught significantly fewer GWSS inside vs. outside of the barrier treatment (χ^2 = 446.2, df=9, $P<10^{-4}$), even those the fewest GWSS were caught on sticky traps where there were no barriers (Figure 3).

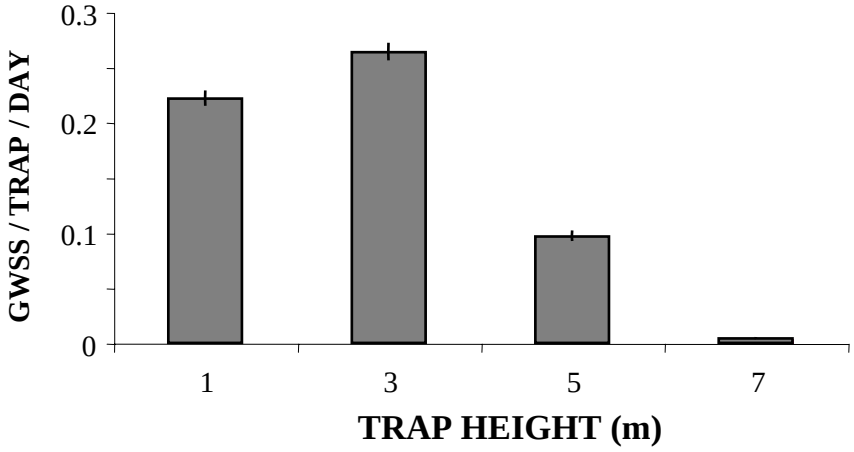

Figure 2. Glassy-winged sharpshooters trapped by height from December 12 2002 through May 29 2003 at Valley Crest Tree Co. Numbers represent Least Square Means + SE across treatment collection date.

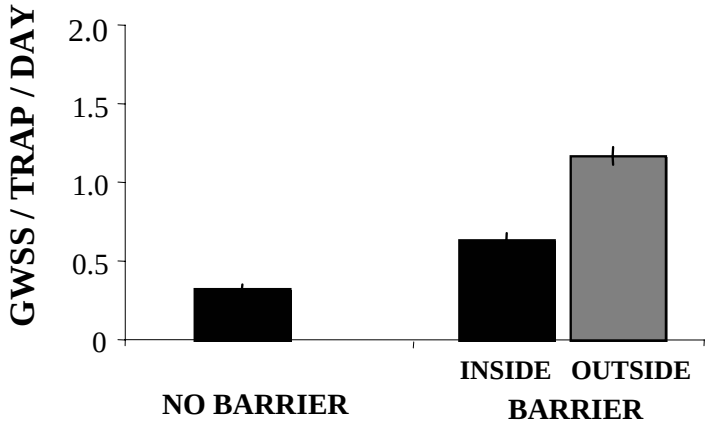

Figure 3. Glassy-winged sharpshooters trapped from December 12 2002 through May 29 2003 at Valley Crest Tree Co on untreated control areas (NO BARRIER) and on the inside and outside of the barrier treatment. Numbers are Least Square Means ± SE across trap height and collection date.

Impact of Barrier on GWSS Flight Behavior

We conducted an experiment to assess the impact of the barrier on GWSS flight direction. In this experiment, GWSS adults were placed on a 1.5 m high platform midway between the barrier and vegetation surrounding the outside of the barrier (citrus or native riparian vegetation), ca 8 m in each direction. Flight direction relative to a clock was noted with 12:00 directly toward the barrier and 6:00 directly away from the barrier. The number of experimental GWSS that flew over the barrier was also noted. We also conducted an experiment to determine the likelyhood that a GWSS making contact with the barrier will either walk or fly over it. In this experiment GWSS were placed on the barrier 1 m above the ground, and we observed the direction of it movement, and if they went over the barrier.

Significantly more GWSS flew away from the barrier (70.5%) than towards it (29.5%) (Figure 4). Only 7.5% of the GWSS released from the platform flew over the barrier (Figure 5), which was 23% of those that flew towards the barrier. GWSS placed on the barrier walked an average of 0.1 m upwards before flying. Ninety four percent of the GWSS placed on the barrier flew away from it, while 6% of GWSS placed on the barrier flew over it.

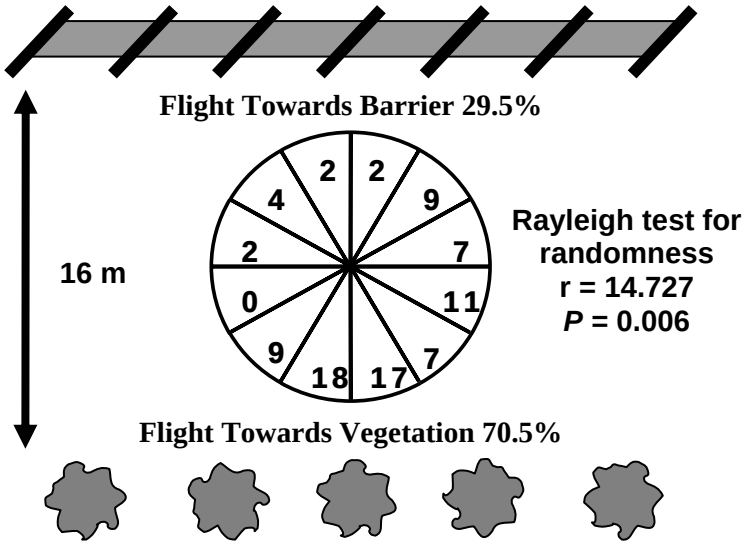

Figure 4. Experimental layout and numbers of glassy-winged sharpshooters observed flying from a 1.5 m high platform in directions relative to a clock. Equal numbers of GWSS were placed on the platform headed towards the barrier and towards vegetation.

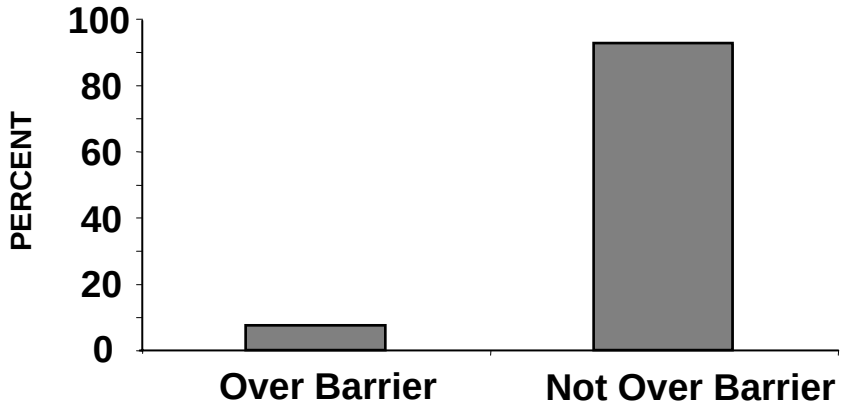

Figure 5. Percentage of GWSS released from a platform (see Figure 5) that flew over the barrier or not.

CONCLUSIONS

The most important effect we observed in this study is the difference in GWSS trap catches on either side of the barrier. This indicates that the barrier is functioning as a means of reducing the influx of GWSS. Other observations support this conclusion. First, we observe over 99% of all GWSS were caught on sticky traps at 5 m or lower, the barrier height, with the largest proportion getting caught at 3 m traps (45%). Second, we observe that when making contact with the barrier, GWSS rarely flew over the barrier, and never walked up more than a few cm. Finally, our studies show that GWSS were either not stimulated to fly in the direction of the barrier, or were repelled by it.

An examination of sticky-trap catches of GWSS show that more were caught on traps at either the outside or inside of the barriers than on traps at the untreated control areas (Figure 3). This is unexpected, but may be an experimental artifact due to placement of barriers in areas of greatest GWSS infestation, or the barrier restraining GWSS movement, and concentrating them in the area of the barrier traps instead of allowing them to fly away from traps. In addition, high rates and distances of GWSS dispersion relative to the length of the barrier and the distance across the nursery yard may further reduce the impact of the barrier on GWSS trap catches in this experiment.

The barrier tactic at ca. $18.00 per linear foot (labor and materials) has a large initial cost relative to other management tactics to reduce the influx of GWSS into a nursery yard. However, the screen material we used (Vineyards NZ LTD, New Zeland) has a 10-year warranty against damage due to the absorption of ultra violet light, and the structure to support the barrier is permanent. In addition, this GWSS management tactic is completely compatible with other tactics, including biocontrol and the use of insecticides.

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FUNDING AGENCIES

Funding for this project was provided by the California Department of Food and Agriculture.

CHARACTERIZATION OF PLANT METABOLITES OF IMIDACLOPRID IN CITRUS TREES AND GRAPEVINES, AND EVALUATION OF THEIR EFFICACY AGAINST THE GLASSY-WINGED SHARPSHOOTER, *HOMALODISCA COAGULATA*

Project Leaders:

Frank J. Byrne
Department of Entomology
University of California
Riverside, CA 92521

Nick C. Toscano
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here for work conducted from July 2003 through October 2003.

ABSTRACT

The toxicities of important plant metabolites of the neonicotinoid insecticide imidacloprid were assessed against the glassy-winged sharpshooter in topical application bioassays. The olefin and 5-hydroxy metabolites were only slightly less toxic than the parent material, whereas the diol and desnitro derivatives exhibited no toxicity. The formation of active imidacloprid metabolites within plants could potentially extend the protective period of imidacloprid applications if the metabolites are formed at the insect feeding sites.

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 *Xylella fastidiosa*-related diseases.

One of the important features of imidacloprid is the toxicological profile of some of its key plant metabolites. Some of these metabolites are closely related to imidacloprid and have been shown to have insecticidal activity against aphids and whiteflies. The most potent of these arises from the oxidative conversion of the parent compound to mono-hydroxy and olefin derivatives (Nauen et al., 1998, 1999). In some cases, the activity of the metabolites exceeds that of the parent compound.

The aims of this study are to determine the extent to which metabolites of imidacloprid 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 imidacloprid to yield equally or more toxic metabolites may also account for the stability of imidacloprid to resistance. From a global standpoint, imidacloprid is used to control a wide range of agricultural and veterinary pests. Despite its widespread use, the compound has remained relatively resilient to the pressures of resistance, a feature rarely seen with the more conventional insecticides when used in a similar manner. Certainly, the success of imidacloprid treatments against the GWSS owes much to its excellent systemic and residual properties as well as to its inherent toxicity; however, it is unknown whether imidacloprid metabolites contribute to this success or not.

OBJECTIVES

1. Determine the metabolic fate of imidacloprid within citrus trees and grapevines;
2. Determine the relative toxicities of imidacloprid and its metabolites to the GWSS.

RESULTS AND CONCLUSIONS

The toxicity of four imidacloprid metabolites to GWSS adults was assessed in topical application bioassays (Table 1). The diol and desnitro derivatives of imidacloprid did not induce a toxic response at the highest dose tested. In contrast, the olefin and 5-OH metabolites were highly toxic, although neither was more toxic than imidacloprid. The olefin had a 2-fold lower toxicity than imidacloprid, while the 5-OH metabolite retained toxicity at a level that was 5-fold lower than the parent compound.

These data concur with those of Nauen *et al*. (1998, 1999), who showed that the olefin and 5-OH metabolites were toxic to both aphids and whiteflies. In the aphid study, the toxicity of the metabolites was confirmed by both oral ingestion and topical bioassays, while in our study we utilized a topical application bioassay only (Figure 1). It is not surprising that all compounds, including imidacloprid itself, were more toxic when delivered via the oral route, as this eliminates any pharmacokinetic effects that might occur with a contact bioassay.

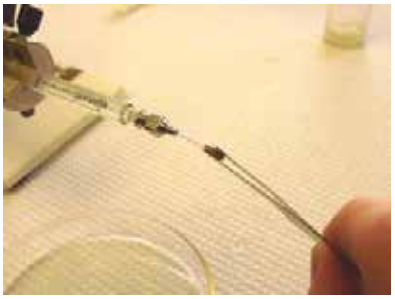

Figure 1. Topical application of imidacloprid to the abdomens of GWSS adults.

Table 1. Toxicity of imidacloprid and its metabolites to the GWSS in topical application bioassays.

Compound	LD50 (ng a.i. per insect)	95% FL	Slope	No. of insects
Imidacloprid	3	1-8	1.0 ±0.2	250
Imidacloprid Olefin	6	3-10	1.4 ±0.3	200
Imidacloprid 5-OH	15	10-21	1.5 ±0.3	200
Imidacloprid Diol	No mortality detected at highest dose tested (100ng)	-	-	200
Imidacloprid DesNitro	No mortality detected at highest dose tested (100ng)	-		200

In this study, confirmation that imidacloprid metabolites are toxic to GWSS by contact assay probably indicates that these materials would be even more toxic if encountered by the insects while feeding on the xylem sap. We are currently evaluating the stability of imidacloprid within xylem sap, and determining the potential for the development of metabolites. It is not known whether there is any metabolism of this insecticide within the xylem system. Should imidacloprid metabolites be found, particularly the olefin or mono-hydroxy derivatives, it will indicate one of the important factors contributing to the overall success of imidacloprid applications against the GWSS.

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FUNDING AGENCIES

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DEVELOPMENT OF GLASSY-WINGED SHARPSHOOTER MIMETIC INSECTICIDAL PEPTIDES, AND AN ENDOPHYTIC BACTERIAL SYSTEM FOR THEIR DELIVERY TO MATURE GRAPE

Project Leader:
Brian A. Federici
Department of Entomology
University of California
Riverside, CA 92521

Cooperator:
Donald A. Cooksey
Department of Plant Pathology
University of California
Riverside, CA 92521

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

ABSTRACT
Transgenic insecticidal crops have been developed and deployed to control major chewing insect pests such as caterpillars and beetles, but not for economically important xylem-feeding sucking insect pests like the glassy-winged sharpshooter, *Homalodisca coagulata* (GWSS). Our goal is to develop novel, highly specific insecticidal proteins derived from the variable binding domains of immunoglobulin molecules. These mimetic peptides are being targeted to bind to the exposed active domains of transport proteins on the surface of the midgut microvillar membrane, enzymes found in GWSS saliva, and proteins associated with *Xylella fastidiosa* acquisition and transmission. Identifying target GWSS proteins is being accomplished through a genomics approach utilizing PCR amplification and cDNA microarray screening to identify, clone, and characterize the GWSS genes encoding these proteins. Targeting these proteins with antibody based mimetic peptides offers a potentially powerful means for controlling Pierce's disease that is environmentally friendly and could reduce crop loss due to GWSS feeding and transmission of *X. fastidiosa*.

INTRODUCTION
Very little is known of the gene expression changes that occur in insects during acquisition and transmission of the pathogens they vector. However, it is unlikely that insect vectors act simply as passive syringes in the process of inoculating their host plants. It has been shown that the acquisition and transmission of some plant pathogens afford distinct advantages to their vectors. Virus acquisition by immature aphids has a profound effect on their viability by affording aphids protection from parasitism. This protection is due to a virus induced alteration of the carbohydrate balance of the host plant that impacts the feeding aphid, making it less attractive as a parasite host (Christiansen-Weniger et al., 1998). Essentially nothing is known of the changes in gene expression that occur in GWSS after acquiring *X. fastidiosa*. We propose to characterize glassy-winged sharpshooter genes associated with *X. fastidiosa* acquisition and transmission. This will be accomplished by screening a GWSS normalized cDNA microarray with probes derived from RNA isolated from *X. fastidiosa* acquiring and transmitting insects. Once identified the gene products will be synthesized and used as antigens for antibody production. Antibody libraries will be screened to identify the mimetic peptides that bind most efficiently to GWSS proteins. These peptides will be used in feeding studies to identify those which block *X. fastidiosa* acquisition and transmission. Thus far, mimetic technology has been used most extensively and successfully in medicine (Clemens, 1996). Examples of medical uses include 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 against disease constituents (Moe et al., 1999). In these applications mimetic technology has been used to treat human cancers (Monzayi-Karbassi and Keiber-Emmons, 2001), diabetes (Deghenghi, 1998), and heart disease (Lincoff et al., 2000). While application of this technology will be new to agriculture, antibody proteins have already been synthesized in plants for the purpose of producing antibodies to be used in medical applications (Larrick et al. 2001; Stoger et al., 2002). Producing transformed lines of crop plants in which the antibody peptides can be synthesized in a tissue-specific manner is also straightforward, since promoters, which will direct expression to the cell wall and vascular structures of plants, have been isolated by other researchers (Shi et al., 1994; Springer, 2000). However, this is of little use in the application of this technology to existing vineyards. While replacement of producing vines by transgenic plants may represent a long-term solution to the problems presented by GWSS and Pierce's disease, it does nothing to relieve the immediate impact of this pathogen-vector system on an important industry in California. Clearly, there is need for the development of alternative methods for introducing such materials to mature plants. One such system would utilize the bacteria, which live as endophytes inside grape xylem. Most endophytic plant bacteria have not been studied extensively, and yet, due to the ease of bacterial transformation, and plant inoculation, these organisms represent ideal candidates for such applications. We are developing such an endophytic bacterial system for grape.

OBJECTIVES

1. Screen a glassy-winged sharpshooter normalized cDNA microarray for genes encoding proteins related to *Xylella fastidiosa*-acquisition and transmission.
2. Identify functional domain peptides using bioinformatics, synthesize them, and utilize them for antibody production.
3. Clone single-chain fragment variable (ScFv) antibody genes into recombinant phage libraries and screen the libraries.
4. Conduct feeding studies to identify mimetic peptides effective in killing or deterring GWSS feeding.
5. Develop an endophytic bacterial system for insecticidal peptide delivery to mature grape xylem.

