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Pierce's Disease Control Program
California Department of Food and Agriculture
1220 N Street, Room 325
Sacramento, California 95814
Telephone: (916) 651-0253
Fax: (916) 651-0275
http://www.cdfa.ca.gov/phpps/pdcp
E-mail: pdcpinfo@cdfa.ca.gov

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Taxonomic Note:
A recent paper (see citation below) has prompted a change in the scientific name of the glassy-winged sharpshooter (GWSS). The scientific name *Homalodisca coagulata* (Say, 1832) has been replaced with the name *Homalodisca vitripennis* (Germar, 1821). Accordingly, this document uses *Homalodisca vitripennis* as the scientific name for GWSS.

- Reference: Takiya, D.M., S.H. McKamey, & R.R. Cavichioli. 2006. Validity of Homalodisca and of H. vitripennis as the name for glassy-winged sharpshooter (Hemiptera: Cicadellidae: Cicadellinae). Annals Entomol. Soc. Amer. 99(4): 648-655.
- Source: Serguei V. Triapitsyn (UC Riverside) and David J.W. Morgan (CDFA).

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Section 1: Vector Biology and Ecology

MODELING SHARPSHOOTER TRANSMISSION OF *XYLELLA FASTIDIOSA*

Project Leader:
Rodrigo Almeida
Dept. Environ. Sci., Policy & Mgmt.
University of California
Berkeley, CA 94720
Rodrigo@nature.berkeley.edu

Researcher:
Matthew Daugherty
Dept. Environ. Sci., Policy & Mgmt.
University of California
Berkeley, CA 94720

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

ABSTRACT

The dynamics of vectored diseases are governed by the interplay of a variety of biological and ecological factors including: vector behavior and demography, pathogen acquisition and inoculation efficiency, host resistance, and the role of environmental factors in mediating these processes. We are studying some of the ecological and biological traits that influence the transmission efficiency of *Xylella fastidiosa (Xf)* to grapes by the blue-green and glassy-winged sharpshooters. In particular, we are examining how sharpshooter abundance, acquisition and inoculation periods on plants, and sex affect transmission efficiency for each of these species. This work will contribute to a more mechanistic understanding of *Xf* transmission that will be used to develop biologically realistic models of disease dynamics, providing a platform for evaluating the efficacy of different Pierce's disease management strategies.

INTRODUCTION

Pierce's disease (PD) epidemiology is complex because of the interplay among several *Xylella fastidiosa (Xf)* insect vectors and host plant species - which likely contribute to variability in transmission efficiency and patterns of disease spread in the field. To date the only quantitative description of *Xf* transmission is that of Purcell (1981). Our goal is to further refine this model via experimental estimation of additional ecological and biological parameters likely to govern transmission efficiency of blue-green and glassy-winged sharpshooters. We are especially interested in how vector abundance, acquisition and inoculation periods, temperature, and vector species, and sex may contribute to heterogeneous transmission efficiencies. Results from the new experiments will be used in conjunction with previously published results on the biology of *Xf* transmission to refine models of *Xf* transmission.

OBJECTIVES

1. Determine the effect of temperature, vector numbers and time on sharpshooter transmission of *Xf*.
2. Develop a model to describe sharpshooter transmission of *Xf* as a function of variables that affect efficiency.
3. Determine if *Xf* colonization of vectors affects their fitness.

RESULTS

This project is being initiated. We will first focus our studies on how sharpshooter abundance and inoculation period affect, independently, transmission – these two variables were treated as interchangeable by Purcell (1981). In this experiment we varied sharpshooter number (1, 2, or 4 adults - each species separately) fully crossed with inoculation access period (0.5, 1, 2, 4 days - access acquisition period constant at 4 days) and measured the probability of transmission to grape seedlings. Data are currently being collected, therefore we have no results to report at this time.

REFERENCES

Purcell, A. H. 1981. Vector preference and inoculation efficiency as components of resistance to Pierce' disease in European grape *Vitis vinifera* cultivars. Phytopathology 71: 429-435

FUNDING AGENCIES

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

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

Project Leaders:

Blake Bextine
University of Texas
Department of Biology
Tyler, TX 75799

Matthew J. Blua
University of California
Department of Entomology
Riverside, CA 92521

Cooperator:

Brian C. Jackson
University of Texas
Department of Biology
Tyler, TX 75799

Reporting Period: The results reported here are from work conducted July 2004 to September 2006.

ABSTRACT

In this report, we describe quantitative aspects of *Xylella fastidiosa* (*Xf*) transmission by the glassy-winged sharpshooter (GWSS). In previous studies, we discovered correlations between the number of *Xf* cells acquired by GWSS and acquisition access period, and total ingestion time on *Xf* sources. On the other end of the disease cycle, correlations were detected between the number of *Xf* cells inoculated into plant stems and the length of inoculation access periods (IAP), and the number of probes. In the study reported here, correlations between the number of cells inoculated into a plant and IAP or number of probes were consistent when the IAP was restricted to 30, 60, 90, or 120 minutes.

INTRODUCTION

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

Recent advancements in technology allow us to examine quantitative aspects of *Xylella fastidiosa (Xf)* transmission with greater sensitivity and at lower titers of cells than with traditional means. This includes two techniques we have mastered in our laboratories. First, we are currently using a quantitative real-time (QRT PCR) technique in conjunction with commercially available DNA extraction kits to detect and quantify low titers (currently ca 1×10^1 cells) of *Xf* in plant and insect tissue. Second, we have developed a low-cost method to rapidly extract DNA from the glassy-winged sharpshooter (GWSS) and plant tissue in 96-well micro-titer plates. In preliminary laboratory experiments *Xf* titer was quantified in plant tissues following inoculation by single infectious GWSS.

It is intriguing that species of sharpshooters differ widely in transmission efficiency. Transmission efficiency ranges from a high of over 90% for the blue-green sharpshooter (*Graphocephala atropunctata*) to 1% for several other including *Oncometopia facialis, Acrogonia virescens,* and *Homalodisca ignorata* (7). Recently, rates of *Xf* transmission efficiency for the GWSS from grapevine to grapevine were found to be as high as 20%. These observations beg two questions: First, what aspects of *Xf* transmission by sharpshooter vectors vary in ways that cause a wide range in efficiencies among vectors? Second, can we exploit an understanding of transmission efficiency to reduce PD spread? We seek to understand quantitative aspects of *Xf* transmission by GWSS. We are hopeful that this unique approach to investigating the transmission of an insect-vectored plant pathogen will lead to new tactics to manage disease spread.

In the pursuit of better understanding the interactions between GWSS and *Xf* during transmission events, we have developed a model system. *Xf* bacterial cultures were scraped from plates and suspended in a sterile suspension. This bacterial suspension was infiltrated into cut *Chrysanthemum grandiflora* stem (Bextine et al. 2004). GWSS were caged in snap cap vials on stems (Figure 1). Survival through the acquisition access period (AAP) indicated effective feeding because starving these insects for 48 h resulted in 100% mortality (Bextine et al. 2004). After the AAP, GWSS were placed on *Xf*-free chrysanthemums for 48 h, so that any detection of bacteria in subsequent inoculation assays would be associated with transmission and not stylet contamination (Figure 2). Surviving GWSS were transferred to sterile vials containing a fresh chrysanthemum stem cutting. The insects were exposed to a stem for an inoculation access period (IAP). GWSS and stems were tested for the presence of *Xf* by QRT PCR. While the rate of *Xf* transmission was higher than previously reported (Almeida and Purcell 2003-a, b, Costa et al. 2000), we feel this is a fair assessment of the insects' ability to move the bacterium from an infected stem to a non-infected one.

Figure 1. GWSS feeding on a cut stem infused with *Xf*.

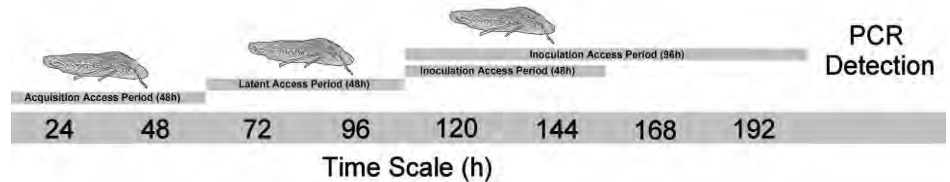

Figure. 2. Artificial Pierce's disease cycle for determination of *Xf* transmission.

OBJECTIVES

Our long-term goal is to understand quantitative aspects of the process of *Xf* transmission by GWSS in order to develop a means of reducing the efficiency with which it spreads the pathogen from an infected plant to a non-infected one. Our specific objectives for this project are to:

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

RESULTS

Quantitative Real-Time Polymerase Chain Reaction

We developed the capacity to quantify as few as 5 *Xf* cells in both in plant tissue and insect vectors by quantitative real-time polymerase chain reaction (QRT PCR) (Bextine et al. 2006, Oliveira et al. 2002). Our QRT PCR diagnostic technique is performed in a Rotor Gene 3000 (Corbett Research, Australia) using iQ Supermix (Bio-Rad Laboratories Inc., Hercules, CA) in 20µl reactions with a new *Xf*-specific primer set (set 6) and probe (BCJ probe) based on the *gyrB* gene.