RESULTS

We are currently funded by the Exotic Pests and Diseases Research Program to construct and screen a GWSS normalized cDNA microarray. The targets of these screening experiments are genes encoding proteins specific for the salivary gland and midgut epithelium. We think these are good candidates for development of mimetic insecticidal peptides. However, although the peptides developed to block the activities of the salivary and midgut proteins should impact the GWSS's ability to transmit *Xylella*, it will be advantageous to identify and target proteins that are specifically involved in the GWSS-*Xylella* interaction. Thus far we have isolated portions of the five genes described in Table 1 from GWSS using degenerate PCR. An alignment of the protein sequence predicted from the V-ATPaseA clone with the protein sequences of V-ATPase A subunits from *Manduca sexta* and *Aedes aegypti* is illustrated below (Figure 1) showing the conserved membrane spanning regions and the extracellular exposed loop (Gamba *et al.*, 1998). Depending on *in situ* hybridization study results, we will use this region for the synthesis of our first mimetic peptide

Table 1. Examples of genes identified and partially cloned from GWSS along with the functions of the proteins they encode

Gene	Function
V-ATPase A subunit	pH balance
V-ATPase c subunit	pH balance
Mal1 (maltase-like)	sugar degradation
LhP1 (trypsin-like)	protein degradation
HCMT1	membrane transport

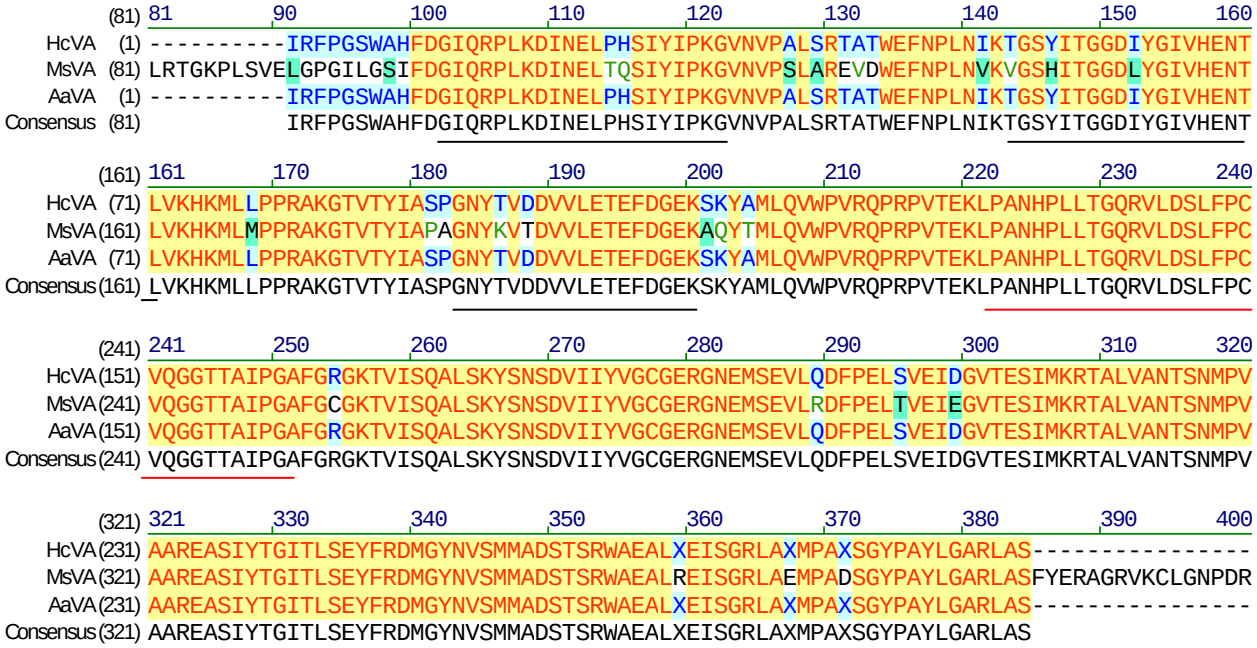


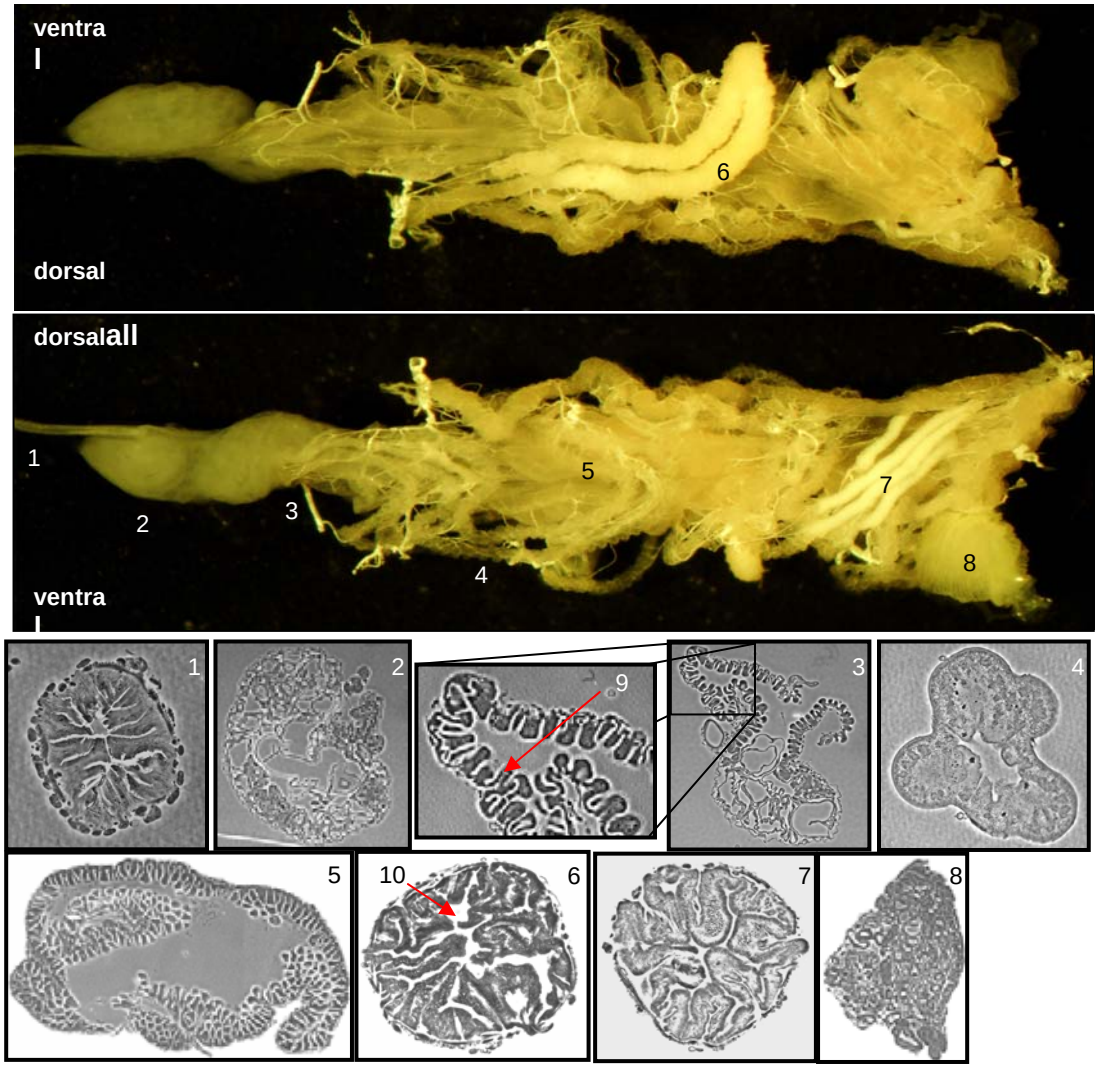
Figure 1. VA = ATPase A, Hc = *Homalodisca coagulata*, Ms = *Manduca sexta*, Aa = *Aedes aegypti*
Membrane-spanning regions ——— Conserved exposed loop ———

In addition we have constructed a normalized cDNA library from total RNA isolated from whole GWSS of both sexes, all life stages, and GWSS that had been allowed to feed on grape infected with *X. fastidiosa*. We are currently assembling a microarray of 10,000 cDNA clones, and dissecting gut, salivary gland, and mouthpart tissues from both clean and *X. fastidiosa* transmitting GWSS for RNA isolation and probe synthesis. We are developing *in situ* hybridization techniques for localization of gene expression studies and have dissected and identified all of the components of the GWSS alimentary canal and initiated ultrastructural studies of these tissues (Figure 2).

CONCLUSIONS

This approach to vector and pathogen control have clear advantages. Chemical insecticides, which target receptors or enzymes in the insect's nervous, hormonal, or cuticular systems, are currently the main agents used for GWSS control. Such insecticides are broad-spectrum and kill beneficial insects as well as the target pest, and represent an environmental hazard as well. Insecticides that will target the insect in such a way as to limit its impact to the

Figure 2. Lateral views of the GWSS alimentary canal. 1. Oesophagus, 2. "Crop-like food storage organ, 3. Upper filter chamber, 4. Caeca, 5. Central filter chamber, 6. Descending midgut,



7. Malpighian tubule, 8. Rectum, 9.Filter chamber microvillar brush border, 10. Descending midgut microvillar brush border membrane.

specific insect pest, and which are readily biodegradable, are very attractive alternatives. In addition to the obvious applied benefits associated with the development of such plant protection technologies, is the tremendous amount of basic biological information such genomics studies acquire. The importance of the accumulation of this information and the genomics tools developed cannot be over estimated. The impact of human genomics projects on the development of new medical technologies gives some indication of what could be in the future for agriculture.

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AREA-WIDE MANAGEMENT OF THE GLASSY-WINGED SHARPSHOOTER IN THE TEMECULA AND COACHELLA VALLEYS

Project Leaders:

Raymond L. Hix
Department of Entomology
University of California
Riverside, CA 92521

Nick C. Toscano
Department of Entomology
University of California
Riverside, CA 92521

Carmen Gispert
UC Cooperative Extension
Riverside County

Reporting Period: The results reported here are from work conducted from December 2002 to October 2003.

INTRODUCTION

Riverside County has two general areas where citrus interfaces with grape: 1) the Coachella Valley with about 14,000 acres of table grapes and 12,000 acres of citrus; and 2) the Temecula valley with 1800 acres of wine grapes in proximity to 1600 acres of citrus. *Xylella fastidiosa* was recently recovered from the Coachella Valley (Perring and Gispert, unpublished), and the glassy-winged sharpshooter (GWSS) is very abundant. In Riverside County, table and wine grapes are the most vulnerable crops to GWSS as a vector of the bacterium *Xylella fastidiosa*, the causal agent of Pierce's disease (PD). Perhaps more than any other source, citrus is viewed as an important year round reproductive host of GWSS, but also one that concentrates GWSS populations over the winter months during the time that grapes and many ornamental hosts are dormant.

Temecula. The Temecula viticultural area was the first in California to be seriously impacted by the glassy-winged sharpshooter (GWSS) *Homalodisca coagulata* and the spread of *X. fastidiosa*, the causative agent for PD. While PD problems were first identified in 1996, it was realized by 1999 that the situation was dire. As a result, this ongoing cooperative demonstration project was initiated in 2000 to examine the impact of area-wide management strategies on GWSS populations and PD incidences in the Temecula Valley. The Temecula advisory committee consists of representatives from wine grape growers, citrus growers, University of California-Riverside, USDA, California Department of Food and Agriculture (CDFA) and the Riverside County Agricultural Commissioner's Office.

In the 2000 season, the opportunity to treat nearly the entire commercial citrus in the Temecula viticultural area was seized upon in an effort to destroy a substantial portion of the regional GWSS population. The emergency treatment of 1300 acres of citrus in Temecula, CA with Admire® (imidacloprid) during Apr and May 2000 represented a pivotal shift toward an area-wide management of GWSS. In March and April 2001, 269 acres of citrus were treated with Admire and an additional 319 acres were treated with foliar applications of Baythroid® on an "as needed" basis. Many grape growers treated their grapes with Admire and/or made foliar applications of Provado®, or Danitol® in 2002. Recommendations were made to remove sick vines in order to remove bacterial reservoirs. Though response was slow initially, growers are aggressively removing sick vines.

Although wine and table grapes are the most vulnerable due to the risk of PD, other crops were scrutinized for contributions to GWSS population growth. Citrus was the most important year long reproductive host of GWSS in Temecula. Citrus also seemed to concentrate GWSS over the winter months when grapes and most ornamental hosts were dormant.

Coachella. The table grape industry in the Coachella Valley is represented by 10,465 acres of producing vines, which generated grapes valued at $108.5 million in 2001. GWSS was identified in the Coachella Valley in the early 1990's, and increases in the numbers of this efficient PD vector over the past 15 months have been documented. In July 2002, the occurrence of *X. fastidiosa* (PD strain) in 13 vines from 2 adjacent vineyards in the southeastern part of the Valley was confirmed. With this discovery, and the increasing numbers of GWSS, there was a real need for an area-wide GWSS and PD management program, to prevent the devastating epidemic like which occurred in Temecula. Clearly, there are no apparent biological or climatological factors that will limit the spread of PD in grapes in the Coachella Valley. Obviously, GWSS had the potential to develop high densities in the Coachella Valley.

OBJECTIVES

The objectives of the Riverside County GWSS Areawide Management programs are:

1. Delineate the areas to be targeted for follow treatments to suppress GWSS populations in the Temecula and Coachella Valleys for the 2004 season.
2. Determine the impact of the 2003 area-wide management program on GWSS populations in citrus, grapes, and other plant hosts in the ecosystem in the 2003 season.
3. Determine the impact of the area-wide program on GWSS adult oviposition and nymphal development.
4. Determine the impact of the GWSS program on beneficial citrus insects, pest upsets and GWSS parasitism.
5. Evaluate the biological and economic effectiveness of an area-wide insecticide program of GWSS.

RESULTS AND CONCLUSIONS

Temecula

Based on trap and visual surveys in late 2002, 501 acres of citrus were targeted in 2003 with Admire and another 109 acres with Danitol® (pyrethroid). The success of the program over 2000, 2001, and 2002 suggests that this level of treatment in citrus every 3 years would keep GWSS populations suppressed in the Temecula viticulture area (Figure 1). This should be coupled with GWSS management within vineyards throughout the valley on a yearly basis. Recommendations were made to remove sick vines in order to remove bacterial reservoirs. Though response was slow initially, growers now aggressively remove sick vines.

Coachella

This program was officially initiated on February 10, 2003. With cooperation of the citrus growers, 10,312 acres of citrus were treated primarily with Admire (7,329.5 acres) and Assail® (acetamiprid) (2734.6 acres) by April 30. Assail treatments were used primarily in flood irrigated citrus groves by speed sprayer, or citrus interplanted in date gardens by handgun applications. By April 10, 2003, all groves with populations of GWSS were treated. Some of these Assail treatments were repeated in the summer as a precaution. Summer GWSS populations were suppressed in Coachella Valley citrus (Figure 2). The ironic fortune in Coachella Valley is that grape growers have been treating grapes for the vine mealybug for about eight years, which probably helped hold back a PD epidemic due to GWSS.

In order for areawide programs to be successful in areas with large acreages of citrus, these groves must be treated before the insects move into deciduous perennial crops and ornamentals after foliage development. If done on an areawide basis, groves don't have to be treated every year. Both organic citrus groves and organic vineyards pose challenges to areawide management programs (Figure 3).

FUNDING AGENCIES

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

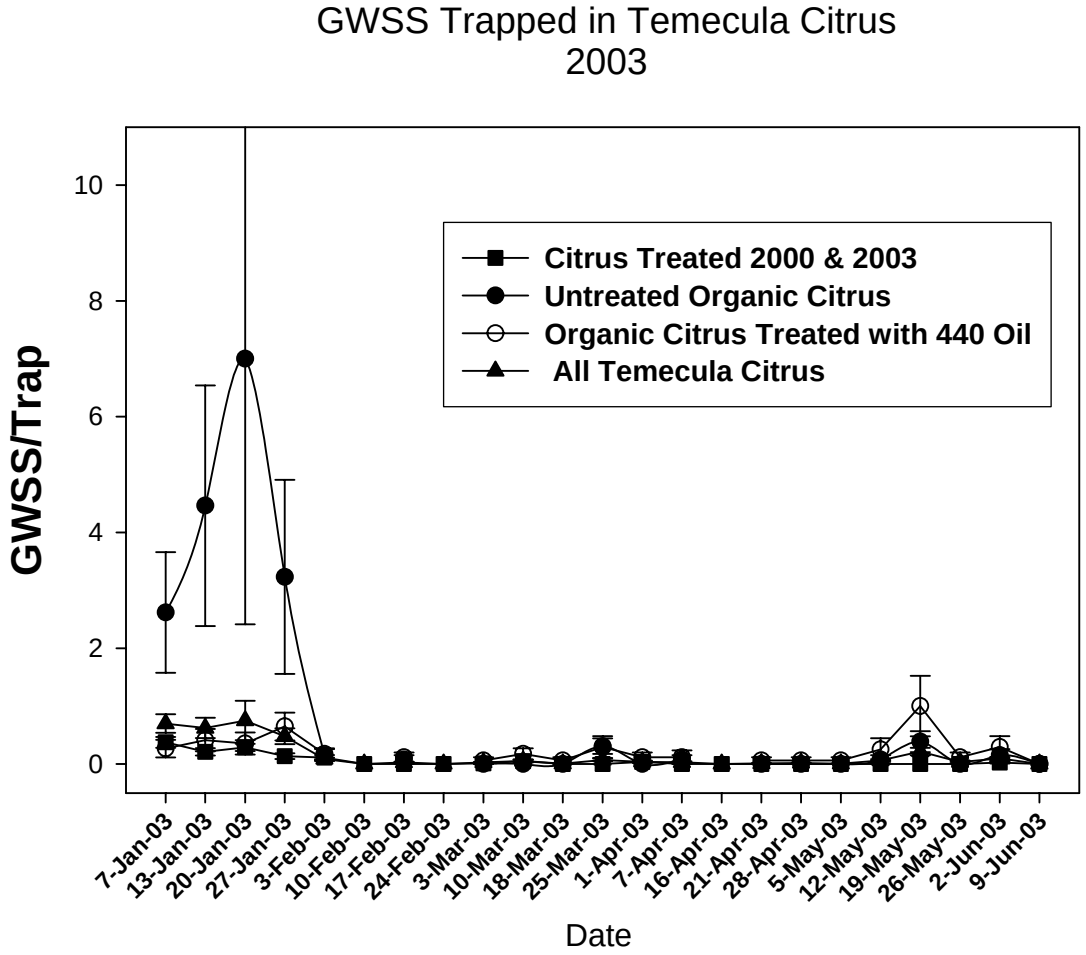

Figure 1. The majority of GWSS trapped in Temecula citrus were associated with untreated organic citrus when compared to organic citrus treated with 440 oil, or Admire in 2000 & 2003. N=250 for All Temecula Citrus (± SEM).

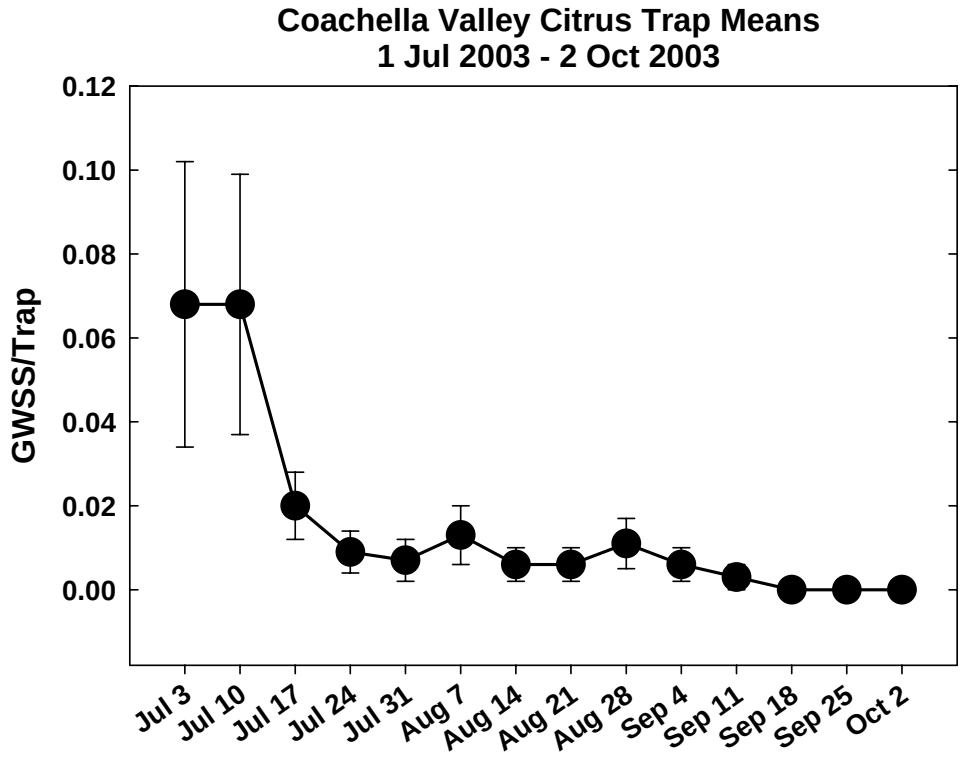

Figure 2. GWSS means for citrus in the Coachella Valley Areawide Program. N = 247 to 344 ± SEM.

Figure 3. GWSS trapped in an organic vineyard represented the largest population in the Temecula Viticulture Area for 2002.

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

Project Leaders:
Shizuo George Kamita
Department of Entomology
University of California
Davis, CA 95616

Bruce D. Hammock
Department of Entomology
University of California
Davis, CA 95616

Cooperators:
David G. Gilchrist
Department of Plant Pathology
University of California
Davis, CA 95616

Bruce Kirkpatrick
Department of Plant Pathology
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted from July 1, 2003 to October 31, 2003.

ABSTRACT
In this project, we are evaluating the effectiveness of peptide antibiotics such as cecropins A, B, and P1 against *Xylella fastidiosa*. In our initial experiments, we have found that cecropins (in particular cecropin A) are effective at inhibiting the growth of *X. fastidiosa*. We are currently attempting to express individual cecropins in an in vitro expression system based on baculovirus expression vectors and insect cells. The baculovirus system will allow us to easily manipulate the coding sequence of the peptide antibiotic in order to optimize their efficacy. In the second phase of our project we will attempt to express the most effective cecropin constructs in transgenic plants and determine whether (i) active cecropin is produced and (ii) if active cecropin is expressed, does feeding by GWSS on these transgenic plants reduce *X. fastidiosa* transmission.

INTRODUCTION
Traditional antibiotics are natural or chemically synthesized small molecules that can selectively kill or stop the growth of bacteria. Antibiotic inhibition of *Xylella.* (at least 17 isolates tested) has been analyzed for six different antibiotics (ampicillin, kanamycin, neomycin, penicillin, streptomycin, and tetracycline) (4, 10). 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 (6, 18). 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 (6, 13). 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* (9, 17). At least ten different cecropins have been isolated from lepidopteran (moths and butterflies) and dipteran (flies) insects (1, 12) and one cecropin has been identified from the small intestine of a pig (11). Cecropins are composed of a single chain of 35-39 common L-amino acids and do not contain disulfide bonds (12). Cecropins are active against many Gram(-) bacteria and some Gram(+) bacteria, but are inactive against eukaryotic cells at concentrations that are antimicrobial (1, 7, 18) and possibly at concentrations up to 300 times higher (17). *X. fastidiosa* is a Gram(-) bacterium (8). In Gram(-) bacteria the antibacterial activities of cecropins A, B, and P1 are up to ten-times greater than tetracycline (1, 2). Cecropins have a unique combination of characteristics (specificity, gene basis, small size, potency against Gram(-) bacteria, etc.) that should make them ideal substances for the control of *X. fastidiosa* in GWSS.

A basic component of Integrated Pest Management (IPM) is the use of cultural practices such as the purposeful manipulation of the environment in order to reduce pest damage (14). This concept is elegantly illustrated in the southern San Joaquin Valley where strips of alfalfa are planted between cotton fields for protection against a key cotton pest, the lygus bug. This practice is successful because the lygus bug prefers feeding and living on alfalfa over cotton (15). The pest species is

effectively trapped in the "trap crop" because of a particular preference and damage to the crop is minimized. In the case of a grape vineyard and Pierce's disease (PD), other plantings adjacent to the grape vineyard may serve as preferred food source and breeding ground for the glassy-winged sharpshooter (GWSS) (16). The goal of our proposed project will be to develop a model system to test the potential of a transgenic, peptide antibiotic-expressing trap plant for the control of *X. fastidiosa* transmission by GWSS. Our approach is highly unique in that we are attempting to reduce transmission frequency through the use of a transgenic trap plant rather generating a transgenic crop plant (i.e., transgenic grape). Perhaps a direct manifestation of this objective would be to generate transgenic, cecropin-expressing grape. We believe, however, that this "direct" approach has several drawbacks including longer generation times, unknown effect on the quality of the fruit or wine, regulatory concerns, and public acceptance. Thus, our approach should be faster in terms of field applicability. However, once the genetic technology is optimized for the trap plant it can later be applied to the target grape crop if there is such a need. Furthermore, should our approach be successful for PD, it will also be applicable to other insect-vectored, bacterial diseases.

OBJECTIVES

1. Identify peptide antibiotics (cecropins) that are effective against *Xylella fastidiosa*.
    i. Determine the antibiotic sensitivity of *X. fastidiosa* to chemically synthesized cecropins.
    ii. Produce modified cecropins using baculovirus expression vectors.
    iii. Determine the toxicity of cecropins against GWSS cells grown in culture.
2. 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

Objective 1

During the four month-long reporting period, we have established procedures for the continual culture and storage of *X. fastidiosa* in our laboratory. In general, our procedures are copied from protocols established in the Bruce Kirkpatrick laboratory. We have tested GYE (glutamate yeast extract) (3), PD3, and PW media for their abilities to support maximal growth of *X. fastidiosa*. In our hands, we have found that PD3 gives the fasted growth of *X. fastidiosa* (Temecula strain) 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 light lawn by 10 days post seeding).

Using the optimized growth conditions, we have examined the minimal inhibitory concentrations (MIC assay) at which cecropins A, B, and P1 are effective in inhibiting the growth of *X. fastidiosa* (see Table 1). On the basis of our preliminary experiments, cecropins A, B, and P1 appear to be effective at partially inhibiting *X. fastidiosa* growth at concentrations of 0.05, 0.25, and 0.5 µM, respectively, at two weeks post inoculation. In general, cecropin A appeared to be 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 degradation. We are currently repeating the preliminary experiments with intermediate concentrations of cecropin and chemical antibiotics.

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Table 1. Effect of Cecropin or Kanamycin on *X. fastidiosa* Growth

	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

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

EFFECTS OF SUB-LETHAL DOSES OF IMIDACLOPRID ON VECTOR TRANSMISSION OF *XYLELLA FASTIDIOSA*

Project Leader:
Alexander H. Purcell
Division of Insect Biology
201 Wellman Hall, MC3112
University of California
Berkeley, CA 94720-3112

Cooperator:
Keiko Okano
Division of Insect Biology
201 Wellman Hall
University of California
Berkeley, CA 94720-3112

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

ABSTRACT
Two agents, soil-applied imidacloprid (Admire 2F) and particle film (Surround™ WP) foliar sprays were applied to grape seedlings to test their effect on vector insects' (glassy-winged sharpshooter, GWSS, and blue-green sharpshooter, BGSS) host preference, under caged laboratory conditions. Imidacloprid at sub-lethal dosages that inhibit feeding performance did not show any repellency effect against either of the insects, but GWSS on the imidacloprid-treated plants moved more frequently. In contrast, the particle film strongly repelled BGSS (GWSS not tested yet), but the insects seldom changed location once they landed and settled on a plant with or without the treatment. The test plants used in the preference tests will be diagnosed for the Pierce's disease bacterium (*Xylella fastidiosa*), in order to evaluate how the above results affect transmission.

INTRODUCTION
Chemical treatments often are used to control plant diseases caused by insect vector-borne pathogens (Perring et al. 1999). Insecticides have proven effective to reduce numbers of glassy-winged sharpshooter (GWSS). The systemic insecticide imidacloprid (Admire 2F, Bayer Co., Kansas City, MO) has been studied in relation to GWSS mainly for its mortality effect (Bethke et al 2001). Although the insecticide seems to be effective in killing GWSS, a recent study in Georgia showed that the imidacloprid application slowed the rate of PD spread but was prone to the high infestation of GWSS (Krewer et al 2001). Therefore, it is important to know how imidacloprid's effect on the insect's behavior affects the pathogen transmission process. The probability of vector transmission depends not only in the number (*n*) of vectors per plant, but on what percentage are infective (*i*), how efficiently infective insects transmit (*E*), and the time spent on a plant (*t*) (Purcell 1981). Imidacloprid has been shown to affect the feeding behavior (Bethke et al 2001) of GWSS and behavioral effects on other vector insects (Quintela and McCoy 1997), so we examined how GWSS transmission of *X. fastidiosa* might be affected by systemic imidacloprid in grape. This knowledge could be useful in optimizing the use of imidacloprid or suggest alternative/supplemental strategies.

The dosage of imidacloprid applied in this experiment was "sub-lethal", which had less than 10% mortality over 24 hour period, as determined from our previous experiments to sharply reduce GWSS' feeding. We tested whether this deterred feeding would repel the insects from imidacloprid-treated grapevines. For comparison, a similar experiment using the particle film (Surround™ WP, Engelhard Corp., Iselin, NJ) was also conducted, as field and laboratory studies have already demonstrated the repellency of particle films for GWSS (Puterka et al 2003).

OBJECTIVES
1. Observe and compare how the Admire 2F or Surround WP application affects a grapevine's acceptance as a host by GWSS and BGSS.
2. Evaluate the above effects on the transmission rate of PD.

RESULTS AND CONCLUSIONS
Preference Test Settings
We set 8 arenas (W60xL60xH45 cm) covered with white mesh, each with 2 plants, inside in a greenhouse insectary. There were 3 different combinations of grape seedlings (*Vitis vinifera*, Pinot Noir): 2 arenas each of No-choice (+/+, both treated) and No-choice (-/-, both untreated), and 4 arenas of Choice (+/-, one treated and one untreated). The treatments applied here were either imidacloprid (Admire 2F, 0.000197gAI/500g soil applied 7 days prior to the experiments) or Surround WP (6%, sprayed for full coverage the day before the experiments).

We released 4 insects (GWSS or BGSS) in the center of each arena for the Admire experiments, and 2 per arena for the Surround experiment. These insects were marked with a spot of color so that each individual could be distinguished. The observations occurred at 10, 30min, 1, 2, 3, 6, 9, 12, and 23rd hour after the release, and the location of the insects were recorded. The experiment was replicated 4 times each. We used Student's t-test for the comparison of the data. The test plants used in the preference tests will be diagnosed for the Pierce's disease (results pending).

Admire 2F Preference Test

GWSS No-choice. A significant mortality difference was observed only at the 23rd hour observations. The number of GWSS per plant was slightly higher for the untreated-only arenas at 2,9.23rd hours ($P<0.05$) (Figures 1 and 2). In the Admire-treated only arenas, insects moved between plants and/or plant and cage more frequently than in the untreated-only arenas ($P<0.05$).

*GWSS Choic*e. The mortality rate and the number of GWSS per plant in the choice arenas were similar to that of the treated-only, no-choice arenas. Within the choice arenas, there were no significant differences between the number of GWSS on the treated plant and that on the untreated one (Figure 3).

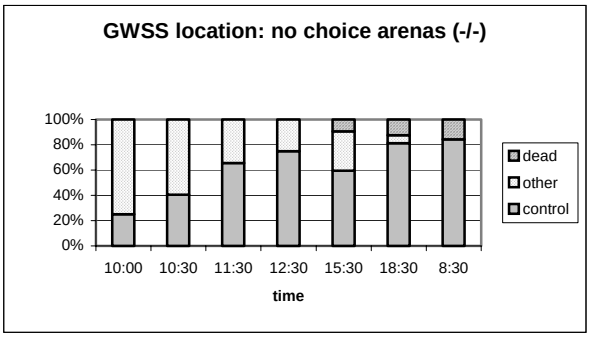

Figure 1. Insect on plant, untreated only.

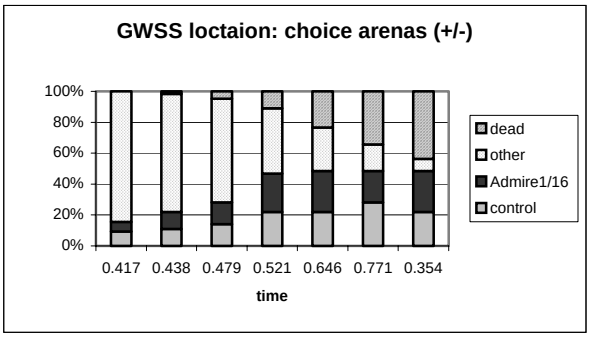

Figure 3. Insect on plant, treated and no-treated.

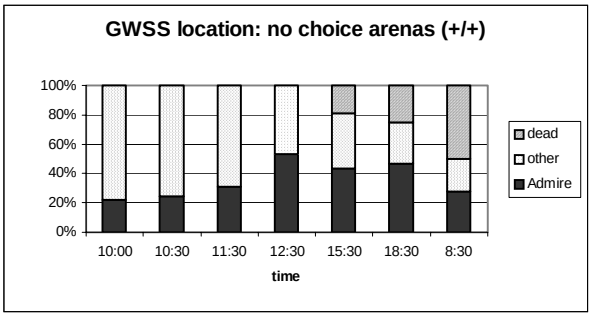

Figure 2. Insect on plant, Admire treated only.

Table 1. GWSS movement.

arena	avg. location changes
choice(+/-)	2.81 (± 0.47)
no-choice (+/+)	4.38 (± 0.52)
no-choice (-/-)	2.5 (± 0.45)

BGSS No-choice: The average mortality rates were about 10% for both of the plant combinations. The numbers of BGSS on plants were similar throughout the day (Figures 4 and 5). In contrast to GWSS, the frequency of the insects moving around did not differ among the plant combinations, and BGSS moved less frequently than GWSS (data not shown).