Inoculation Access Period

A bacterial suspension was made by suspending bacterial cultures scraped from plates in sterile phosphate buffered saline (PBS) by gentle shaking. 10 cm sections of cut *Chrysanthemum grandiflora* were artificially inoculated with *Xf* by forcing the bacterial suspension through by attaching a 10cc syringe to the proximal end of the stem and applying pressure until fluid was seen coming out of the distal end. Field-collected GWSS were allowed to feed from these stems for an acquisition period of 48 h. The insects were then exposed to a clean plant for a latent period of 24 h to ensure that detected *Xf* were not due to contaminated stylets. Surviving GWSS were then allowed to feed on sterile 5 cm cut *C grandiflora* stems for an IAP of 30, 60, 90, or 120 minutes. Finally, GWSS were removed from the cutting and DNA was extracted by vacuum extraction techniques followed by the Qiagen DNeasy tissue kit. Plant inoculation targets were crushed in a lytic buffer in an Agdia bag, then DNA was extracted using the Qiagen DNeasy tissue kit. QRT-PCR was run to detect *Xf* cells, using (set6 primers and BCJ probe1) and optimized run conditions. SYBR green melt curves were used to verify amplification products.

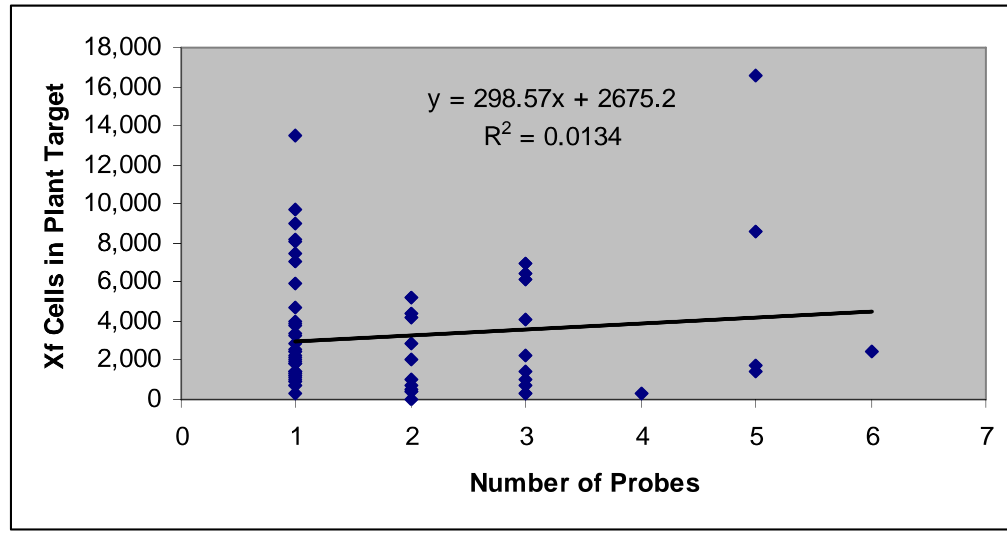

Figure 3. *Xf* cells vs. the number of probes into the plant target by GWSS.

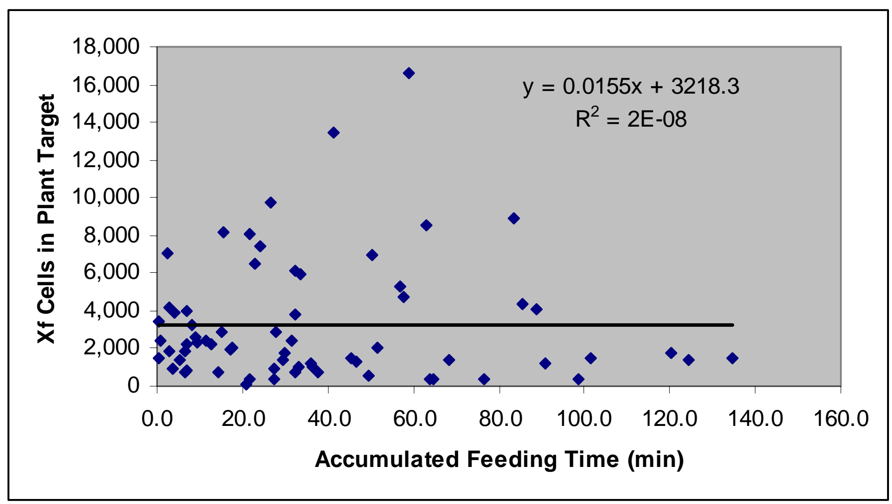

Figure 4. *Xf* cells vs. the total accumulated feeding time of GWSS on the plant target.

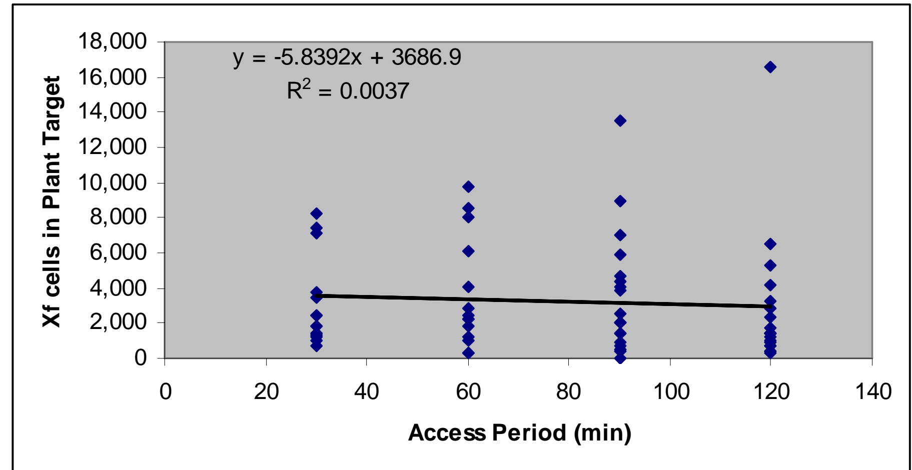

Figure 5. *Xf* Cells vs. Access Period of GWSS to plant target.

Further replicates were relatively consistent with the preliminary data. We are currently finishing the data set, so full statistical evaluation has not occurred. However, there was a positive correlation between the number of probes into a target plant and the number of *Xf* cells transmitted (Figure 3). There appears to be little correlation between accumulated feeding time and the number of *Xf* cells in the plant target (Figure 4). In other words, prolonged feeding by GWSS does not result in a greater number of *Xf* cells being transmitted. Finally, there was a slightly negative correlation between the access period and the number of *Xf* cells in the plant target (Figure 5). This is not unexpected, as the maximum access period was so short that the number of probes was not well correlated with the access period, with some insects feeding for the whole period, some probing extensively but feeding little, and some not feeding at all. Over longer access periods (hours and days), there will be more opportunities to probe, and thus greater transmission of *Xf* is expected.

CONCLUSIONS

Overall, the high degree of variability in transmission rates indicates that transmission is a complex event. Whether or not *Xf* is sheared from the foregut walls may not be easily predicted based on time feeding or number of probes alone (evidenced by low R^2 values), although trends may be shown. As we finish this data set and statistical analysis is applied to these events, we will determine if one acquisition probe is as good as 6 probes or if the positive correlation is due to an artifact of the methods used. It is also important to note that in our study, the distribution of *Xf* in stems is homogeneous, as opposed to an aggregated distribution of *Xf* in stems which is what we would expect from an diseased plant in the field. Additionally, significant amounts of bacteria may be transmitted to a plant by an insect that has probed only once, or fed for only a matter of minutes; pesticides that require insects to feed on the plant may prevent secondary transmission (acquisition from an infected grapevine and transmission to another within the same field) and lower the vector population, but will not entirely prevent transmission. With regard to number of cells vs. access period or accumulated feeding time, the critical time period might occur before 30 minutes. In our initial studies, we looked at these transmission events on a scale of 24, 48, and 96

hours. In this reporting period, we looked at shorter periods (30, 60, 90, or 120 minutes). In other words, the statistically significant slopes of the line generated if time periods might be between 1 to 10 minutes.

Disease epidemics involving *Xf* depend on the transmission of the bacterium from an infected host to an uninfected host by an insect vector. Successful transmission is a function of two major events: acquisition and inoculation. Our studies examine the interactions that involve GWSS feeding behaviors that are associated with *Xf* acquisition and inoculation, and the probability that these behaviors will lead to plant infection. The quantitative aspects of our studies are unique, due in part to technologies that allow us to quantify low numbers of *Xf* cells. Understanding these associations will allow the development of plant protection tactics that take advantage of critical "weak links" in the transmission process.