BGSS Choice: The mortality rate was similar to the no-choice arenas. Within the choice arenas, there was no difference between the number of BGSS on the Admire-treated and untreated plants (Figure 6). The location change of the insects did not differ from that of the no-choice arenas (data not shown).

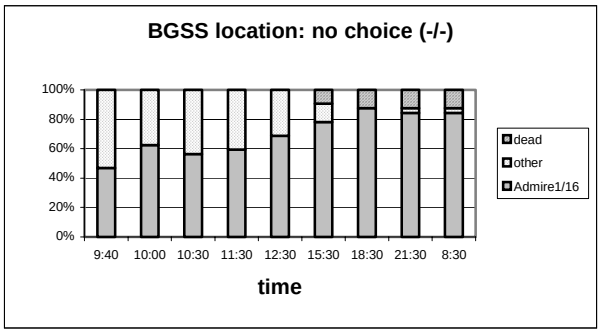

Figure 4. Insect on plant, untreated only.

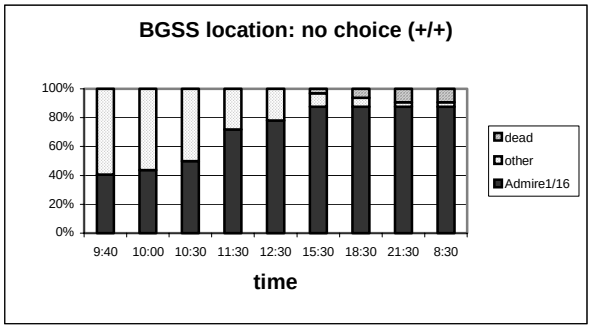

Figure 5. Insect on plant, treated only.

Figure 6. Insect on plant, treated and untreated.

Surround WP Preference Test

BGSS No-choice: The mortality was significantly higher in the treated-only arenas at 6, 12, and 24 hours ($P<0.05$). As the consequence, the number of BGSS on the Surround-treated plants was lower than on the untreated plants (at 1st and 24th hours, $P<0.05$, at 12th hour, $P<0.001$). However, the difference between the two started even before significant mortality differences were observed (Figures 7 and 8). Once landed on the plant, the insects were not likely to change their locations. This was more so for the Surround-treated plants, where the location change occurred only twice out of 16 BGSS in 4 days.

BGSS Choice: BGSS mortality was intermediate that of the two types of no-choice arenas and did not show significant difference with either of those. The numbers of insects on plant were different from that of Surround-only arenas, at 6th and 24th hours ($P<0.05$). Within the choice arenas, there was a strong tendency of the insects landing and staying on the control plants throughout the observations ($P<0.001$) (Figure 9). Again, the insects tended not to change their location once they had landed on the plant, and there was only one incident where a BGSS switched plants from Surround-treated to untreated, out of 32 insects in 4 days (data not shown).

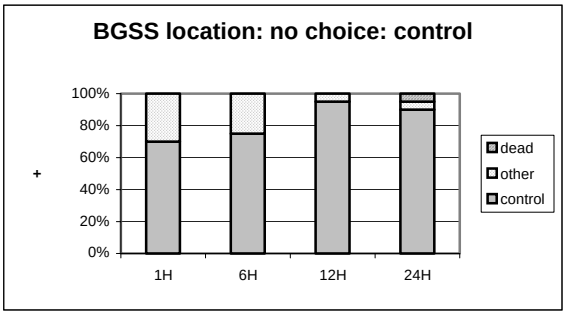

Figure 7. Insect on plant, untreated only.

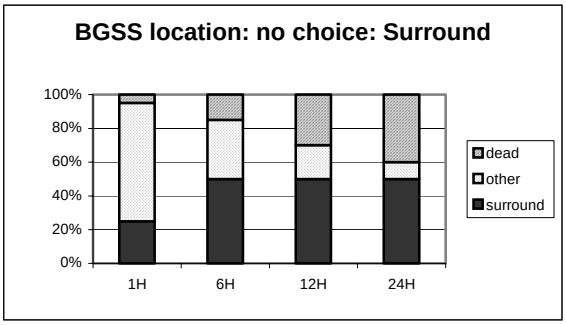

Figure 8. Insect on plant, Admire-treated only.

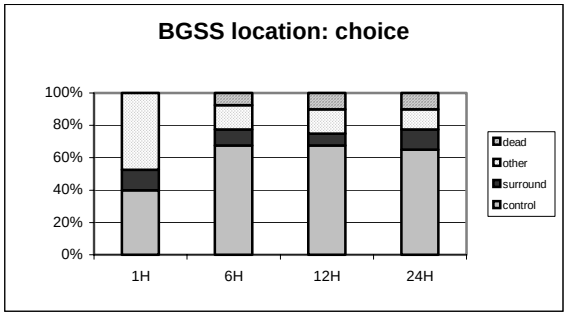

Figure 9. Insect on plant, treated and untreated.

CONCLUSIONS

The sub-lethal imidacloprid application did not affect the initial landing of either sharpshooters. In contrast to the repellency of Surround, imidacloprid showed no repellency at the 1st hour observations. GWSS tended to move on and off the plant more than BGSS. However, GWSS in imidacloprid-treated only arenas changed location more frequently than those in untreated-only arenas, whereas BGSS activity did not differ among different arenas. With choice or no-choice, the BGSS landed on some Surround-treated plants, and most of them stayed on the same plant, or some even on the same spot throughout the experiment. When we diagnose these test plants, we will consider how the factors such as the lowered number of insect per plant either by mortality or repellency, different level of insect mobility, and feeding deterrence may have affected the pathogen transmission.

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FUNDING AGENCIES

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PESTICIDE SCREENING AGAINST THE GLASSY-WINGED SHARPSHOOTER, *HOMALODISCA COAGULATA* (SAY), USING COMMERCIALLY AVAILABLE BIORATIONAL, ORGANIC, AND REDUCED RISK PESTICIDES

Project Leaders:

Richard Redak
Department of Entomology
University of California
Riverside, CA 92521

James Bethke
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted from July 1, 2001 to January 1, 2003.

ABSTRACT

Herein we summarize the results of multiple insecticide trials designed to evaluate biorational, organic and reduced risk insecticides. Our results indicated that few of the biorational/organic/reduced products tested would be applicable for glassy-winged sharpshooter control or eradication (poor mortality, slow knock down activity, poor residual activity). None of these products tested achieved 70% mortality in both adult and nymphal trials. Rotenone and 10% rosemary oil (Ecotrol) may be the only exception when used strictly against adults; however, their level of control is below that of the pryrethroids, chloronicotinyls, organophosphates, and carbamates. With the possible exception of rotenone and pyrethrins (PyGanic), conventional pyrethroids, organophosphates, and chloronicotinyls were more effective than all other compounds tested.

INTRODUCTION

Glassy-winged sharpshooter (*Homalodisca coagulata* (Say), Homoptera: Cicadellidae) has become a serious pest of California's commercial nursery production and backyard landscape environment. This is in large part due to the intra-state quarantine measures restricting movement of nursery products from the southern portions of the state, by which sharpshooter populations have become established to the northern and central portions of the state that currently do not have established populations of this insect. Once sharpshooters are detected in "uninfested" northern counties, eradication programs are immediately initiated to limit the further spread of this insect. Current eradication programs rely on the application of traditional registered chemical insecticides. Compounds currently being used in the eradication programs include pyrethroids, carbamates, organophosphates and neonicotinoids. The aforementioned pesticides can possess serious human health and environmental risks if used incorrectly or for prolonged periods of time in a single area. One solution to avoiding these risks would be the use of so-called biorational pesticides. Such compounds traditionally are thought to possess lower risks to both humans and the environment.

OBJECTIVES

The objective of the research presented here was to determine the efficaciousness of products that are alternatives to conventional pesticides for the urban control (eradication) of glassy-winged sharpshooter. The California Depart-ment of Food and Agriculture initially selected products for evaluation. At the time of project initiation 24 products were selected by CDFA. During the period of evaluation an additional 11 compounds were added to the list by CDFA. Additionally, we included 13 compounds for evaluation that were not included in the above list of 35 products. We included these later compounds as they represented additional chemical classes not represented in the original listing. In total, 62 separate pesticides and formulations were evaluated. We included some conventional products such as an organophosphate, several pyrethroids, and several miticides (potential ovicides) for comparative purposes. In addition, we tested several chloronicotinyls, of which many are classified as reduced risk for certain applications. Three compounds, sabadilla, pyrellin, and bordeaux oil mix were originally listed for testing but were not included in the trials conducted here. These compounds were either unavailable (sabadilla), too costly to consider (pyrellin) or an nonstandardized mix of other compounds already included in the trials (bordeaux oil mix-a mixture of light oils and baking soda). One other compound, sulfur, is still under evaluation. For a complete list of products that were on the CDFA's initial list and additional products tested see Table

RESULTS

Table 2 shows the summary results of all trials and for all products. Only the highest level of efficacy obtained for any particular products is shown. The chloronicotinyls, insect growth regulators, organophosphates, and pyrethroids, were, for the most part, effective against glassy-winged sharpshooters. Many (most?) of these compounds have been field tested against various stages of glassy-winged sharpshooters in other trials by this lab and others (data not shown). The pyrethroids, as a class, provided exceptional control against adults and nymphs but had limited activity against eggs; although fenpropathrin and deltamethrin showed excellent activity against emerging nymphs from eggs. As shown in other studies, the chloronicotinyls are very effective at killing adult and nymphal stages of glassy-winged sharpshooters. Chloronicotinyls also appear to be reasonably effective against sharpshooter egg masses, causing mortality as the sharpshooter nymphs begin to hatch. The insect growth regulators, many of which are also reduced risk depending on the application, are effective against nymphs; however their efficacy is only apparent after a 1-2 week interval. Insect growth regulators would not be effective against the egg or adult stage, or if quick knock down to prevent subsequent spread is desired.

In general, oils, fertilizers, surfactants, biologicals, botanicals, fungicides, repellants, gut poisons, and miticites, all failed to induce adequate mortality for any stage of sharpshooter upon which they were evaluated; they should not be considered as useful products in an urban eradication program. There are some minor exceptions: fenpyroximate (FujiMite), Rotenone, pyrethrins (in the form of Pyganic), neem (in the form of Azatin), and high concentrations of rosemary oil (Ecotrol). Fenpyroximate, a traditional miticide, proved minimally efficacious against adults and nymphs at >70% mortality. Pyganic and Azatin have not been tested against adults, but are effective against nymphs at >90% mortality; Azatin appears to be most effective as an insect growth regulator against the nymphal stage. The residual activity of 10% rosemary oil (Ecotrol), observed over an extended 5-day period, provided significant mortality to adult GWSS. An evaluation of 10% rosemary oil for control of nymphs is underway. Pending the outcome of that trial, we may move forward with long term residual activity (multi-week) trials with this compound. Rosemary oil at lower concentrations (5%, Hexacide) did not induce significant mortality.

The most promising organic product evaluated was rotenone. Rotenone caused 100% mortality to adults when treated directly on the plants as a residual; however, it did not induce significant mortality when directly applied to the adults. This suggests to us that both trials should be repeated; it is extremely rare that a compound will show lower toxicity in a direct contact trail than in a residual trial. Currently, to our knowledge, rotenone is not listed for use in California as an insecticide, has a high potential for environmental damage relative to other products tested, and in reality is not likely to be used broadly in an urban setting for sharpshooter control. Consequently, we chose not to evaluate this compound further than the initial mortality trials. We are currently conducting nymphal trials with rotenone.

CONCLUSIONS

Few of the biorational/organic/reduced products tested would be applicable for glassy-winged sharpshooter control or eradication. None of these products achieved 70% mortality in both adult and nymphal trials. Rotenone and 10% rosemary oil (Ecotrol) may be the only exception when used strictly against adults; however, their level of control is below that of the pryrethroids, chloronicotinyls, organophosphates, and carbamates (not tested here). For practical health and safety purposes, we do not feel rotenone will ever be used on a broad scale for eradicating or managing sharpshooter populations. Additionally, as rotenone breaks down rapidly, we anticipate it to fail in long term trials. Rosemary oil appears to be only effective after relatively long exposure times (5 d), making its successful use in eradication unlikely. Pryethrins (Pyganic) and neem (formulated as Azatin XL) may be effective against nymphs, but their efficacy occurs over a prolonged period of time. As they are not effective against adults, and function long term as growth regulators, their utility for quickly eradicating a population is also unlikely. Similarly, other insect growth regulators tested here (buprofizen, novaluron, and pyriproxifen) induced nymphal mortality over the development time of the insect, but showed little immediate mortality against adults. We do not recommend that these compounds be utilized for eradication purposes.

In conclusion, with the possible exception of rotenone and pyrethrins (PyGanic), conventional pyrethroids, organophosphates, and chloronicotinyls were more effective than all other compounds tested. These classes of compounds were not subsequently subjected to long-term trials here as they have been previously evaluated under these conditions (They were included here for comparative purposes.).

Table 1. Organic, biorational, and reduced risk pesticides evaluated for efficacy of control against the glassy-winged sharpshooter.

Class of Chemical	Common Name	Trade Name	Rate per 100 gal water	Biorational, Organic, Reduced Risk
barrier/repellant	kaolin clay	Surround	16.20 kg	Yes
biological-bacterial	*Bacillus thuringiensis*	Dipel Pro DF	8.00 oz	Yes
biological-bacterial	*Bacillus thuringiensis*	Gnatrol	16.00 oz	Yes
biological-bacterial	spinosad	Conserve SC	22.00 oz	Yes
botanical-plant extract	azadiractin/neem	Ornazin	8.00 oz	Yes
botanical-plant extract	azadiractin/neem	Triact 70	1.00 gal	Yes
botanical-plant extract	capsaicin	Hot Pepper Wax	8.00 oz	Yes
botanical-plant extract	garlic oil, black pepper	Garlic Oil	10.00gal	Yes
botanical-plant extract	pyrethrins	PyGanic	16.00 oz	Yes
botanical-plant extract	pyrethrins, PBO, silicon dioxide	Diatect V	6.00 lb	Yes

Table 1. Organic, biorational, and reduced risk pesticides evaluated for efficacy of control against the glassy-winged sharpshooter.

Class of Chemical	Common Name	Trade Name	Rate per 100 gal water	Biorational, Organic, Reduced Risk
botanical-plant extract	pyrethrins, silicon dioxide	Diatect II	6.00 lb	Yes
botanical-plant extract	rosemary oil 10%	Ecotrol	16.00 oz	Yes
botanical-plant extract	rosemary oil 5%	Hexacide	16.00 oz	Yes
botanical-plant extract	rotenone	Rotenone	20.00 lb	Yes
botanical-plant extract	sodium lauryl sulfate/acetic/citric acids	Sharpshooter	3.75 gal	Yes
chloronicotinyl	acetamiprid	Tristar 70WP	2.25 oz	No
chloronicotinyl	acetamiprid	Tristar 70WP	1.12 oz	No
chloronicotinyl	acetamiprid	Tristar 70WP	0.50 oz	No
chloronicotinyl	dinotefuran	Valent 10112 20 SG	0.25 lb	No
chloronicotinyl	flonicamid	Flonicamid	60.00 g	No
chloronicotinyl	flonicamid	Flonicamid	40.00 g	No
chloronicotinyl	imidacloprid	Forte 200 SL	7.10 ml	No[2]
chloronicotinyl	imidacloprid	Marathon II	50.00 ml	No[2]
chloronicotinyl	imidacloprid	Marathon II	3.40 oz	No[2]
chloronicotinyl	thiamethoxam	Flagship	4.00 oz	No
foliar fertilizer	liquid amino acid chelate	Metalosate	82.70 ml	Yes
fungicide	baking soda	Arm & Hammer	8.00 oz	Yes
inorganic gut poison	boric acid	Boric Acid	-	Yes
inorganic gut poison	sodium aluminoflouride	Prokil	30.00 lb	Yes
insect growth regulator	azadiractin/neem	Azatin XL[1]	16.00 oz	Yes
insect growth regulator	buprofizen	Talus	2.86 lb	Yes
insect growth regulator	cyromazine	Citation	2.66 oz	Yes
insect growth regulator	novaluron	Pedistal	8.00 oz	Yes
insect growth regulator	pyriproxifen	Distance	16.00 oz	Yes
insect growth regulator/miticide	fenpyroximate	Fujimite	4.00 pt	Yes
miticide/ovicide	abamectin	Avid	10.00 oz	No
miticide/ovicide	hexythiazox	Hexygon	2.00 oz	No
oil	methylated seed oil	Phase	16.00 oz	Yes
oil	Natur-Chem	Unknown	1.00 gal	Yes
oil	Natur-Chem	Unknown	2.00 gal	Yes
oil	paraffinic oils	Ultrafine oil	1.00 gal	Yes
organophosphate	chlorpyrifos	Dursban Pro	16.00 oz	No
pyrethroid	bifenthrin	Talstar	10.00 oz	No
pyrethroid	bifenthrin	Talstar	19.20 oz	No
pyrethroid	cyfluthrin	Tempo/Decathlon	1.90 oz	No
pyrethroid	deltamethrin	DeltaGard 5SC	8.00 oz	No

Table 1. Organic, biorational, and reduced risk pesticides evaluated for efficacy of control against the glassy-winged sharpshooter.

Class of Chemical	Common Name	Trade Name	Rate per 100 gal water	Biorational, Organic, Reduced Risk
pyrethroid	fenpropathrin	Tame	5.00 oz	No
pyrethroid	fenpropathrin	Tame	10.67 oz	No
pyrethroid	lambda-cyhalothrin	Scimitar	5.00 oz	No
pyrethroid	permethrin	Astro	4.00 oz	No
repellant	DEET/vanilla scent	Bugspray 1 X	-	Yes
repellant	DEET/vanilla scent	Bugspray 1/2 X	-	Yes
repellant	DEET/vanilla scent	Bugspray 1/4 X	-	Yes
repellant	peppermint oil/vanilla scent	Buggins 0.01X	-	Yes
repellant	peppermint oil/vanilla scent	Buggins 1X	-	Yes
soap	potassium salts	Insecticidal Soap	1.00 gal	Yes
surfactant	buffering acids/ silicone defoamer	No Foam B	24.00 oz	Yes
surfactant	organosilicone	L 77	16.00 oz	Yes
surfactant	organosilicone, latex polymer	Tactic	16.00 oz	Yes
surfactant	soy phospholipids	LI 700	32.00 oz	Yes
surfactant	terpine resin, fatty acids	Parasol	8.00 oz	Yes
surfactant	terpine resin, fatty acids	Umbrella	8.00 oz	Yes

Table 2. Summary results of all trials conducted[1]. Details of individual trials are available upon request

Class of Chemical	Common Name	Trade Name	Rate per 100 gal water	Mortality within 24 hr after contact with treated plant (unless otherwise noted)			Mortality over nymphal development time[2]	Topical Adult Mortality in 24 hr
				Eggs	Nymphs	Adults		
barrier/repellant	kaolin clay	Surround	16.20 kg	NA[3]		6.0		
biological-bacterial	*Bacillus thuringiensis*	Dipel Pro DF	8.00 oz	NA		10.0		
biological-bacterial	*Bacillus thuringiensis*	Gnatrol	16.00 oz	NA		4.0		
biological-bacterial	spinosad	Conserve SC	22.00 oz			4.0		
botanical-plant extract	azadiractin/neem	Ornazin	8.00 oz		10.0	10.0		16.0
botanical-plant extract	azadiractin/neem	Triact 70	1.00 gal		25.8	16.0		20.0
botanical-plant extract	capsaicin	Hot Pepper Wax	8.00 oz		IP[5]	6.0		16.0
botanical-plant extract	garlic oil, black pepper	Garlic Oil	10.00gal		IP	14.0		29.0
botanical-plant extract	pyrethrins	PyGanic	16.00 oz	11.6	IP	IP	94.0	
botanical-plant extract	pyrethrins, PBO, silicon dioxide	Diatect V	6.00 lb	2.8	16.0	50.0		25.0
botanical-plant extract	pyrethrins, silicon dioxide	Diatect II	6.00 lb	0.6	8.0	36.0		18.0
botanical-plant extract	rosemary oil 10%	Ecotrol	16.00 oz	8.8	IP	54 , 100[4]		8.0

Table 2. Summary results of all trials conducted[1]. Details of individual trials are available upon request

Class of Chemical	Common Name	Trade Name	Rate per 100 gal water	Mortality within 24 hr after contact with treated plant (unless otherwise noted)			Mortality over nymphal development time[2]	Topical Adult Mortality in 24 hr
				Eggs	Nymphs	Adults		
botanical-plant extract	rosemary oil 5%	Hexacide	16.00 oz	61.6	18.6	16.0		4.0
botanical-plant extract	rotenone	Rotenone	20.00 lb	2.5	IP	100.0		63.0
botanical-plant extract	sodium lauryl sulfate/acetic/citric acids	Sharpshooter	3.75 gal	0.0		4.0		13.0
chloronicotinyl	acetamiprid	Tristar 70WP	2.25 oz	100.0	93.3	100.0		
chloronicotinyl	acetamiprid	Tristar 70WP	1.12 oz	100.0		100.0		
chloronicotinyl	acetamiprid	Tristar 70WP	0.50 oz	100.0		100.0		
chloronicotinyl	dinotefuran	Valent 10112 20 SG	0.25 lb	73.6		100.0		
chloronicotinyl	flonicamid	Flonicamid	60.00 g			6.0		
chloronicotinyl	flonicamid	Flonicamid	40.00 g			10.0		
chloronicotinyl	imidacloprid	Forte 200 SL	7.10 ml	86.8		100.0		
chloronicotinyl	imidacloprid	Marathon II	50.00 ml	100.0	100.0	98.0		100.0
chloronicotinyl	imidacloprid	Marathon II	3.40 oz		90.0			
chloronicotinyl	thiamethoxam	Flagship	4.00 oz	33.3				
foliar fertilizer	liquid amino acid chelate	Metalosate	82.70 ml	NA		6.0		
fungicide	baking soda	Arm & Hammer	8.00 oz	NA		10.0		
inorganic gut poison	boric acid	Boric Acid	-	NA		8.0[6]		
inorganic gut poison	sodium aluminoflouride	Prokil	30.00 lb	NA	14.0	22.0		26.0
insect growth regulator	azadiractin/neem	Azatin XL	16.00 oz				98	
insect growth regulator	buprofizen	Talus	2.86 lb	10.8		6.0	98.0	35.0
insect growth regulator	cyromazine	Citation	2.66 oz			12.0		
insect growth regulator	novaluron	Pedistal	8.00 oz	0.0		16.0	100.0	19.0
insect growth regulator	pyriproxifen	Distance	16.00 oz	26.2		10.0	92.0	16.0
insect growth regulator/miticide	fenpyroximate	Fujimite	4.00 pt		72.0	78.3		91.0
miticide/ovicide	abamectin	Avid	10.00 oz	54.4				
miticide/ovicide	hexythiazox	Hexygon	2.00 oz	49.0				
oil	methylated seed oil	Phase	16.00 oz	4.9	22.0	20.0		9.0
oil	Natur-Chem	Unknown	1.00 gal	3.9		4.0		
oil	Natur-Chem	Unknown	2.00 gal	8.7		2.0		
oil	paraffinic oils	Ultrafine oil	1.00 gal	23.4	15.0	8.0		18.0
organophosphate	chlorpyrifos	Dursban Pro	16.00 oz			100.0		
pyrethroid	bifenthrin	Talstar	10.00 oz	45.3	100.0	100.0		100.0
pyrethroid	bifenthrin	Talstar	19.20 oz	89.9	100.0			
pyrethroid	cyfluthrin	Tempo/Decathlon	1.90 oz	43.2	95.0	100.0		

Table 2. Summary results of all trials conducted[1]. Details of individual trials are available upon request

Class of Chemical	Common Name	Trade Name	Rate per 100 gal water	Mortality within 24 hr after contact with treated plant (unless otherwise noted)			Mortality over nymphal development time[2]	Topical Adult Mortality in 24 hr
				Eggs	Nymphs	Adults		
pyrethroid	deltamethrin	DeltaGard 5SC	8.00 oz	100.0		100.0		
pyrethroid	fenpropathrin	Tame	5.00 oz			100.0		
pyrethroid	fenpropathrin	Tame	10.67 oz	100.0	96.7			
pyrethroid	lambda-cyhalothrin	Scimitar	5.00 oz		100.0	100.0		100.0
pyrethroid	permethrin	Astro	4.00 oz	95.7	90.0			
repellant	DEET/vanilla scent	Bugspray 1 X[6]	-	NA	NA	12.0		
repellant	DEET/vanilla scent	Bugspray 1/2 X	-	NA	NA	2.0		
repellant	DEET/vanilla scent	Bugspray 1/4 X	-	NA	NA	4.0		
repellant	peppermint oil/vanilla scent	Buggins .01X	-	NA	NA	4.0		
repellant	peppermint oil/vanilla scent	Buggins 1X	-	NA	NA	2.0		
soap	potassium salts	Insecticidal Soap	1.00 gal	17.8		24.0		21.0
surfactant	buffering acids/ silicone defoamer	No Foam B	24.00 oz			4.0		
surfactant	organosilicone	L 77	16.00 oz	4.3		8.0		28.0
surfactant	organosilicone, latex polymer	Tactic	16.00 oz	0.1		8.0		50.0
surfactant	soy phospholipids	LI 700	32.00 oz	12.5		8.0		14.0
surfactant	terpine resin, fatty acids	Parasol	8.00 oz	3.9				10.0
surfactant	terpine resin, fatty acids	Umbrella	8.00 oz		13.3	10.0		20.0

FUNDING AGENCIES
Funding for this project was provided by the California Department of Food and Agriculture.

TOWARD A STANDARDIZED TREATMENT PROTOCOL TO ELIMINATE GLASSY-WINGED SHARPSHOOTER EGG MASSES IN COMMERCIAL NURSERY STOCK

Project Leaders:
Richard Redak
Department of Entomology
University of California
Riverside, CA 92521

James Bethke
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:
Bordier's Nursery
Monrovia Nursery

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

ABSTRACT
Carbaryl, fenpropathrin, deltamethrin and acetamiprid were evaluated for their ability to kill glassy-wing sharpshooter nymphs emerging from the egg mass under standard commercial nursery operations (including transportation). Carbaryl provided the best overall results resulting in near absolute control (>98%) of emerging nymphs on all plant types (trees, shrubs, bedding plants). Fenpropathrin also achieved very good control. Control of emerging sharpshooters was poorest on bedding plants; this is largely due to the tremendous number of densely packed leaves within a small plant canopy and the resultant difficulty in achieving adequate coverage of the insecticides.

INTRODUCTION
Glassy-winged sharpshooter (*Homalodisca coagulata* (Say), Homoptera: Cicadellidae) has become a serious pest of California's commercial nursery production. This is in large part due to the intra-state quarantine measures restricting movement of nursery products from the southern portions of the state, in which sharpshooter populations have become established, to the northern and central portions of the state that currently do not have established populations of this insect. Currently, movement of nursery products from areas with established sharpshooter populations to areas without established populations requires labor-intensive pesticide applications and visual inspections for viable stages of the insect at both points of origin and destination. Currently, there are no state-approved standardized disinfestation protocols for the prevention and elimination of glassy-winged sharpshooters within nursery crops. The objective of the research presented here is to evaluate a standard set of pesticide treatment protocols to prevent the commercial shipment of viable egg masses of glassy-winged sharpshooters on representative nursery products.

OBJECTIVES
The objective of the research presented here is to evaluate a standard set of pesticide treatment protocols to prevent the commercial shipment of viable egg masses of glassy-winged sharpshooters on representative nursery products. Trials were conducted simultaneously on trees, crape myrtle (*Lagerstroemia indica*, L. 'Zuni"), shrubs, photinia (*Photinia fraseri* Lindl.), and bedding plants, periwinkle (*Vinca minor* L. 'Bowls'). Compounds evaluated for quarantine use include carbaryl, fenpropathrin, deltamethrin and acetamiprid.

RESULTS
Treatment Effects (See Figures 1 and 2).

Lagerstroemia: Carbary (Sevin) provided absolute control for both viable egg masses and emerging nymphs. Applications of carbaryl resulted in 0% viable egg masses and consequently 0% emergence of eggs. There was no successful emergence of a sharpshooter from carbaryl treated plants. Fenpropathrin (Tame) also showed excellent activity with only 1.25% of the egg masses showing any viability with 0.37% of the eggs successfully hatching. Acetamiprid (Tristar) allowed 30.2% of the egg masses to remain viable. This resulted in 8.5% of the eggs on a plant hatching. For acetamiprid, although one-third of the egg masses showed viability, within an egg mass, very few eggs successfully emerged. Deltamethrin showed relatively poor control for preventing successful egg hatch.

Photinia: Carbaryl again provided the best overall control resulting in 1.3% of the egg masses deposited displaying viability. Only 0.8% of the eggs deposited successful emerged on the carbaryl treated plants. Fenpropathrin showed good control with 6.1% of the egg masses showing viability yielding 1.6% of the total eggs successfully emerging. Similar to the results for *Lagerstroemia*, acetamiprid was somewhat intermediate in eliminating viable egg masses (12.2% of the egg masses remained viable) and preventing successful emergence (5.9% of the eggs successfully emerged). Deltamethrin was not significantly different from the control in eliminating viable egg masses or reducing successful emergence.

Vinca: Leaves on the *Vinca* were damaged easily, became necrotic, and fell off prior to the final assessment. Therefore, many of the masses found in the initial count had been damaged and had fallen off the plant. None-the less, as with the above

species, carbaryl provided the best control resulting in 1.8% of the egg masses remaining viable and 1.6% of the eggs successfully emerging. Fenpropathrin allowed 25% of the egg mass to remain viable resulting in 32.3% of the eggs to successfully emerge. Acetamiprid allowed 18.2% of the egg masses to remain viable and 16.6% of the eggs to emerge. Deltamethrin was not successful in eliminating viable egg masses or preventing emergence; the deltamethrin treatment was not significantly different from the control treatment in preventing successful emergence from the eggs.

In all cases control treated plants displayed greater than 90% successful emergence of nymphs from eggs and greater than 95% of the egg masses remaining viable.

CONCLUSIONS

Carbaryl provided the best overall results resulting in near absolute control (>98%) of emerging nymphs on all plant types. Fenpropathrin also achieved very good control. Control of emerging sharpshooters was poorest on *Vinca.* This is largely due to the tremendous number of densely packed leaves within a small plant canopy. Adequate coverage of insecticidal materials is very difficult is such plant types and will be problematic. The likelihood of egg masses moving through a quarantine and treatment program on small canopy, densely foliated plants, we suspect will be much greater and consequently may deserve special considerations.

Surprisingly, deltamethrin did not prevent significant emergence from egg. This is in contradiction to our earlier lab/field trial showing deltamethrin caused significant mortality immediately after emergence. In the latter case, leaves containing experimental egg masses were returned to the lab and observed for emergence from the egg. As nymphs emerged from the chorion, they died. In the experiment presented here, the experimental conditions of this trial (handling and caging) prevented us from observing what happened to the nymphs upon emergence. The actual mortality (as opposed to emergence) was most likely higher as large numbers of dead nymphs were observed in the bags below the caged leaves, and many dead nymphs were observed on the leaves that fell off as they were being removed for assessment. Although the number of viable egg masses that resulted in successful egg emergence was high for the deltamethrin trials, we suspect that the overall mortality induced by this compound was extremely high (based on previous lab trials); but the experimental conditions utilized here prevented us from observing this phenomena. We will adjust our methodology for the winter experiment such that we will try and assess the direct, post-emergence mortality of all treatments.

Counting egg masses and/or eggs was determined four times throughout this experiment: July 14 (initial assessment), July 15 (a double check reassessment), July 17 (after treatment) and July 28-30 (final counts). Initially undetected, egg masses were found on each occasion following the initial July 14 assessment. The occurrence of these additional egg masses may be explained in two ways. First, undetected adults may have been present throughout the trial. This explanation requires that adults survive and remain undetected throughout the trial after 2 disinfestation periods (July 14 and 15), and pesticide applications (July 18). This is unlikely in the extreme as each plant and leaf were examined for adults and nymphs during both July 14 and 15 counts. If either adults or nymphs were found at this point, they were immediately removed. Second, it is highly unlikely that adults or late instar nymphs survived treatment applications or were able to cross sleeve cages to access experimental plants. A much more likely explanation is that the additional egg masses were undetected during the first three counts and were only discovered as each leaf on each plant was examined under a microscope at the end of the trial. At this time, masses are more easily seen because the edge of the egg mass had turned necrotic, a dark outline of the open end of the egg mass. In total, an additional 113 egg masses were detected during the fourth observation period. This accounts for approximately 12-14% of the total number of egg masses observed. The fact that a field crew of 13 professional entomologists and CDFA personnel all highly trained to detect sharpshooter egg masses missed 12-14% of the egg masses present on 300 plants is worrisome and draws into question the utility of the statewide inspection programs. The implications for current egg mass monitoring programs are significant; they may be missing a tremendous number of egg masses (~10% of the total). If this is the case, simply prophylactically treating nursery stock prior to shipment will be more effective in limiting the spread of sharpshooters in the egg stage than inspections.