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DISPERSAL AND MOVEMENT OF THE GLASSY-WINGED SHARPSHOOTER AND ASSOCIATED NATURAL ENEMIES IN A CONTINUOUS, DEFICIT-IRRIGATED AGRICULTURAL LANDSCAPE

Project Leaders:

Russell L. Groves
Department of Entomology
University of Wisconsin
Madison, WI 53706

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

James Hagler
USDA-ARS, ALARC
21881 Cardon Lane
Mariscopa, AZ 85239

Collaborators:

Rodrigo Krugner
Department of Entomology
University of California
Riverside, CA 92521

Hannah Nadel
USDA-ARS, CDPG, SJVASC
9611 S. Riverbend Ave
Parlier, CA 93648

David Morgan
CDFA Mt. Rubidoux Field Station
Rubidoux, CA 92501

Joseph Morse
Department of Entomology
University of California
Riverside, CA 92521

Drake Stenger
USDA-ARS, SJVASC
9611 S. Riverbend Ave.
Parlier, CA 93648

Robert Luck
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted May 2005 through August 2006.

ABSTRACT

A combination of field and laboratory experiments in this study have been designed to advance our understanding of the operative host-plant factors utilized by adult glassy-winged sharpshooters (GWSS) and associated natural enemies as long-range cues to locate feeding and oviposition hosts in a complex agricultural landscape. Specifically, a second year of field studies have been conducted to determine the influence of continuous deficit irrigation regimes implemented in sweet orange cv. 'Valencia' oranges on the population dynamics of GWSS and other associated natural enemies. Dispersal and population dynamics of GWSS were monitored under continuous irrigation treatments receiving 60%, 80%, and 100% of evapo-transpiration (ET_c) rates. Similar to the results obtained in our 2005 season, citrus trees irrigated at 60% ET_c had warmer leaves, significantly higher xylem matric potential, and fewer adult and immature GWSS than experimental trees irrigated with 80% and 100% ET_c. Mean numbers of adult and nymphal GWSS collected from beat samples and observed in visual inspections were numerically higher in the 80% versus 100% ET_c treatments. In caged experiments using sweet orange cv. 'Washington navel' and avocado cv. 'Hass' maintained under different continuous deficit irrigation illustrated GWSS population shifts that occurred between plants. Adult GWSS showed a preference for contact with surplus-irrigated plants of both species compared with plants under continuous deficit irrigation, with a stronger response evident in the avocado trials. During preliminary nutrition trials with overwintered adults, GWSS that landed on plants showed a slight preference for settling on plants fertilized with ammonium versus nitrate averaging over 3 trials. An olfactometer system for studying the response of GWSS to host-plant volatiles has been built and the airflow dynamics adjusted to equally integrate odor fields from humidity or volatile sources. However, evaluation of the data (number of insects landing on the target) to date shows no conclusive differences among a variety of treatments, suggesting that GWSS may not use olfactory cues during host location, or that olfaction is used only in conjunction with visual cues. Identifying how the dispersing lifestages of GWSS locate and exploit specific host species will begin to provide the necessary information required to develop strategies for control of this highly mobile insect and the spread of *Xylella fastidiosa* into susceptible crops.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) is a highly polyphagous and mobile insect utilizing numerous plant species as both feeding and oviposition hosts (Adlerz, et al. 1979, Daane et al. 2003, Groves et al. 2003). Recent research has documented that different host plant species are not equally utilized by all GWSS lifestages. Mizell and Andersen (2003) report that host plant xylem chemistry plays a key role in the regulation of GWSS feeding and oviposition over a wide range of host plant species. Similarly, Daane and Johnson (2003) concluded that ornamental landscape plant species greatly influence GWSS seasonal population biology. Specifically, ornamental species which favorably support adult GWSS oviposition and feeding do not equally support comparable nymphal populations. Although significant new information has become available regarding the sequence of hosts in which GWSS populations thrive, little is understood about the host-location strategies of GWSS, which are critical behavioral responses that assist the insect in locating suitable hosts. Successful insect-host associations depend upon an insect's ability to locate a suitable host(s) in a complex, heterogeneous landscape. Mechanisms of host location in many phytophagous insects are often mediated by long-range, semiochemical cues arising from their host plant(s), which vary by plant physiological conditions including nutrition (available nitrogen and carbohydrate), xylem water potential, and plant age or developmental stage (Finch 1986). Similarly, we have an incomplete understanding of host-selection cues utilized by the mymarid egg parasitoids of GWSS, which may involve the host (GWSS egg mass), the host plant, or a combination of both. Ongoing experiments in this study will increase our understanding of the operative host-plant factors utilized by adult GWSS and associated natural enemies as cues to locate feeding and oviposition hosts in a

complex agricultural landscape. Identifying how the dispersing lifestages of GWSS locate and exploit specific host species will begin to provide the necessary information required to develop strategies for control of this highly mobile insect and further limit the spread of *Xylella fastidiosa (Xf)* movement into susceptible crops.

OBJECTIVES

1. Evaluate host-plant factors utilized by adult GWSS and associated natural enemies as long-range cues to locate feeding and oviposition hosts in a complex agricultural landscape.
2. Monitor adult GWSS movement and host selection behavior, ovipositional preference, and nymphal population performance on host plants maintained under continuous irrigation deficits

Objective 1

Olfactometer

An olfactometer system for studying the response of GWSS to host-plant volatiles has been built and the airflow dynamics adjusted to equally integrate odor fields from humidity or volatile sources. Bioassays have been performed using spring adult GWSS collected from infested areas in Bakersfield and Fillmore, CA area. Behavioral responses of the adult insect replicates tested have been evaluated using the Noldus *Observer XT* software in an effort to measure the accuracy of taped recordings. Preliminary bioassays compare the level of GWSS responses to singly presented, humidified odor plumes. Preliminary tests demonstrate no differential response between the sexes; and to date, no sexual difference has been found in other leafhoppers orienting to plants or plant odors. However, evaluation of the data (number of insects landing on the target) to date shows no conclusive differences among a variety of treatments, suggesting that GWSS may not use olfactory cues during host location, or that olfaction is used only in conjunction with visual cues. To date, most trials were made with field-collected GWSS. Fifty trials of *Gonatocerus ashmeadi* behavior under a variety of choice treatments have been recorded in the Y-tube olfactometer, but analysis by the Noldus *Observer* is scheduled for fall and winter.

Objective 2

Laboratory Choice Bioassays

Field-collected GWSS adults were caged with a choice of two plants grown under two nutritional treatments, and with a choice of two plants grown under two water-deficit treatments. The nutritional study was conducted on citrus plants, while the water-deficit study was conducted separately on citrus and avocado to allow a comparison of the behavior of GWSS toward host plants with different water-deficit responses. During preliminary nutrition trials with overwintered adults, GWSS that landed on plants showed a slight preference for settling on plants fertilized with ammonium averaging over the 3 trials. However, means of 3 additional trials (12 replicates) conducted with summer adults did not show any apparent preference for either treatment. The mean number of GWSS egg masses deposited on the citrus under the two nutritional treatments were also similar (3.25 per NO_3 plant, 2.67 per NH_4 plant). Up to and during the water-deficit trials, the water-deprived avocado plants showed signs of stress, including loss of stem and leaf turgor and leaf excision, whereas the citrus plants showed no obvious signs of stress. The GWSS showed a preference for contact with surplus-irrigated plants of both species compared with plants under CDI and GDI, with a stronger response evident in the avocado trials. The recent results are consistent with preliminary data gathered previously from overwintered adults. However, although the number of egg masses deposited on plants was higher on surplus-irrigated avocado compared with CDI and GDI plants, GDI citrus had more egg masses than surplus-irrigated citrus. The latter represented a change from the preliminary data, where surplus-irrigated plants of both species had consistently higher numbers of egg masses. The preliminary trials were repeated because the overwintered GWSS suffered heavy mortality.

Laboratory no-choice bioassays

Preliminary results from 10 GWSS of each sex confined in sachets on citrus receiving supplemental fertilization (NH_4 versus NO_3) revealed no significant differences in excreta production between the treatments, suggesting no differential feeding rates on plants of either treatment. This agrees with results from choice tests. However, mortality was high in the sachets, so the test will be repeated with improved sachets. More excreta was produced on average on deficit-irrigated citrus and avocado than on surplus-irrigated plants, but the differences were not significant. Further replication is planned to confirm the observed trend, especially because it appears to conflict with results of choice tests. Secondary conclusions were drawn from the data, including that females produced significantly more excreta than males across all treatments, suggesting greater feeding rates by females. Furthermore, excreta production was significantly higher in the afternoon than in the morning, suggesting that feeding is a diurnal activity. Lastly, excreta production on avocado was <10% of the amount produced on citrus, suggesting that avocado is a less preferred host or that avocado branches are not suitable feeding sites.

CDI and GWSS Population Dynamics

Studies were conducted on the Citrus Experiment Station, UC Riverside, in Field 5 and the continuous deficit-irrigation schedules evaluated in this experiment included trees irrigated at 100, 80, and 60% *ETc* throughout the 2005-06 crop year.