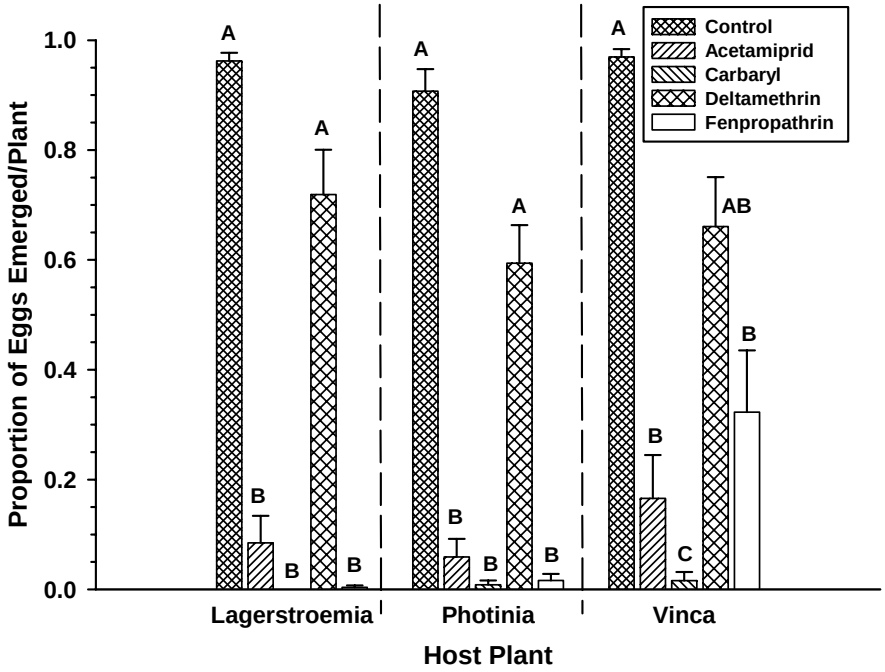

Figure 1. Effect of insecticide application on the proportion of glassy-winged sharpshooter eggs successfully hatching from an egg mass. Different letters over bars, within a species, indicate significant differences among treatments (Kruskal-Wallis test, followed by Nemenyi multiple range test, $P<0.05$)

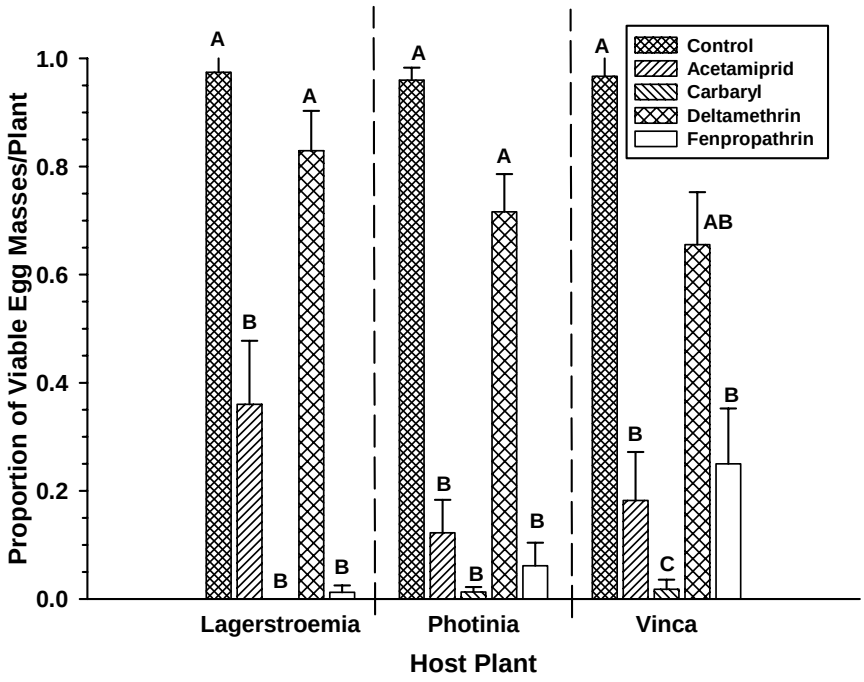

Figure 2. Effect of insecticide application on the proportion of viable glassy-winged sharpshooter egg masses. Different letters over bars, within a species, indicate significant differences among treatments (Kruskal-Wallis test, followed by Nemenyi multiple range test, $P< 0.05$)

FUNDING AGENCIES

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

IMPACT OF LAYERING CONTROL TACTICS ON THE SPREAD OF PIERCE'S DISEASE BY THE GLASSY-WINGED SHARPSHOOTER

Project Leaders:
Richard Redak
Department of Entomology
University of California
Riverside, CA 92521

Matthew J. Blua
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The result reported here are from work conducted from October 2002 to October 2003.

ABSTRACT
A variety of plant and insect treatment combinations were evaluated as to their ability to prevent/limit Pierce's disease of grapes. A combination of neonicotinoids (imidacloprid plus acetamiprid) and kaolin film provided the best protection against glassy-winged sharpshooter, reducing densities by approximately 90%. The same treatment combination reduced sharpshooter oviposition by 75%. Egg parasitism was unaffected by any of the treatments. The layering of neonicotinoids and kaolin limited the incidence of PD to 30% after 18 months, but PD incidence climbed to above 70% in all treatments after 30 months. Antibiotic therapy (metalosate), alone or in combination, did not affect PD incidence.

INTRODUCTION
Solutions to managing and controlling Pierce's disease of grapes are often conceptualized as ways of breaking at least one two-way interaction among the insect, plant, and bacteria components that are required for successful disease spread and propagation. Hypothetical solutions may also involve altering the abiotic and biotic environment within which these interactions take place. On the basis of our understanding of Pierce's disease epidemics, as well as other insect transmitted plant pathogen systems, one single control tactic (especially focused upon the insect) will not be sufficient to substantially reduce vector populations such that the incidence of disease is below an economically acceptable level. One management and control strategy that potentially may be utilized to limit the damage brought about by Pierce's disease involves layering separate vector and disease management tactics together such that vector population densities are reduced, their interactions with grapevines are inhibited or disrupted, and the interface between grapevines and the disease organism, *X. fastidiosa*, is disrupted. Here we report on our efforts to simultaneously implement (i.e. "layer") various control strategies currently available to limit the spread of Pierce's disease transmitted by the glassy-winged sharpshooter, *Homalodisca coagula.*

OBJECTIVES
Our specific objectives are to determine the ability of a variety of treatment and treatment combinations on 1) their ability to reduce glassy-winged sharpshooter density and feeding and 2) their ability to reduce the rate of spread of Pierce's disease in newly planted vineyards. The research site was established in April of 2001 at the Agricultural Operations facility located on the campus of the University of California, Riverside. One thousand grape vines were acquired from SunRidge Nursery in early may and planted on May 16, 2001. The variety utilized in this study is Chardonnay 04 on S04 rootstock. Vines were planted with 6 ft spacing between plants and 12 ft spacing between rows and watered with drip irrigation. At total of 10 rows of 100 vines per row was planted. Treatment and treatment combinations evaluated are 1) imidacloprid at full rate, 2) imidacloprid at 1/2 rate, 3) a combination of imidacloprid plus acetamiprid, 4) metalosate, 5) kaolin, 6) imidacloprid-acetamiprid combination plus kaolin, 7) imidacloprid-acetamiprid combination plus metalosate, 8) metalosate + kaolin, 9) imidacloprid-acetamiprid combination plus kaolin plus metalosate, and 10) control (water only). Treatments involving acetamiprid could not be evaluated until Fall of 2002.

RESULTS
Results indicated that there was a significant difference among treatments with respect to the number of sharpshooters found on experimental plants for 2001, 2002 and 200 (Figure 1). As expected plants treated only with metalosate (a potential prophylactic treatment for Pierce's disease) supported similar numbers of sharpshooters as untreated control plants. Overall plants treated with kaolin demonstrated reduced numbers of sharpshooters relative to the untreated controls during 2001and 2002. Plants treated with imidacloprid exhibited the lowest numbers of sharpshooters. For 2001 and 2002, there were no significant differences in the numbers of sharpshooters found on plants treated with kaolin as compared to the numbers found on insecticide treated plants. Kaolin failed to lower sharpshooter numbers in 2003. No experimental treatment has yet resulted in complete protection from sharpshooters; consequently, all treated plants remain at risk of exposure to *X. fastidiosa*. With the exception of metalosate, all treatments were reasonably effective in reducing sharpshooter numbers throughout the fall season. Within each year, differences among treatments were lost as sharpshooter numbers naturally declined at the end of fall. In all three years, a combination of imidacloprid, acetamiprid and kaolin was most effective at reducing overall sharpshooter numbers; however, it should be noted that a significant number of sharpshooters was found on all treated plants throughout the growing season.

Experimental treatments were also evaluated as to their impacts on sharpshooter oviposition (relative number of egg masses deposited per plant) and sharpshooter egg parasitism by Mymarid parasites. Plants treated with a combination of imidacloprid

plus acetamiprid plus kaolin significantly reduced the number of egg masses deposited on plants. All other treatments were not different from the control with respect to oviposition. None of the treatments significantly affected egg parasitism by Mymarid parasites relative to the control (Figure 2).

Several treatments have significantly reduced the incidence of Pierce's disease symptoms in experimental plants during the first 18 months of the study (Figure 3). Imidacloprid at full rate, kaolin, kaolin plus metalosate, imidacloprid plus acetamiprid plus kaolin, imidacloprid plus acetamiprid plus metalosate, imidacloprid plus acetamiprid plus kaolin plus metalosate all significantly reduced the incidence of Pierce's disease relative to untreated controls. Other treatments and treatment combinations (including just metalosate) did not significantly reduce the incidence of PD. Unfortunately, the incidence of PD was relatively high (30%) even in the most successful treatment. Furthermore, after 30 months, all treatments were showing very high levels of PD (Figure 3).

CONCLUSIONS

A combination of neonicotinoids (imidacloprid plus acetamiprid) and kaolin film provided the best protection against glassy-winged sharpshooter reducing densities by approximately 90%. The same treatment combination reduced sharpshooter oviposition by 75%. Egg parasitism was unaffected by any of the treatments. The layering of neonicotinoids and kaolin limited the incidence of PD to 30% after 18 months, but PD incidence climbed to above 70% in all treatments after 30 months. Antibiotic therapy (metalosate), alone or in combination, did not affect PD incidence. This study was done under very high densities of GWSS. Given the positive results for reducing PD incidence after a single year, further studies are required to evaluate treatment layering as a viable option to manage PD under conditions of lower insect vector density.

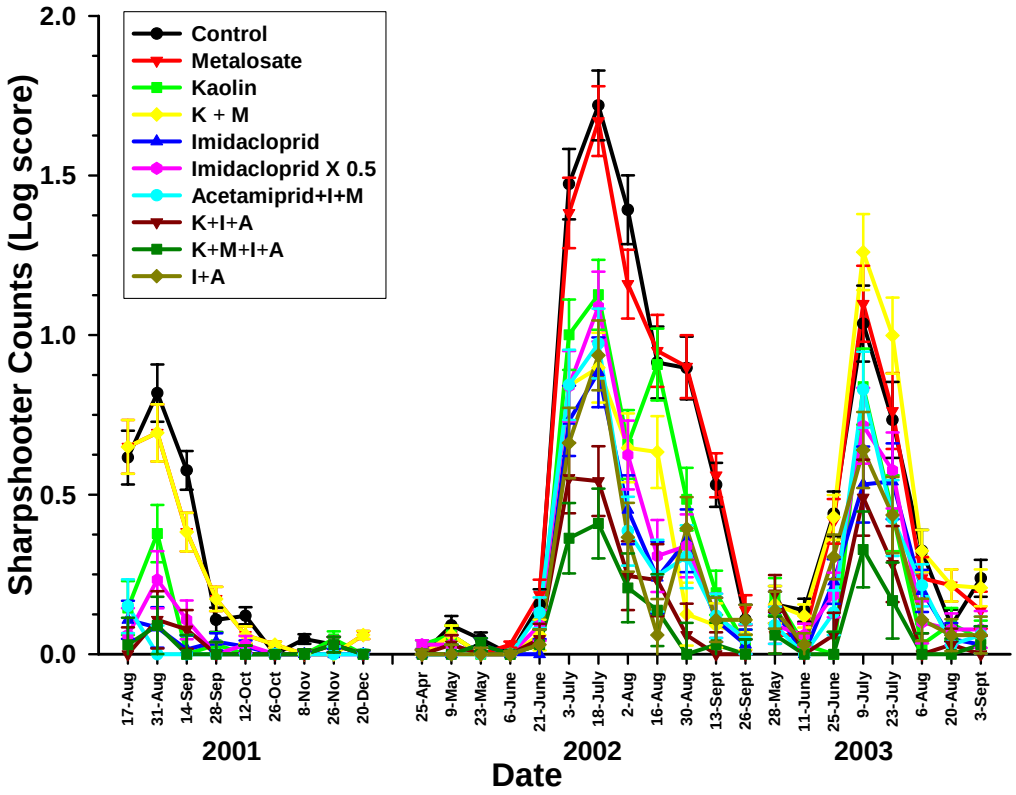

Figure 1. Effect of treatments on numbers of glassy-winged sharpshooters detected in grape plants.

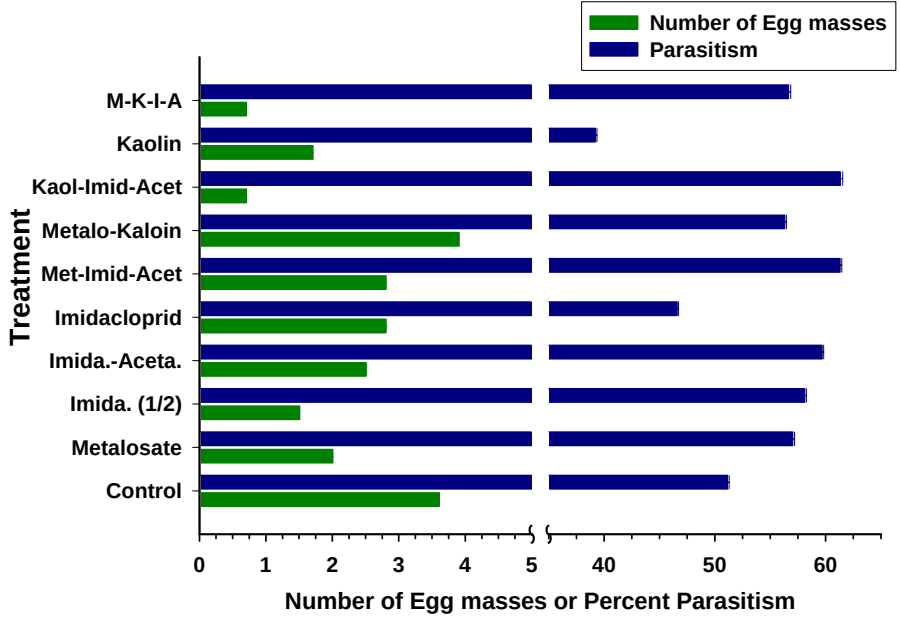

Figure 2. Effect of treatments on sharpshooter oviposition and egg parasitism.

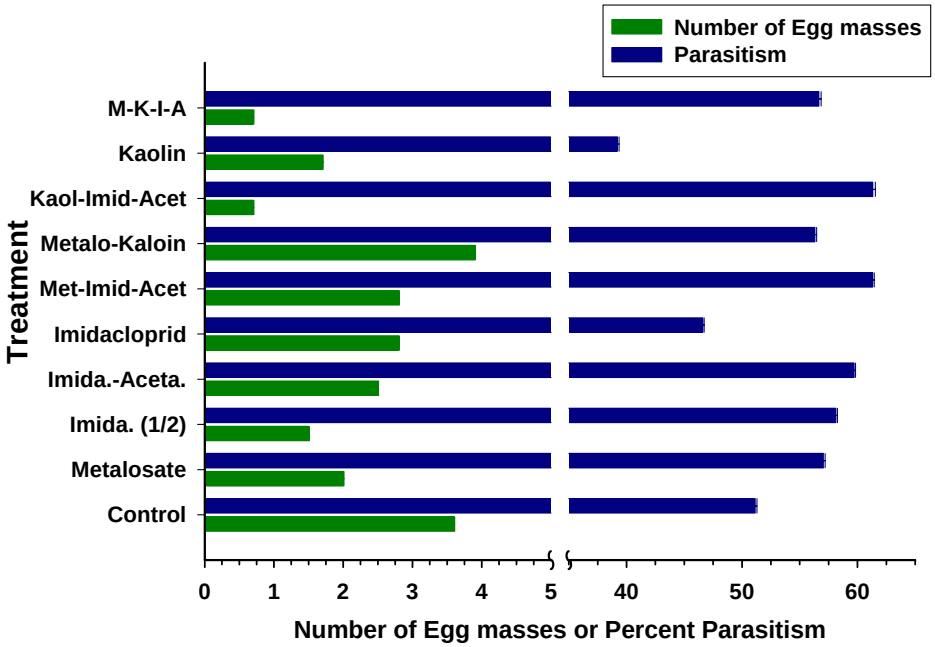

Figure 3. Effects of treatments on incidence of Pierce's Disease.

FUNDING AGENCIES
Funding for this project was provided by the California Department of Food and Agriculture.

IMPACT OF NEONICOTINOIDS AND AN INSECT GROWTH REGULATOR ON NATURAL ENEMIES OF THE GLASSY-WINGED SHARPSHOOTER AND OTHER CITRUS PESTS

Project Leader:
Nick C. Toscano
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:
Nilima Prabhaker & Frank Byrne
Department of Entomology
University of California
Riverside, CA 92521

Steven Castle & Steve Naranjo
USDA-ARS
Western Cotton Research Lab
Phoenix, AZ 85040

Reporting Period: The research on this project was initiated on October 2003.

INTRODUCTION

Relatively few data are available on the direct effects of selective insecticides against natural enemies. The work proposed here will investigate the various toxicological effects against parasitoids and predators of glassy-winged sharpshooter (GWSS) and other citrus pests of three neonicotinoids, imidacloprid (Admire), acetamiprid (Assail) and thiamethoxam (Actara) and one insect growth regulator (IGR), pyriproxyfen, (Esteem). These insecticides were selected because they are currently being used or tested against GWSS immatures and adults and other citrus pests. Direct effects of conventional insecticide applications on parasitoid and predator populations are known, but the effects of systemic neonicotinoid insecticides such as Admire are not documented and evidence needs to be established through laboratory and field testing. These tests will determine the impact of these selected insecticides on the natural enemies of the GWSS and other pest in citrus and grapes. This information will especially useful in GWSS area wide programs or in vineyards where GWSS is present.

The main objective of this research is the development and use of reliable and simple test methods for detecting the susceptibility and tolerance of the egg parasitoids of GWSS to Admire, Assail, and Platinum. Bioassay techniques for testing the responses of GWSS to these insecticides have already been established. The development of bioassay techniques (foliar and systemic) for testing the GWSS natural enemies, will be specific for each insecticide chemistry.

OBJECTIVES

1. Monitor citrus orchards in Kern, Riverside, San Bernardino, and Ventura counties, California, to determine relative abundances of select parasitoids and predators.
2. Develop appropriate methods for establishing baseline toxicity to Actara, Admire, Assail, and Esteem against selected beneficial insects on citrus and grapes.
3. Determine the lethal effects of Actara, Admire, Assail, and Esteem on survivorship of three parasitoids, *Gonatacerus ashmeadi, G. triguttatus, Aphytis melinus,* and a predator, *Rodolia cardinalis* through residues on citrus leaves and contaminated hosts.
4. Determine the sub-lethal effects of the three neonicotinoids and Esteem on emergence, development and foraging behavior of the natural enemies with insect hosts.

RESULTS

The funding for this project was received on October 14, 2003, therefore research has just been initiated. Work is in progress and we are presently investigating the impact of Admire to *G. ashmeadi* and *Aphytis melinus* on field treated citrus leaves.

FUNDING AGENCIES

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

LABORATORY AND FIELD EVALUATIONS OF IMIDACLOPRID, THIAMETHOXAM, AND ACETAMIPRID AGAINST THE GLASSY-WINGED SHARPSHOOTER ON GRAPEVINES

Project Leaders:

Nick C. Toscano
Dept of Entomology
University of California
Riverside, CA 92521

Frank J. Byrne
Dept of Entomology
University of California
Riverside, CA 92521

Steven J. Castle
Western Cotton Research Laboratory
USDA-ARS
Phoenix, AZ

Cooperators:

Jianlong Bi
Dept of Entomology
University of California
Riverside, CA 92521

Nilima Prabhaker
Dept of Entomology
University of California
Riverside, CA 92521

Mac Learned
Bayer CropScience
Paso Robles, CA

Carmen Gispert
UC Cooperative Extension
Riverside County
Riverside, CA

Reporting Period: The results reported here are for work conducted from July 2003 through October 2003.

ABSTRACT

In a comparison of Admire application rates in Temecula vineyards, a 16 oz. per acre treatment proved very effective at attaining xylem sap imidacloprid levels that were necessary to protect vines from sharpshooter feeding. An 8 oz. per acre rate proved to be too inconsistent in terms of the level of protection afforded, while a 32 oz. rate produced unnecessarily high levels of imidacloprid that did not extend the period of protection much beyond that afforded by the 16 oz. rate. In Coachella Valley vineyards, the 16 oz. rate produced levels of imidacloprid within the xylem sap that were extremely short-lived, raising concerns about the stability of this material within this region.

INTRODUCTION

The neonicotinoid insecticide imidacloprid (Admire) has played a significant role in reducing glassy-winged sharpshooter (GWSS) populations in citrus orchards and vineyards in Southern California area-wide management programs. In Temecula, where the first area-wide management program incorporating imidacloprid was initiated in response to a severe Pierces disease (PD) epidemic, remnant GWSS infestations are now associated primarily with untreated tracts of vegetation such as organic citrus, while densities in conventional orchards and vineyards are almost undetectable (Hix et al., 2002). In southern Kern County, GWSS population densities have been similarly reduced as a result of the General Beale Road project, while areas such as Riverside/Redlands that have not yet participated in area-wide management programs still retain high GWSS populations. More recently, the introduction of an area-wide program in Coachella Valley has demonstrated yet again the capacity of imidacloprid treatments to deal very effectively with GWSS infestations on both citrus and grapevines.

Upon completion of an earlier study investigating the uptake, distribution and persistence of systemic applications of two neonicotinoid insecticides, imidacloprid and thiamethoxam, in citrus trees and grapevines (Toscano et al., 2002), we identified several key areas of research that were still required. In this report, we include data from our studies on the uptake and distribution of imidacloprid within grapevines, focusing on the impact of vine age and application rate on the rate of uptake and persistence over time. Data are presented for vineyards in Temecula Valley and Coachella Valley.

OBJECTIVES

1. Determine the impact of soil type and irrigation on the uptake and residual persistence of imidacloprid and thiamethoxam in grapevines;
2. Determine the best combination of application rates and number of applications of imidacloprid in order to maximize and extend protection to vineyards;
3. Determine the absorption, distribution and residual persistence of foliar applications of acetamiprid within grapevines.

RESULTS

Admire Application Rates and Uptake/Persistence

In this component of the study, conducted in two Temecula Valley vineyards, we compared different application rates of Admire applied by drip chemigation at rates of 8, 16 and 32 oz. per acre. The primary goal was to determine the rate of uptake and persistence of the material in vines treated at each rate, thereby giving an indication of the degree of protection afforded grapevines. During the trials, xylem fluid was extracted using a pressure bomb on at least a weekly basis over a

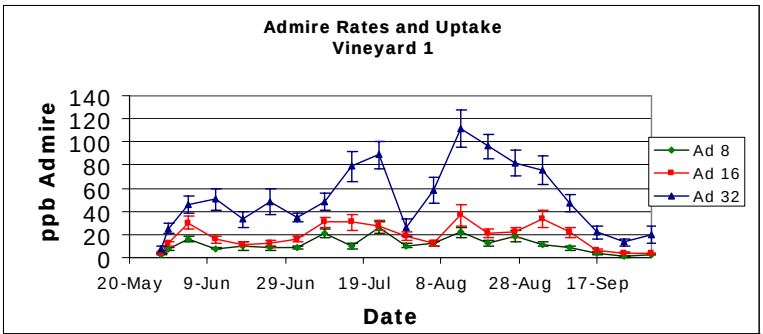

Figure 1. Profiles of imidacloprid titers in the xylem sap of grapevines treated with 8, 16 and 32 oz. per acre Admire on May 28th, 2003

4-month period and the titers of imidacloprid determined using an ELISA technique (Toscano et al., 2002).

In Vineyard I (Figure 1), which consisted of 4-year old Cabernet vines, the 8 oz per acre rate produced an average titer of imidacloprid within the xylem sap of about 10 ppb throughout the experimental period. This is close to the minimum level of material that we believe is necessary to give protection against GWSS feeding. At this low rate, however, many vines were not protected adequately, while others received very good protection. The 16 oz. per acre rate proved more effective in terms of maintaining xylem sap imidacloprid levels equal to or greater than the 10 ppb threshold. Vines remained protected for up to three months. During a similar trial in 2002 in the same vineyard (Toscano et al., 2002), a four month period of protection was afforded the same vines when they were treated with a 16 oz. per acre rate of Admire. In 2002, the vineyard had been treated one month earlier and this may have had a significant impact on the levels of imidacloprid taken up due to more active growth of the vines and a lower overall plant volume through which the material had to disperse (see later section on vine age).

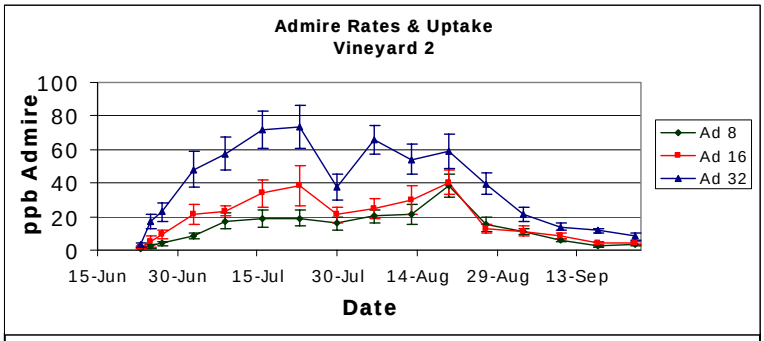

Figure 2. Profiles of imidacloprid titers in the xylem sap of grapevines treated with 8, 16 and 32 oz. per acre Admire on June 19th, 2003

In Vineyard II (Chardonnay grapes) vines were at least 20 years old. Similar rates were applied as in Vineyard I, and the progressive increases in application rates resulted in a concomitant rise in imidacloprid levels within the xylem sap. Although the initial rate of uptake was slower for all three rates in Vineyard II, the overall degree of protection attained from the 8 and 16 oz. rates were better. In both vineyards, the 32 oz. rate resulted in titers of imidacloprid within the xylem sap that were far in excess of those required to protect the vines from sharpshooter feeding.

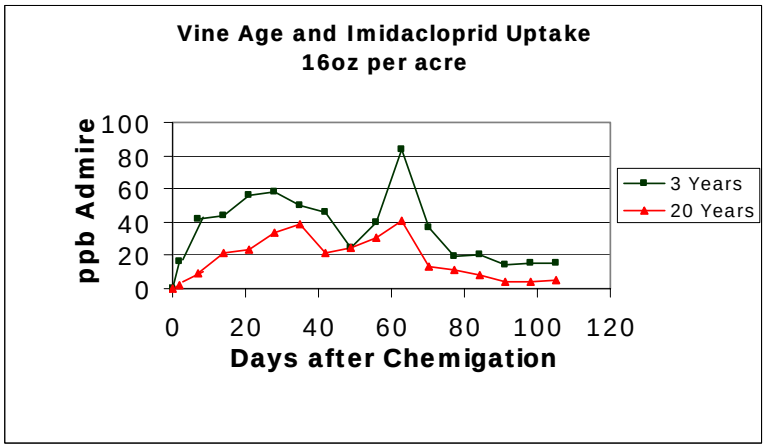

Figure 3. Profiles of imidacloprid titers in the xylem sap of 2-year old and 20-year old grapevines treated with 16 oz. per acre Admire on June 19th, 2003

Vine Age and Admire Uptake/Persistence

The most obvious effect of vine age on the uptake and persistence of Admire applied at 16 oz. per acre was in the rate of initial uptake (Figure 3). In a comparison of uptake into vines aged 2 and 20 years, the imidacloprid titers within xylem sap increased more rapidly in the younger vines. Titers were also consistently higher in the younger vines throughout the evaluation period. It should be noted, however, that the uptake dynamics of Admire applied at 8 and 16 oz. per acre to the 20-year old vines (Vineyard II, Figure 2) were similar, if not somewhat better, than in the 4-year old vines (Vineyard I, Figure 1). Thus, it appears that any 'age-effect' is more likely to be manifested in newly established vines which have substantially less 'volume' through which the insecticide must be transported

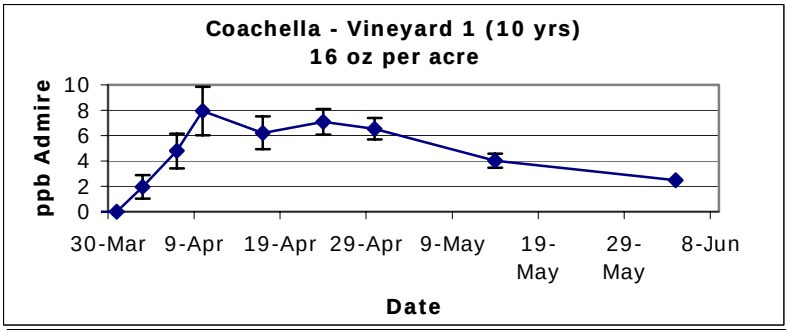

Figure 4. Profile of imidacloprid titers in the xylem sap of grapevines treated with 16 oz. per acre Admire on Mar 31st, 2003

Admire Applications in Coachella Valley

We monitored the levels of imidacloprid in the xylem sap of table grapevines treated with 16 oz. per acre in six vineyards in Coachella Valley. In this study, the growers applied the materials as part of their standard agronomic practices. In short, we found that the behavior of imidacloprid in this region was remarkably consistent. However, in comparison with the Temecula vineyards, the peak titers of imidacloprid within the xylem sap of Coachella grapevines were extremely low and the overall persistence of the material was very short (Figures 4

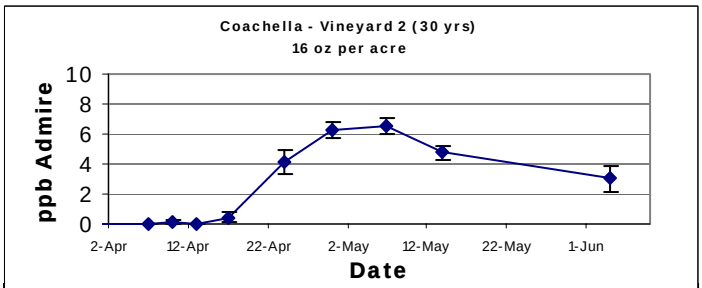

Figure 5. Profile of imidacloprid titers in the xylem sap of grapevines treated with 16 oz. per acre Admire on Apr 3rd, 2003

and 5). In each of the six vineyards tested, a single application of 16 oz. per acre of Admire resulted in xylem sap levels of no more than 8 ppb, which is very close to the critical level required for protection against sharpshooter feeding. In extremely old vines, there was also a significant delay in the initial uptake of material (Figure 5).

CONCLUSIONS

Our studies continue to provide growers with a better understanding of the behavior of Admire within vineyards in the Southern California area. Certainly, there are significant differences between the Temecula and Coachella Valley vineyards and we are investigating the possible causes of these anomalies in our remaining study objectives. What is becoming clear for Temecula

growers is that a single application of 32 oz. per acre is producing an unnecessarily high load of imidacloprid within the plant, whereas a 16 oz. rate can provide adequate control for almost the same period of time. An early season application of 16 oz. per acre, followed by a later application at the same rate, would appear to be the most attractive option for achieving effective xylem sap levels of imidacloprid for protection against sharpshooters. GWSS are known to feed on vines throughout the year, including during the winter dormant phase, so by using the two-16 oz. application strategy growers would be able to extend the window of protection well beyond that afforded to them with a single application of 32 oz. per acre.

The titers of imidacloprid within the xylem sap of vines in Coachella Valley vineyards treated at 16 oz. per acre are of concern. Growers in this region are not receiving the same levels of protection for their vines as Temecula growers. We are investigating several possibilities that may account for these differences, particularly the impact of soil type.

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CHEMICAL CONTROL OF THE GLASSY-WINGED SHARPSHOOTER: ESTABLISHMENT OF BASELINE TOXICITY AND DEVELOPMENT OF MONITORING TECHNIQUES FOR DETECTION OF EARLY RESISTANCE TO INSECTICIDES.

Project Leaders:

Nick C. Toscano, Nilima Prabhaker, & Frank J. Byrne
Department of Entomology
University of California
Riverside, CA 92521

Steven J. Castle
USDA-ARS
Western Cotton Research Lab.
Phoenix, AZ 85040

Reporting Period: The results reported here are from work conducted from October 2002 to October 2003

ABSTRACT

Our research focused primarily on chemical control of the glassy-winged sharpshooter, *Homolodisca coagulata*, a pest of citrus and grapes in California. A number of insecticides from various classes of chemistry were incorporated into this study to evaluate their effectiveness against this pest as well as to establish baseline susceptibility data to each insecticide. The use of insecticides involves some resistance risks in most pests. As such, part of our research was undertaken to monitor the occurrence of any shifts in susceptibility to the principal control agents by comparing the toxicity values for the last two years. Comparison of susceptibility shifts will enable us to analyze the practical implications of any tolerances detected by incorporating such information into well-coordinated recommendations for delaying or combating resistance development in future management programs of this pest.

INTRODUCTION

The first step in examining the effectiveness of insecticides against glassy-winged sharpshooter (GWSS) populations is to monitor for baseline susceptibility. Monitoring results will give comparisons for presence of any cross-resistance patterns between different classes of insecticides. The initial step for monitoring resistance is through development of appropriate bioassay techniques that can establish baseline susceptibility data among various populations. Our goal for the first year was to study the effectiveness of a spectrum of selected insecticides and determine regional differences, if any, to these insecticides. To accomplish this, simple and suitable bioassay techniques were developed to detect toxicological responses of the GWSS to various insecticides. Three techniques, petri-dish, Leaf-dip and Systemic bioassay techniques were described in the previous report (Toscano et al. 2001). Using these techniques, baseline data were determined to selected insecticides against the GWSS over a period of two years. The present report compares the toxicological responses of GWSS to a wide range of chemistry over the past two years.

Once baseline susceptibility data is established, any changes in susceptibility to insecticides through early detection can be invaluable in helping to curtail resistance from progressing to higher levels and frequencies. Knowledge on the rate of resistance development in GWSS to selected insecticides is lacking at the present time. It is well known that resistance does not evolve at the same rate for all pests that come under selection pressure. Many factors, whether genetic, biological/ecological or operational, can influence the rate at which resistance develops in a pest. In the case of GWSS, one method to estimate the potential for resistance risk is to artificially select resistant strains under greenhouse conditions.

In addition to conventional bioassay methods to measure the effectiveness of insecticides, we have proposed to measure biochemically the sensitivity levels of sharpshooter acetylcholinesterases (AChEs) to inhibition by organophosphate (OP) insecticides. Insensitivity of the AChE target-site can seriously impair the effectiveness of OPs in pest control programs. Monitoring populations of GWSS that have been exposed, directly or indirectly, to OPs such as Lorsban® (chlorpyrifos) will enable us to detect resistant AChE alleles should they arise.