Effect of irrigation deficit on citrus trees
Citrus trees irrigated with 60% *ETc* had warmer leaves than the trees irrigated with 100% and 80% *ETc* throughout the season. Although no difference in leaf temperature was evident between the 100% and 80% treatments, results suggested that citrus trees under the 80% deficit irrigation treatment had physiological changes at detectable levels. To support our observations, pressure chamber measurements also showed a difference in water potential between treatments. From mid-May to early June, mid-day leaf water potentials were not different among treatments. Perhaps the unusual 2005 rainy season contributed to an accumulation of soil water, which was exploited by deeper roots and resulted in undetectable differences in water potential among treatments using the pressure chamber. A clear pattern in water potential measurements was observed from early June to late July. Pressure chamber measurements steadily increased and were higher in the 80% and 60% *ETc* treatments than the 100% *ETc* treatment. However, after July 25th, we initiated pre-dawn sampling and observed that no difference existed between the 100% and 80% *ETc* treatments, but these differed from the 60% *ETc* treatment.

Effect of irrigation deficit on insect distribution
Beat net samples documented the presence of GWSS adults and beneficial insects. The numbers of GWSS collected were quite low, never exceeding one adult for every sample. The most common beneficial arthropods were spiders, adult lacewings, and lady beetles. GWSS population densities increased from early June to late July, followed by a decline that continued through early September.

Examining yellow trap counts, GWSS adults collected were slightly higher in the 100% and 80% *ETc* treatments compared to the 60% *ETc* treatment. Few GWSS were found on sticky traps from mid-April to late June. The yellow sticky traps showed a steady increase in insect activity from late June to a peak in late July, which was followed by a sharp decrease in the numbers of insects caught in all treatments. During the early and middle part of the peak, more GWSS were caught on traps located in the 100% and 80% *ETc* treatments than in the 60% *ETc* treatment. However, during the latter part of the peak, the number of GWSS caught on traps located in the 60% *ETc* treatment was higher than the numbers caught in the 100% and 80% Et_c treatments. During this period (mid-August), trap catches averaged two GWSS per trap per week.

Population density patterns found during our visual counts follow the patterns observed for both trap counts and beat samples. Visual counts show an increase in the number of GWSS per tree from late June to a peak in mid-July. Throughout the season, more GWSS adults were found on trees located in the 100% and 80% *ETc* treatments than in the 60% *ETc* treatment. The number of egg masses collected were quite low, never exceeding one mass for every observation. However, there seems to be two periods of egg deposition. One period corresponded to the smaller first GWSS generation (May) and another period corresponded to the second larger generation (July). In the second oviposition period, however, few fresh GWSS egg masses were found and more egg masses were observed on trees located in the 100% and 80% *ETc* treatments than in the 60% *ETc* treatment.

In summary, our measurements of plant condition suggested that there were no differences in leaf temperature and water potential between trees irrigated at 100% and 80% *ETc*. Trees irrigated with 60% *ETc* had warmer leaves, higher water potential, and consequently hosted a smaller number of GWSS than the well irrigated trees. Patterns of insect number throughout the season for trap catches, beat samples, and visual inspection were somewhat correlated, suggesting a solid estimate of population density. Interestingly, relatively more GWSS adults and egg masses were found on trees irrigated at 80% *ETc* than 100% *ETc* perhaps due to a less dilute concentration of available carbohydrate.

CONCLUSIONS
We believe that findings from this project have generated significant new information regarding the host selection behavior and movement patterns of GWSS in California. Combined results from lysimeter studies and field studies evaluating population dynamics illustrate that GWSS populations varied between plants maintained under varying, CDI treatments. Further, trees irrigated with 60% ET_c had warmer leaves, higher water potential, and also hosted fewer GWSS than the well irrigated trees. Patterns of adult GWSS capture throughout the 2005 sampling interval (July – August), estimated from a combination of yellow traps, beat samples, and visual inspections, suggest comparatively higher population densities of GWSS in CDI treatments 80 and 100% ET_c. Furthermore, higher counts of GWSS adults and egg masses were found on trees irrigated at 80% ET_c compared with the 100% ET_c treatment. A more complete understanding of the operative host-plant cues that influence GWSS population dynamics may result in the deployment of strategies to focus control efforts, enhance the efficacy of biological control, and effectively limit the spread of *Xf* induced diseases to susceptible crops.

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

Project Leaders:

Marshall W. Johnson	Kent Daane	Russell Groves	Elaine Backus
Department of Entomology	Division of Insect Biology	USDA-ARS, PWA,	USDA-ARS, PWA,
University of California	Department of ESPM	SJVASC	SJVASC
Kearney Agricultural Center	University of California	9611 S. Riverbend Ave	9611 S. Riverbend Ave
Parlier, CA 93648	Berkeley, CA 94720	Parlier, CA 93648	Parlier, CA 93648

Cooperators:

Youngsoo Son	David Morgan	Kris Lynn-Patterson
Department of Entomology	Calif. Dept. Food & Agric.	University of California
University of California	Mt. Rubidoux Field Station	Kearney Agricultural Center
Kearney Agricultural Center	4500 Glenwood Dr. Bldg E	9240 S. Riverbend Ave.
Parlier, CA 93648	Riverside, CA 92501	Parlier, CA 936348

Reporting Period: The results reported here are from work conducted October 2005 through September 2006.

ABSTRACT

The purpose of this project is to define specific environmental constraints that influence glassy-winged sharpshooter (GWSS) population dynamics and overwintering success. The production of xylem excreta was used as a measure of GWSS feeding rates. GWSS individuals held at 8.3 to 31.1°C exhibited a positive linear relationship between xylem excreta per adult and temperature. A low temperature feeding threshold was estimated to be 13.3°C. A Logan Type I model described the relationship between temperature and daily excreta production (mg). The percentage of adults that produced xylem excreta was significantly different among tested temperatures ($P < 0.0001$), but not between sexes. From 24.6–35.1°C, all adults produced xylem excreta, but the percentage of adults producing excreta declined as temperature decreased. At temperatures ≤ 13.3°C, only 1 of 41 adults tested produced xylem excreta. Using percent data from 8.3–24.6°C, a linear increase in the percentage of adults that produced xylem excreta was observed and provided a lower threshold temperature of 10.0°C, where no xylem excreta were produced. Results from these experiments will be coupled with climatological data to help to spatially define where GWSS can be expected to persist in the agricultural landscape and identify where continued management efforts can be directed to limit introductions into currently non-infested areas.

INTRODUCTION

Climate appears to play a significant role in the geographic distribution of diseases caused by *Xylella fastidiosa* (*Xf*) in California and throughout the southeastern U.S. (Purcell 1997). Similarly, populations of glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, in the southeastern US appear to be constrained by climatic factors that limit the pest's establishment and persistence (Hoddle 2004). Presently, limited information exists on the overwintering biology and ecology of GWSS in the San Joaquin Valley of California. Our results from Year 1 of this project indicated that survival and feeding activity of GWSS adults were significantly influenced by temperature and exposure duration. In particular, low temperatures caused rapid mortality. Access to host plants for feeding was a critical factor for survival at high temperatures (≥ 20°C). In Year 2, models were developed to approximate the influences of temperature on GWSS survival with changes in exposure duration. Additional studies focused on the impacts of temperature on GWSS feeding rates with the aim of determining the thresholds below which feeding stops and to further determine the critical duration of time spent in this non-feeding state, which may result in increased mortality. The results below and future experiments will advance our ability to define the specific environmental constraints that influence GWSS population dynamics and overwintering success by increasing our present understanding of the overwintering requirements of GWSS with a focus on critical environmental and host species factors that may limit population distribution in the Central Valley of California.

OBJECTIVES

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

RESULTS

Objective 1:

(1) Effects of temperature on the survival of GWSS adults

Based on laboratory data collected in Year 1, the time to 50% mortality (LT_{50}) of GWSS adults was estimated at each tested constant temperature (-1.0, 4.0, 8.3, 18.8, 24.6 and 40.1 $^{\circ}$C ± 1°C) and feeding regime [water only (WO), host plant only (HPO), no plant or water (NPW)] using the methods of Kim and Lee (2003). The model estimated that the longest time to 50% mortality at the optimum survival temperature (for each feeding regime) occurred at 12.2 days (9.6°C), 11.4 days

(6.7°C), and 74.9 days (21.1°C) under the regimes of WO, NPW, and HPO, respectively (Figure 1.). Regardless of feeding regime, the skewed bell-shaped curve of temperature-dependent model indicated that GWSS survival was more seriously impaired by exposure to temperatures below the optimum temperature than temperatures above the optimum temperature.

An ongoing experiment was designed to determine the effect of fluctuating diurnal temperatures on adult GWSS survival during the winter season. Temperature-controlled incubators were set to simulate the hourly temperature cycles in three geographically distinct locations in California: Riverside (Riverside County; daily maximum ca. 16°C), Oakville (Napa County; daily maximum ca. 13°C), and Buntingville (Lassen County; daily maximum ca. 9°C) during the month of January. To date, under the simulated winter regime of Buntingville, GWSS adults experienced 100% mortality within 2 weeks, 50% mortality was observed at 5 days, and no xylem excreta production was observed. In contrast, at the end of the first month under the simulated regimes of Riverside and Oakville, 70.0 and 66.7%, respectively, of the GWSS individuals have survived with xylem excreta production observed in both treatments. These initial findings imply that short-term exposure to temperatures above the feeding threshold could provide adequate feeding time to permit GWSS survival in contrast to areas where the temperatures remain below or close to the feeding threshold. These studies will continue until all insects die or greater than 50% mortality is recorded.

(2) Effects of temperature on the feeding of GWSS adults

Laboratory experiments were continued on the effects of temperature on xylem excreta production by GWSS adults using the Parafilm sachet method (Pathak et al. 1982), which assumes a positive correlation between feeding activity and xylem excreta production (Paguia et al. 1980, Padgham and Woodhead 1988). GWSS adults were individually confined inside a Parafilm sachet (7.5 x 6.5 cm) that was attached to the main stem of a host plant ('Frost Eureka' lemon plant) and sealed after enclosing the insect. Plants were transferred to environmental chambers (10 L: 14 D hours) at six constant temperatures: 8.9, 13.3, 18.8, 21.7, 24.6, 31.1, 35.1, and 40.8°C ± 1°C. After 48 hours feeding, GWSS xylem excreta production (mg) was determined by weighing sachets on an electronic balance before and after removal of excreta. One insect per host plant was considered a replicate, and each treatment had ten to twelve replicates per sex. Treatment effects were determined using ANOVA (P = 0.05) and treatment means separated using the Student-Newman-Keuls (SNK) test. Data presented herein are mean values (± SEM) unless otherwise noted.

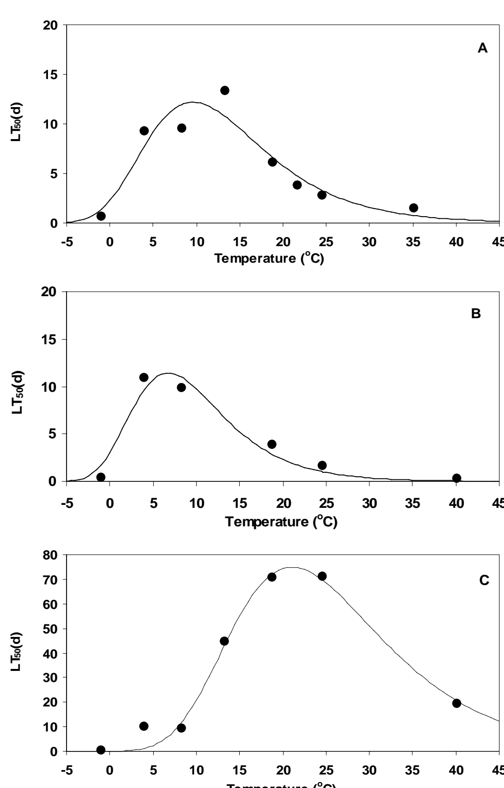

Figure 1. Effects of temperature on time to 50% mortality (LT_{50}) at constant temperatures under different feeding conditions: (A) water only; (B) no water or host plant; and (C) host plant.

Forty out of the 194 GWSS individuals tested did not survive the 2-day feeding period in the Parafilm sachets. Six adults that produced xylem excreta in the sachet died from drowning (1, 1, 1, and 2 adults at 21.7, 24.6, 31.7, and 35.1°C, respectively). Response variables from the drowned adults were not included in the data analysis. No adults survived at 40.8°C, whereas survival was higher than 80% within the range of 8.3–35.1°C (Table 1). There was no difference in the survival rates of males and females (χ^2 = 0.48; df = 1; P > 0.05), but temperature was a significant factor influencing survival (χ^2 = 119.02; df = 7; P < 0.0001). Xylem excreta production by GWSS individuals that survived the 2-day trial was highly dependent upon temperature (F = 38.53; df = 5, 129; P < 0.001), but there was no significant difference in xylem excreta between males and females (F = 0.1933; df = 1, 129; P > 0.05) (Table 1). Therefore, data from males and females were pooled for regression analysis. The highest xylem excreta production was 4,963.0 ± 1,317.5 mg per adult at 31.1°C, while no xylem excreta was observed at 8.3°C. Intriguingly, there was high variation in the excreta production among individuals held at 35.1°C (e.g., the lowest and highest excretion amounts for an individual ranged from 7.2 and 25,241.7 mg, respectively). Hourly excreta production (Table 1) was influenced by temperature (F = 25.80; df = 6, 153; P < 0.001).

Data from individuals (males and females pooled) held at 8.3 to 31.1°C indicated a positive linear increase in xylem excreta per adult as temperature increased (Y = 102.74– 2350.5, R^2 = 0.7314; df = 1, 5; F= 10.89; P < 0.05). A low temperature feeding threshold was estimated to be 13.3°C. A Logan Type I model (Logan et al., 1976) was used to describe the relationship between temperature and daily excreta production (mg) (R^2 = 0.987; F = 75.14; df = 3, 6; P < 0.01) (Figure 2). Xylem excreta production increased gradually up to 21.7°C and then sharply increased to the temperature of maximum production (2,833 mg) at 33.0°C. Feeding activity in terms of xylem excreta production abruptly declined between the temperature of maximum production and the upper threshold of production (36.4°C).

The percentage of adults that produced xylem excreta was significantly different among the tested temperatures ($\chi^2 = 131.58$; $df = 7$; $P < 0.0001$), but not between sexes ($\chi^2 = 1.69$; $df = 1$; $P > 0.05$). From 24.6–35.1°C, all adults produced xylem excreta, but the percentage of adults producing excreta declined as temperature decreased (Figure 3). At temperatures ≤ 13.3°C, only 1 of 41 adults tested produced xylem excreta during the 2-day period. Using percent data from 8.3–24.6°C, a positive linear increase in the percentage of adults that produced xylem excreta was observed ($Y = 6.3986x - 63.778$, R^2=0.9379; df =1, 5; $P < 0.01$) and provided a lower threshold temperature of 10.0°C, where no adult would produce xylem excreta. This lower threshold (10.0°C) is more conservative than the 13.3°C threshold obtained from the excreta amount model.

Table 1. Xylem excreta production by GWSS adults during 2-d feeding on 'Eureka' lemon tree at constant temperatures.

Temp. (°C)	N[1]	Mean ± SEM production (mg) of xylem excreta per surviving adult						
		n	Males	n	Females	n	Total	Excreta/h
8.3	23	11	0.0 ± 0.0c	10	0.0 ± 0.0e	21	0.0 ± 0.0e	0.0 ± 0.0d
13.3	22	10	0.0 ± 0.0c	10	47.8 ± 47.8de	20	23.9 ± 23.9e	0.5 ± 0.5d
18.8	23	11	105.6 ± 98.1c	12	340.7 ± 323.3cd	23	228.2 ± 173.1d	4.8 ± 3.6d
21.7	21	10	325.6 ± 140.9b	10	712.2 ± 310.0bc	20	518.9 ± 171.6c	10.8 ± 3.6c
24.6	25	12	1766.5 ± 987.6a	12	2302.0 ± 1027.6ab	24	2034.2 ± 699.2b	42.4 ± 14.6b
31.1	27	12	2993.9 ± 931.9a	12	6932.0 ± 2384.3a	24	4963.0 ± 1317.5a	103.4 ± 27.4a
35.1	25	10	4156.6 ± 1286.4a	12	4025.0 ± 2064.1ab	22	4084.8 ± 1240.9ab	85.1 ± 25.9a
40.8	24	0	—[3]	0	—	0	—	—

Means followed by same letter within each column not significantly different (Student-Newman-Keuls test, P<0.05).
[1] The number of tested adults (both males and females).
[2] Hourly xylem excreta production of adults (combined males and females).
[3] No data observed due to adult mortality.

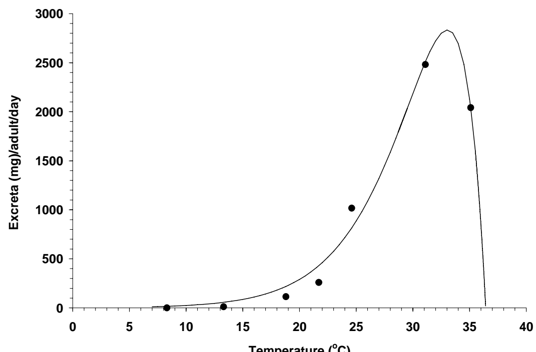

Figure 2. Relationship between temperature (°C) and daily mean xylem excreta (mg) production per GWSS adult on 'Eureka' lemon tree based on Logan et al. (1976) model.

Figure 3. Linear relationship between temperature and percentage adults producing xylem excreta between temperatures 8.3–24.6°C.