OBJECTIVES

1. Develop reliable bioassay technique(s) to evaluate baseline toxicity of insecticides from major classes of insecticides against all life stages of GWSS.
2. Monitor all life stages of the GWSS populations collected from insecticide-treated citrus orchards and vineyards in Riverside, Redlands, San Joaquin Valley and Temecula to determine baseline susceptibility to various insecticides.
3. Investigate the rate of development of resistance to a selected organophosphate (OP), pyrethroid and a neonicotinoid by artificial selection in the greenhouse.
4. Develop electrophoretic techniques to identify esterase profiles in individual GWSS of all life stages including eggs.
5. Develop a microplate assay to measure the levels of sensitivity of GWSS acetylcholinesterase (AchE) variants to inhibition by organophosphate (OP) insecticides commonly used for their control.
6. Monitor GWSS populations throughout California to determine the degree of phenotype variation in esterase and AchE enzymes and how this relates to current pesticide practices.

RESULTS

Objective 1. To assess the effectiveness of various insecticides against the GWSS, a number of bioassay techniques were developed, including foliar as well as systemic applications. Use of appropriate techniques are critical to evaluate insecticidal activity.

Bioassay for contact insecticides

Petri-dish Bioassay: Petri dishes of 60 mm were used for this assay. Agar beds were prepared in the petri dishes for maintenance of citrus leaves for upto a week. Excised citrus leaf discs of the same size as the petri dish were dipped for 30 sec in five concentrations of each insecticide and allowed to dry for an hour before placing on the agar beds. Tests were conducted using 10 contact insecticides: esfenvalerate, cyfluthrin, bifenthrin, fenpropathrin (Pyrethroids), chlorpyrifos, dimethoate (Organophosphates), endosulfan (Cyclodiene), acetamiprid, thiamethoxam (Neonicotinoids), and Pymetrozine. Five GWSS were exposed to the treated leaves in each petri dish covered with a plastic top. Each test was replicated 6 times and included water-only dipped controls. Mortality was assessed after 24, 48 and 96 h. In the case of acetamiprid and thiamethoxam, mortality was assessed after only 4 and 16 h followed by 24 h because of their potency to the insects. No condensation was observed in the petri dishes while maintaining insects for exposure to the treated leaves even over a week.

Leaf-dip Bioassay: Leaf-dip bioassays of attached leaves on citrus plants were conducted in the greenhouse. Five serial dilutions of each of the 9 insecticides were used for dipping the attached leaves and were allowed to dry for an hour. Five adults or immatures were placed in clip cages and attached to the treated leaves. Mortality assessment was similar to that of the Petri-dish bioassays.

Bioassay for systemic insecticides

Sy*stemic Bioassay*: To assess the toxicity of imidacloprid action systemically, the leaf-dip method was modified to accomodate a system that allows excised leaves to take up imidacloprid through the petioles. The uptake and systemic translocation of imidacloprid was accomplished by two methods, a) leaf uptake and b) stem uptake. Excised leaves and stems with two terminal leaves were placed in serial dilutions of imidacloprid in aquapiks to allow uptake for 24 h. Following the 24 h uptake, exposed leaves or stems were placed in aquapiks containing water only. Both citrus and cotton leaves were utilized for this study. Exposure time of the insects to imidacloprid was similar to that of the contact insecticides. Mortality was checked after 24, 48 and 96 h.

Objective 2. To monitor all life stages of the GWSS populations collected from insecticide-treated citrus orchards in Riverside, Redlands and San Joaquin Valley to determine baseline susceptibility to various insecticides. GWSS populations were sampled using sweepnets and bucket samplers from citrus orchards in each of these locations.

For both the Petri-dish and the Leaf-dip technique, toxicity was determined based on the effects of exposure time and location effect. Results showing a two-year comparison of toxicity data to various insecticides using the three techniques are presented in Table 1. In general, GWSS populations are quite susceptible to most insecticides tested. Considerable variation in susceptibility to insecticides has been observed by both techniques. The Petri-dish technique provided stable LC_{50}s with no or low control mortality in 24 h. Mortality increased in the controls over time. Monitoring data for chlorpyrifos indicated a difference of 13- and 15-fold between the two techniques in 24 and 48 h respectively during the first year of testing. Similarly, monitoring data for chlorpyrifos and dimethoate indicated a difference of 10- and 15-fold between the two techniques in the following year. However, no significant changes in responses of GWSS to chlorpyrifos were observed from year to year. No significant differences in LC_{50}s were observed to fenpropathrin using the Petri-dish technique between the Riverside and Redlands populations (LC_{50} = 0.019 to 0.042 ppm). However, a significant difference in LC_{50} was observed to esfenvalerate with the Petri-dish test. Esfenvalerate was more potent to GWSS from Redlands (LC_{50} = 0.00003 ppm) compared to the insects from Riverside (LC_{50} = 0.002 ppm) showing a significant difference of 90-fold. Similarly, Acetamiprid was also quite toxic to GWSS from Redlands with an LC_{50} of 0.003 ppm compared to 0.01 ppm for the Riverside insects using the petri-dish technique. Also the GWSSs from Redlands were more susceptible to Baythroid (LC_{50} = 0.004 ppm) indicating a 10-fold difference compared to Riverside insects (LC_{50} = 0.038 ppm). In all tests mortality increased with time, higher mortality in 48 h compared to 24 h. A slight shift was observed to Esfenvalerate towards lower sensitivity in the second year. Insects from Redlands appear to be more sensitive than other populations. Similarly, Acetamiprid was also quite toxic to GWSS from Redlands with a lower LC_{50} compared to the Riverside or Ventura populations. Among the neonicotinoids, thiamethoxam appears to be slightly less toxic to GWSS populations in 2002 with insects from Redlands showing more sensitivity to Acetamiprid than the previous year. No significant differences in responses of GWSS from various locations to endosulfan were observed. Both IGRs (pyriproxyfen and buprofezin) were non-toxic to the older stages. In conclusion, a two-year comparison of toxicity studies shows that GWSS are still quite susceptible to all insecticides tested so far with small variations between populations from different regions.

Results for imidacloprid toxicity as shown by LC_{50}s indicated no significant difference in 24 h between the Riverside and Redlands insects. However, with longer exposure time, a significant difference was observed between the responses of GWSS from the two locations (LC_{50} = 0.0008 ppm for Redlands insects vs. 0.015 ppm for Riverside insects).

Toxicity studies of all stages of GWSS to various insecticides are still underway and will be completed by next summer. Pyriproxyfen appears to be fairly toxic to the GWSS eggs when less than 24 h old. Treatment of GWSS eggs with pyriproxyfen appears to be critical and should be aimed at eggs less than 24 h old. Buprofezin appears to be toxic to the younger stages of GWSS.

Objective 3. Selection of GWSS strains that are tolerant to an OP (chlorpyrifos), a Pyrethroid (fenpropathrin) and a neonicotinoid (imidacloprid) was undertaken and selected strains were maintained for two generations. Further selection is underway to maintain survivors for a few more generations for further studies.

Objective 4. Develop electrophoretic techniques to identify esterase profiles in individual GWSS of all life stages including eggs. An electrophoretic technique has been developed which enables the esterase profiles of individual insects and eggs to be determined. With this technique, the GWSS and STSS (smoke-tree sharpshooter) can be readily distinguished (Figure 1). There was some degree of esterase polymorphism present in both species, and highly active esterases were also identified. The latter could potentially have a role in the detoxification of pyrethroids, as occurs in many other important insect pests such as aphids and whiteflies.

Objective 5. Develop a microplate assay to measure the levels of sensitivity of GWSS acetylcholinesterase (AchE) variants to inhibition by organophosphate (OP) insecticides commonly used for their control. During the past season, we have continued to monitor AChE sensitivity levels within GWSS populations. To date, we have not detected any resistant alleles in populations (Table 2). The lack of resistance alleles is encouraging for growers and is an indication that current OP use (especially Lorsban) has not selected for resistant populations.

Objective 6. Monitor GWSS populations throughout California to determine the degree of phenotype variation in esterase and AchE enzymes and how this relates to current pesticide practices. To ascertain the likely involvement of esterases in the differential expression of pyrethroid toxicity to GWSS, we compared GWSS populations from Riverside and Redlands. For two consecutive years, the Redlands population was consistently more susceptible to esfenvalerate (Toscano et al., 2002). Frequency distributions of esterase activity showed that esterase levels were generally higher in the Riverside population, suggesting a link between esterase activity and susceptibility to this pyrethroid (Figure 2). We are currently studying the esterase band(s) contributing to the extra activity in the Riverside insects.

CONCLUSIONS

Bioassay techniques were developed to determine the relative toxicity of insecticides to GWSS. Baseline toxicity was established to conventional chemistry, three neonicotinoids and two IGRs. Based on these results, GWSS populations appear to be susceptible to all insecticides tested so far. However, monitoring data showed geographical differences in LC_{50}s, e.g. Redlands populations were more susceptible to most compounds than Riverside populations. These results suggest that the bioassay techniques were suitable and sensitive in detecting even small differences in responses of GWSS to various insecticides. Monitoring results of GWSS also demonstrated seasonal variations in their responses to various insecticides. Selection for resistance to specific insecticides was accomplished for 2 generations. The biochemical basis of GWSS responses to certain insecticides was determined using a microplate assay to measure acetycholinesterase activity.

FUNDING AGENCIES

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

Table 1. A two year comparison of toxicological responses of GWSS to various insecticides.

Insecticide Class	Insecticides	Sample Location	2001		2002	
			LC_{50} (ppm) Petri dish	LC_{50} (ppm) Leaf dip	LC_{50} (ppm) Petri dish	LC_{50} (ppm) Leaf dip
Organophosphates	Chlorpyrifos	Riverside	0.001	0.013	.0038	.0124
		Redlands	0.001	0.015	.0067	.0562
		Ventura	0.005	0.032	0.00208	.045
	Dimethoate	Riverside			0.0091	.038
		Redlands			.0176	.0932
		Ventura			.02986	.0699
Pyrethroids	Bifenthrin	Riverside			0.018	0.046
		Redlands			0.012	0.055
		Ventura			0.006	0.0812
	Cyfluthrin	Riverside	0.038		.00230	.00179
		Redlands	0.004		.0221	.252
		Ventura			.00439	.33841
	Esfenvalerate	Riverside	0.0027	0.022	0.006	0.0102
		Redlands	0.00003	0.00004	0.0009	.0042
		Ventura			0.009	.034
	Fenpropathrin	Riverside	0.042	0.168	.044	.551
		Redlands	0.019	0.012	.0812	.376
		Ventura			.0202	.1431
Cyclodiene	Endosulfan	Riverside	0.006		.00832	0.0723
		Redlands	0.003		.00349	.01953
		Ventura			.00104	.0089
Neonicotinoids	Acetamiprid	Riverside	0.01	0.091	0.005	0.072
		Redlands	0.003	0.008	0.0009	0.014
		Ventura	0.04	0.097	0.025	0.074
	Imidacloprid	Riverside		1.64		0.08
		Redlands		0.61		0.034
		Ventura		1.92		0.121
	Thiamethoxam	Riverside	0.0037	0.0085	0.003	0.004
		Redlands	0.0004	0.0012	0.002	0.008
		Ventura	0.0052	0.0093	0.009	0.020

Figure 1. Esterase profiles of individual adult GWSS (lanes 1-7) and STSS (lanes 8-14).

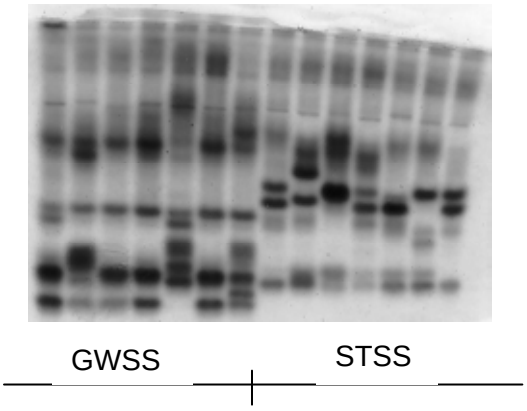

Table 2. Survey of AChE sensitivity in individual GWSS collected from southern California orchards.

Source	Insects tested	Sensitive to 10µM paraoxon (%)
Riverside	343	100
Mentone	94	100
Redlands	47	100
Temecula	53	100
Piru	47	100

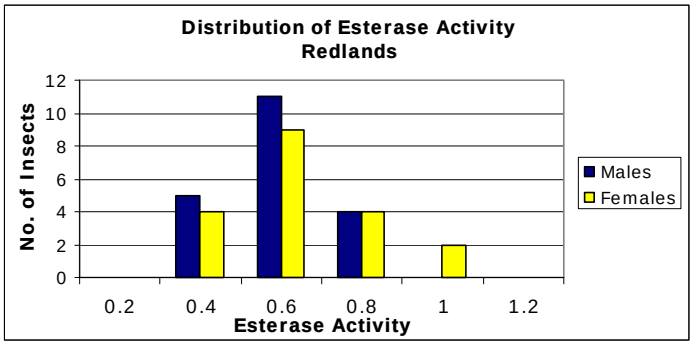

Figure 2. Frequency distributions of esterase activity in individual GWSS adults collected in 2002 from citrus orchards in Redlands.

HOST PLANT INFLUENCE ON THE REPRODUCTIVE ECOLOGY OF THE GLASSY-WINGED SHARPSHOOTER AND ITS SUSCEPTIBILITY TO INSECTICIDES

Project Leaders:

Nick C. Toscano
Department of Entomology
University of California
Riverside, CA 92521

Nilima Prabhaker
Department of Entomology
University of California
Riverside, CA 92521

Steven J. Castle
USDA-ARS
Western Cotton Research Lab.
Phoenix, AZ 85040

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

INTRODUCTION

Few examples of certain pests in the literature of susceptibility changes according to dietary intakes, i.e. different host plants, leads to the question of whether plant species utilized by glassy-winged sharpshooter (GWSS) are potentially influencing its susceptibility to insecticides. As a widely polyphagous species, GWSS likely encounters a diversity of plant compounds even though it feeds principally on less-concentrated xylem fluid. Secondary plant compounds are translocated within vascular tissues and are ingested by vascular feeders. If insecticide toxicities to GWSS vary according to host plant, then the magnitude of potential differences should be elucidated to more clearly understand the performance of insecticides being used against GWSS. Knowledge of susceptibility differences due to host plants, or any other factor that could potentially alter susceptibility, is essential to avoid misleading conclusions concerning the relative effectiveness of a particular insecticide. The more that is understood about how a principal method of control operates in the environment against the pest, the stronger will be an IPM program based on the recognition of how well each component performs. The key to accomplishing efficiency in chemical control is to know the ecology of the target pest, make appropriate insecticide selections according to the conditions of the crop, and time the application to attack the life stage(s) most vulnerable to the specific activity of the insecticide.

OBJECTIVES

1. Evaluate the influence of various host plants on the reproductive status of GWSS adult females, and determine if GWSS selectively utilizes particular host plants for oviposition.
2. Determine differences in susceptibility to select conventional insecticides and neonicotinoids on treated foliage from five host plants.
3. To determine if the host plant effect on susceptibility to insecticides varies after colonization on respective host plants vs. testing on foliage of host plants.
4. Investigate if any susceptibility differences in GWSS reared on various host plants are related to detoxification enzymes, amino acids and sugars.

RESULTS AND CONCLUSIONS

The award for this proposal was received on October 14, 2003.

Objective 1: The influence of various host plants on the reproductive status of GWSS

Based on observations in 2001 in Riverside, California citrus, there were 2 major peaks of reproductive activity among GWSS adults. The first was during the period from late February to mid-April when mating and oviposition was heavily concentrated in citrus only, while the second was during late July through September when reproduction occurred in both citrus and in ornamental plants and many weedy species located outside of citrus. This objective will be pursued in February 2004 and July 2004 at which time collections of adults will coincide with each period of reproductive activity, spring and summer. Adult collections that are made at that time will be sexed, paired and released into 75 x 75 x 75 cm cages that contain four plants potted separately in 20 cm containers. The four plant species to be used will be Valencia orange, okra, basil, and bush bean. Preliminary observations of GWSS colonies during 2001 showed that all four plants species were accepted as feeding hosts, but with definite preferences for basil and orange over okra and bean.

Objective 2: Differences in susceptibility to select conventional insecticides and neonicotinoids

Differences in susceptibility to select conventional insecticides (Asana, Lorsban) and neonicotinoids (Admire, Assail) on treated foliage has been determined on two hosts at this time and will be continued on other host plants during spring and summer. Insects from Redlands appear to be more susceptible in general to the above insecticides compared to Riverside populations. Insects from other other locations have not been tested for comparison at this time.

Objective 3: The host plant effect on susceptibility to insecticides

To determine if host plant effect on susceptibility to insecticides varies after colonization of GWSS on respective host plants vs. testing on foliage of host plants, three GWSS populations are being established on orange, lemon and cotton at this time. After the colonized insects have been maintained for 2-3 generations on these hosts, tests will be conducted on these insects as well as on field collected insects to compare susceptibility differences to select insecticides. In spring more collections of GWSS populations will be made to allow establishment on the remaining hosts, okra, lemon mint and soybean.

Objective 4: Susceptibility differences in GWSS reared on various host plants
Investigation of objective 4 to see if any susceptibility differences in GWSS reared on various host plants are related to detoxification enzymes, amino acids and sugars will follow after successful completion of objective 3.

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