Objective 2

Due to concerns about maintaining live GWSS adult females in field cages in quarantined areas in the San Joaquin Valley, our studies were postponed until we could redesign the methods so that potential escape of GWSS individuals would pose minimal risk to the agricultural community. Methods were modified in the following manner: only GWSS males will be used that have been reared on *Xylella*-free host plants; replications have been reduced to 5 cages each at two test locations (Bakersfield and the Kern National Wildlife Refuge, Kern County); test plants will be double-caged with sticky traps and imidachloprid-treated plants in the outer cage; and the observation period will be reduced from 5 to 2 months. This study will be conducted in December 2006 and January 2007.

CONCLUSIONS

Findings from our studies clearly indicate that survival and feeding activity of GWSS adults are significantly influenced by temperature and exposure duration. In particular, low temperatures (< 10°C) caused rapid mortality. Availability of host plants was a critical factor for survival at high temperatures (≥ 20°C). This project has a high probability of success in terms

of generating significant new information regarding the thermo-biology of GWSS in California. Models generated from these data will allow for the spatial estimation of GWSS overwintering success.

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FUNDING AGENCIES

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WEB GEOGRAPHIC INFORMATION SYSTEMS FOR PIERCE'S DISEASE AND GLASSY-WINGED SHARPSHOOTER MAPPING IN CALIFORNIA

Project Leader:	**Researcher:**
Maggi Kelly	Mindy Syfert
Dept. Environ. Sci., Policy & Mgmt.	Dept. Environ. Sci., Policy & Mgmt.
University of California	University of California
Berkeley, CA 94720	Berkeley, CA 94720

Reporting Period: The results reported here are from work conducted October 2005 to June 2006.

ABSTRACT
The establishment of the non-native glassy-winged sharpshooter (GWSS) in California has seriously affected the epidemiology of Pierce's disease (PD) throughout the agricultural landscape. Geographic Information Systems (GIS) offers the opportunity to aid in the management of PD as well as in epidemiological research. We developed a web-based GIS site with spatial and temporal data relating to PD/GWSS based on feedback from a web-based survey emailed to 2005 PD research symposium participants. The survey focused on participants' interests in PD-related data, spatial analysis, and additional comments. The resulting webGIS displays various data layers of value to PD/GWSS researchers, including climatic variables and proximity analyses. Our survey results indicate an interest among PD/GWSS researchers in temporal analyses and some interest in data sharing. In addition, the data survey provides insight to PD researchers' attention to investigating PD patterns at a landscape scale and spatial modeling. However, there still exist some barriers preventing access to all statewide PD and GWSS data that will have to be overcome in order to develop and maintain a comprehensive statewide PD/GWSS webGIS system.

INTRODUCTION
Pierce's disease (PD), caused by the *Xylella fastidiosa* (*Xf*) bacterium, has been present in California for about 100 years. However, the introduction of the non-native glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, in recent years has increased the ability for the bacterium to spread. GWSS and PD have the ability to invade areas outside of their natural range and pose serious threats to the health of agricultural crops such as alfalfa, almond, citrus, coffee, grape, peach, plum and oleander (Hoddle, 2004). In particular, the GWSS vector has contributed to above normal grapevine losses in Southern California and could influence grapevine losses in Northern California.

Past studies indicate a linkage between the local environment and PD incidence and spread. For instance, Hoddle (2004) indicated the improbability of GWSS colonizing areas north of California due to the climatic condition of cold stress. Fiel and Purcell (2001) found temperatures below 12 to 17° C and above 34 ° C to negatively affect *XF* growth in vitro and in potted grapevines. Also, proximity to citrus crops or riparian habitats appears to influence PD incidence (Perring et al. 2001; Purcell and Saunders, 1999). The intersection of Geographical Systems (GIS) and the Internet has allowed the provision and visualization of geospatial data over the web possible. Web-based GIS (webGIS) provides insight into relationships between environmental variables at multiple scales to aid in natural resource management (Kearns et al., 2003). The ease of web-based access to spatial data is particularly advantageous for individuals to develop epidemiological hypotheses about distribution and spread at several scales – from vineyard to county to regional. This paper documents our progress in developing one for the PD/GWSS community.

OBJECTIVES
The goals for this project were twofold:
1. Provide researchers with a web-based tool to access spatial and temporal data relating to PD/GWSS in California at the landscape scale
2. Provide initial spatial analysis of known crop relationships to GWSS movements.

To achieve these goals, we developed a web-based GIS site and conducted a web-based survey to acquire user input about data needs relating to GWSS/PD research.

METHODS
WebGIS
We developed a webGIS site titled "Pierce's Disease & GWSS Mapping" utilizing ESRI's ArcIMS software (Figure 1). Determining which data to include in the webGIS was based on an initial evaluation of publicly available GIS data relevant to GWSS/PD. The GIS data were then downloaded from the web, processed, and integrated in the webGIS and finalized after survey responses were assessed. In addition to collecting data from the web, we developed and included a "Growing Degree-Days for 2005" and "Weather Stations" spatial data layers (Figure 2) based on a non-spatial degree-days model by UC Statewide Integrated Pest Management Program (http://axp.ipm.ucdavis.edu/WEATHER/ddretrieve.html).

Figure 1. Opening page of PD/GWSS Mapping website, located at:

Figure 2. 2005 growing degree-days and weather stations data layers displayed with identify tool results

The data collected for the webGIS were grouped into three categories: base data, climate data, and vegetation data. A fourth category is based on data layers we developed based on a spatial analysis of proximity to crops. A fifth category of PD/GWSS data will be included when the data are publicly available.

The webGIS site is completely functional with zoom tools, navigation tools, measurement tools, feature selection tools and an identify attributes tool. An example of the identify tool is seen in figure 2, which is particularly useful for users to obtain information for an exact area of interest. The webGIS site also has a metadata link for users to view important details about each data layer.

Data Survey

A data survey was developed for participants to answer questions and tell us which data and/or analysis they thought to be potentially useful in understanding PD. The survey provided user input in determining data content to include in the webGIS as well as assessing future research topics.

The data survey was presented to 2005 PD research symposium participants in the form of a website (http://giifserv.cnr.berkeley.edu/website/PD/Survey/PDfeedback.htm). The survey had a total of eight questions, focusing on data interests, possible spatial analysis interests, and text boxes for participants to give additional suggestions at the end of each question. The survey included a link to our webGIS site for participants to view data examples from those listed in the survey. The survey responses were collected and stored in a database.

RESULTS

Data Survey

We had a 20% response rate to our survey. An estimated 175 people were sent out emails requesting their input and a total of 32 responded. All 32 survey participant responses were assessed in early June 2006.

The data with the highest interest is shown in figure 3. We were particularly interested in responses relating to climate data, which generated considerable interest. Climatic factors max/min temperatures, temperatures, growing degree days, number of frost day, precipitation, humidity, and dew point resulted in high levels of interest. Also apparent in figure 3 is the higher interest in monthly data (44%-72%) instead of annual data (38%-47%) when applicable. In general, there was more interest in Daymet data (1km resolution) over PRISM data (4km resolution), except where PRISM offers a dew point, a climatic variable Daymet does not include. In addition, there was a general consensus to have most of the base data listed in the survey included in the webGIS. The survey yielded the highest data interest in riparian vegetation (84%).

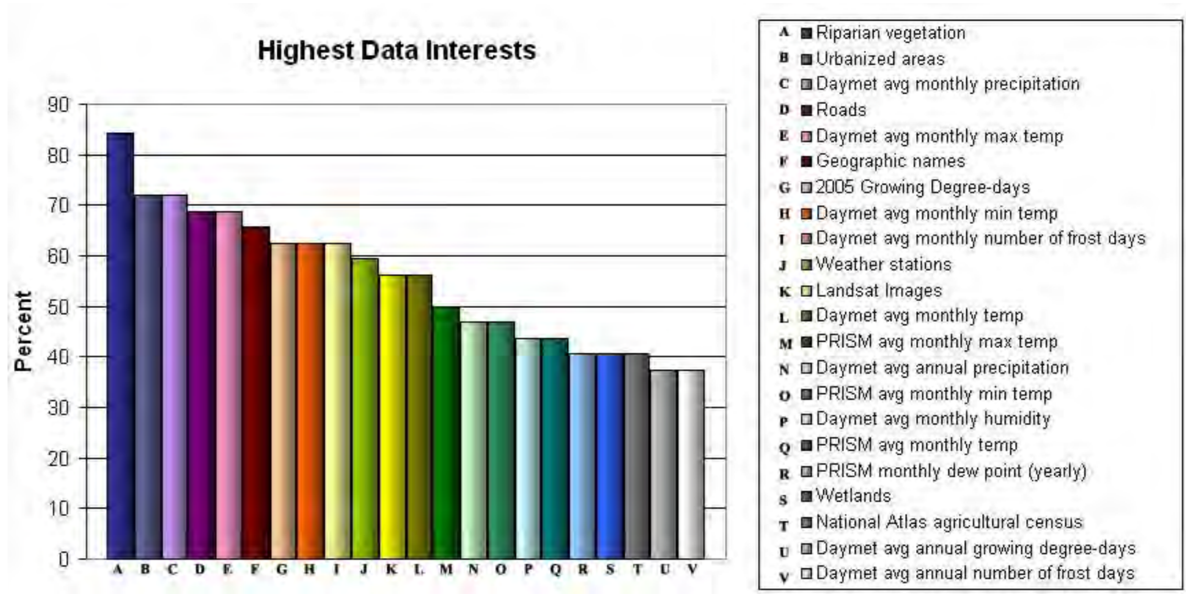

Figure 3. Data with interests levels above 35%

The spatial modeling questions relating to proximity and a change over time analyses received much attention. The respondents' interest in temporal analysis (66%) and proximity analysis (97%) was very high. This high level of interest in spatial analyses directed us to perform a first round proximity analyses.

Spatial Analysis

Due to high interest from survey participants, an initial proximity analysis was performed based on information about GWSS movement and its connection to PD incidence. It has been indicated that vineyards within close proximity to citrus orchards

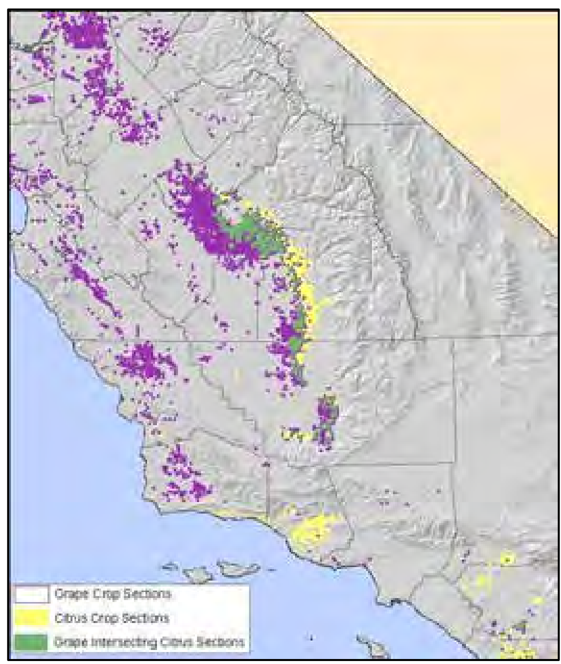

Figure 4. Map highlighting grape crop sections intersecting citrus crop sections (shown in green)

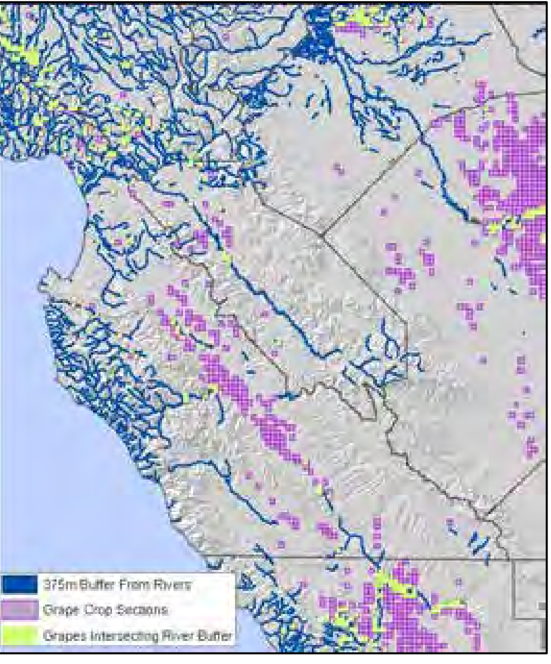

Figure 5. Map highlighting grape crop sections intersecting buffered rivers (shown in light green)

are susceptible to PD incidence (Perring et al. 2001). In addition, Purcell and Saunders (1999) indicated a connection between PD incidence and proximity to riparian vegetation. An initial proximity analysis was conducted in which grape and citrus crops data layers were used for the analysis; the crop data layers, supplied by CDFA, are based on based on 2003 Pesticide Use Report (PUR) and aggregated to PLSS sections. A new data layer was created with all grape sections that intersect with citrus sections. A total of 20 % of the grape sections that intersect citrus sections, known as the citrus-grape interface, indicated a high potential for PD incidence. Another data layer with a 375m buffer from streams was created to

represent possible riparian habitat, a host habitat for GWSS. The results of these data intersections are available to researchers via the webGIS site (http://giifserv.cnr.berkeley.edu/website/PD/viewer.htm).

CONCLUSIONS

Our webGIS site will be a resource of spatial data for researchers to assess spatial phenomenon such as climatic effects on PD/GWSS. The data survey provides insight to PD researchers' interest in investigating PD patterns at a landscape scale and spatial modeling. There is particular interest in temporal analysis, which is a potential topic for future research. In addition, a little over half (54%) of the survey participants are interested in sharing their data with the rest of the research community. This indicates another area of research: the development of tools for researchers to upload their data to our webGIS, making it a central resource for accessing data for PD. We also hope to continue working on the development of the webGIS site by including enhanced query tools such as, select by attribute and allowing the user to define thresholds (eg. growing degree-days threshold). These areas of development would advance the PD/GWSS webGIS site to a more comprehensive statewide resource to assess spatial data at multiple scales.

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FUNDING AGENCIES

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ANALYSIS OF THE BACTERIAL COMMUNITY INHABITING GLASSY-WINGED SHARPSHOOTER FOREGUT BY CULTURE-DEPENDENT TECHNIQUES AND DGGE

Project Leaders:

Paulo T. Lacava
Dept. of Genetics
University of São Paulo
Piracicaba, SP 13400-970
ptlacava@esalq.usp.br

Francisco Dini-Andreote
Dept. of Genetics
University of São Paulo
Piracicaba, SP 13400-970

José Luiz Ramirez
Dept. of Entomology
University of California
Riverside, CA 92521

Thomas A. Miller
Dept. of Entomology
University of California
Riverside, CA 92521

Cooperators:

Jennifer Parker
Dept. of Entomology
University of California
Riverside, CA 92521

Welington L. Araújo
Dept. of Genetic
University of São Paulo
Piracicaba, SP 13400-970

João Lucio Azevedo
Dept. of Genetic
University of São Paulo
Piracicaba, SP 13400-970

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ABSTRACT

The glassy-winged sharpshooter (GWSS) is an important vector of *Xylella fastidiosa* (*Xf*), the bacterial pathogen that causes several economically important plant diseases, including citrus variegated chlorosis (CVC), oleander leaf scorch (OLS) and Pierce's disease (PD) of grapevines. In recent years there has been an increasing interest in the potential use of biological control agents to halt the spread of *Xf*. One such strategy is the exploration of symbiotic microorganisms to reduce the spread of the pathogen (symbiotic control). In a symbiotic control strategy a bacterium symbiont that occupies the same niche as the pathogen must be identified. The study of the bacterial community of GWSS foreguts by isolation and DGGE revealed the presence of several potential symbiotic candidates such as *Bacillus* sp., *Pseudomonas* sp., *Methylobacterium* sp. and *Curtobacterium flaccumfaciens* (*C. flaccumfaciens*). Members of genus *Methylobacterium* and *C. flaccumfaciens* are frequently isolated as endophytes from citrus plants with CVC symptoms and without disease symptoms. Recently, an interaction between *Methylobacterium*, *C. flaccumfaciens* and *Xf* was strongly indicated, reinforcing that these bacteria could interact inside the host plant and vector insect. In the future, the genus *Methylobacterium* and *C. flaccumfaciens* could be an interesting candidate in a strategy of symbiotic control to *Xf*.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) is one of the main vectors of *Xylella fastidiosa (Xf)*. It is a xylophagous insect that has a wide array of host plants, including many ornamental and crop plants (Purcell and Hopkins 1996, Purcell and Saunders 1999). One new potential management strategy for Pierce's disease (PD) of grapevine is the use of symbiotic control. Symbiotic control exploits the interactions among a pathogen-transmitting organism, its bacterial symbionts, and the pathogenic organism itself (Beard 2002). For symbiotic control to be effective in limiting the spread of PD, a culturable symbiont that inhabits the pre-cibarium and cibarium of GWSS is required, since these areas are colonized by the pathogen, *Xf*. A previous biochemical analysis of the GWSS foregut microdiversity encountered three bacterial species that meet these requirements: *Chryseomonas* spp, *Ralstonia* spp, and *Alcaligenes* spp (Bextine 2004). The *Alcaligenes* species were of particular interest because they were frequently isolated from wild GWSS (Kuzina 2004). Although *Alcaligenes* spp. can colonize GWSS, this specie does not colonize grapevines well (Bextine 2005). According to Bextine et al (2005), the amount of colonization by *Alcaligenes xylosoxidans* subsp. *denitrificans* decreased in the following order: orange (*Citrus sinensis* ''sweet orange'') > chrysanthemum (*Chrysanthemum grandiflora* cv. ''White Diamond'') > periwinkle (*Vinca rosea*) > crepe myrtle (*Lagerstroemia indica*) > grapevine (*Vitis vinifera* cv. Chardonnay). Therefore, steps in a symbiotic control strategy should be directed in finding a bacterial symbiont that colonizes well the pre-cibarium and cibarium of GWSS as well as the target host plant: grapevines. The CelectrophoreticCT separation of PCR products of variable regions of genes encoding 16S rDNA (by use of primers homologous to conserved regions of the gene) in a polyacrylamide matrix over a denaturing gradient (DGGE) is a technique recently introduced in microbial ecology (Muyzer et al. C1993CT). The denaturing gradient can be achieved either chemically with urea and formamide in DGGE. This technique is reported to be interchangeable, giving comparable fingerprints of microbial communities. Recently, Reeson et al. (2003) demonstrated the efficiency of DGGE to study the bacterial communities associated to insects (wasp *Vespula germanica*).

OBJECTIVES

1. The aim of this work was to characterize the bacterial community inhabiting GWSS foreguts by using isolation in culture medium and DGGE techniques.

RESULTS

The study of bacterial community by isolation and DGGE (Figures 1, 2, and 3) revealed the presence of several genera of bacteria such as *Bacillus* sp., *Cryocola* sp., *Microbacterium* sp., *Micrococcus* sp., *Pedobacte* sp.r, *Pseudomonas* sp., *Methylobacterium extorquens*, *C. flaccumfaciens*, *Baumannia cicadellinicola*, and *Wolbachia* spp. Members of genus

Methylobacterium are frequently isolated as endophytes from citrus plants with CVC symptoms and recently, an interaction between *Methylobacterium* spp., *Curtobacterium flaccumfaciens* and *Xf* was strongly indicated (Araújo et al. 2002, Lacava et al. 2004) reinforcing that these bacteria could interact inside the host plant and vector insect. Besides, Lacava et al. (2004) suggests that the CVC symptoms in citrus plants could be a result of the population balance between *Methylobacterium* spp., *C. flaccumfaciens* and *Xf*.

CONCLUSIONS

Our study of bacterial diversity associated with GWSS foreguts was initiated by using culture-dependent methods as well as based on sequence (DGGE) polymorphisms of 16S rRNA gene, using total DNA extracted from GWSS foreguts. The diversity profiles obtained with isolation methods indicated a low bacterial diversity. On the contrary, a high bacterial diversity in GWSS foreguts was observed with the use of DGGE (culture independent technique). The preliminary results show that DGGE is suitable for the analysis of the bacterial diversity in GWSS and in the future, bacteria such as *Methylobacterium* spp. *C. flaccumfaciens*, found as part of the bacterial community of GWSS, could be used as potential candidates in a symbiotic control strategy against *Xf*. Further analyses of the data collected in the present study are still being conducted and they will be presented at the symposium.

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FUNDING AGENCIES

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Figure 1. Petri dishes containing bacterial colonies isolated from GWSS heads.

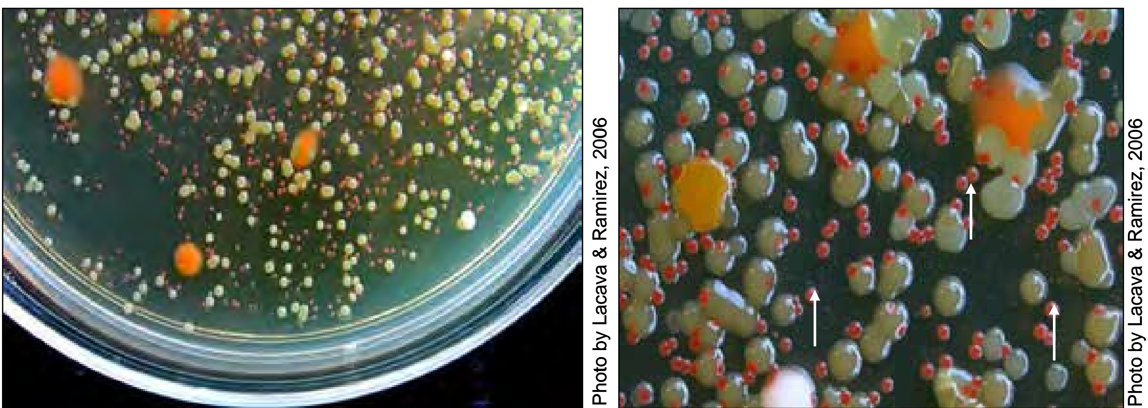

Figure 2. Petri dishes containing bacterial colonies isolated from GWSS heads. The white arrows (right photo) indicate *Methylobacterium extorquens* isolated from GWSS heads.

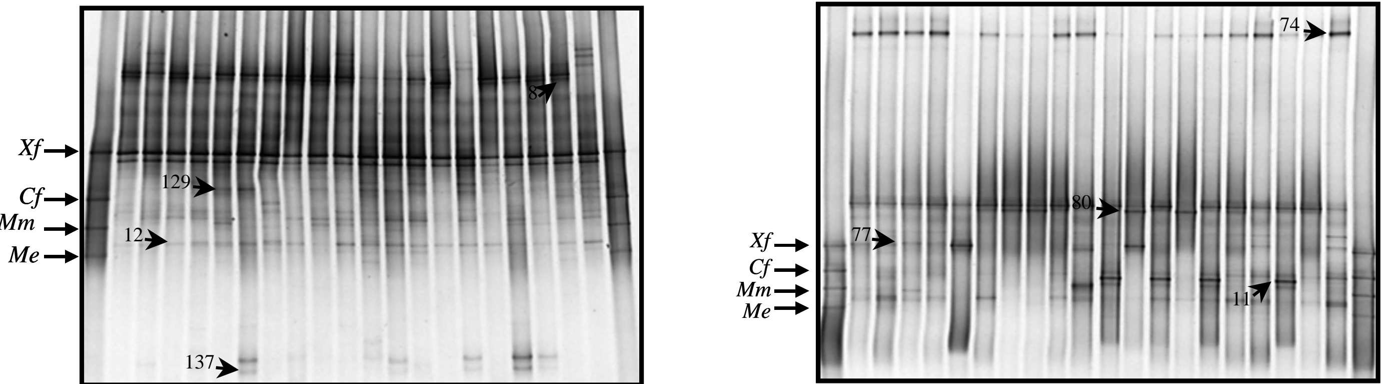

Figure 3. DGGE fingerprints of the bacterial communities from GWSS. **(A)** Young GWSS collected in asymptomatic *Citrus* sp. ; **(B)** Adults of GWSS collected in PD infected grapes and then transferred and reared in healthy grapevines. *Xf: Xylella fastidiosa* (PD strain); *Cf: Curtobacterium flaccumfaciens; Mn: Methylobacterium mesophilicum; Me: Methylobacterium extorquens.*

SPECIES DIVERSITY, DISTRIBUTION, AND ABUNDANCE OF XYLEM FLUID FEEDING HEMIPTERA IN VINEYARDS THROUGHOUT TEXAS

Project Leaders:
Isabelle Lauzière
Department of Entomology
Texas A&M University
Texas Agricultural Experiment Station
Fredericksburg, TX, 78624

Forrest Mitchell
Department of Entomology
Texas A&M University
Texas Agricultural Experiment Station
Stephenville, TX, 76401

Cooperator:
Simon Sheather
Department of Statistics
Texas A&M University
College Station, Texas, 77843

Reporting Period: The results reported here are from work conducted for three years (2003-September 2006).

ABSTRACT

A survey of xylem fluid feeding insects (Hemiptera: Auchenorrhyncha) exhibiting potential for transmission of *Xylella fastidiosa* (*Xf*) the bacterium causing Pierce's disease (PD) of grapevine was initiated in Texas in 2003 and continues to the present. Twenty five insect species belonging to 4 families and 14 genera were collected from yellow sticky traps and identified. Among these, three species, two leafhoppers and one spittlebug, comprised over 90% of the xylem fluid feeding insects caught. *Homalodisca vitripennis* (formerly *H. coagulata*), the most commonly known vector of PD of grapevine in the U.S. is the most common and abundant insect captured across the state. Insect abundance varied significantly over seasons, with most of captures taking place between June and August, and per location, with 5 to 15 species caught at any given vineyard. Residual populations found overwintering near vineyards will be the focus of future studies. A grape strain and an ornamental strain of *Xf* have been detected in insects processed by real time PCR. These initial observations provide critical information to vineyard managers for timely applications of insecticides prior to insect feeding and vectoring to susceptible grapevines.

INTRODUCTION

Pierce's disease (PD) of grapevine has become the most limiting factor to grape production in Texas, as it has in California during the past decade. It is transmitted by certain insects which feed on xylem fluid of plants where the bacterium develops. During the 1990's, the grape growing region of Central Texas witnessed an increase in the incidence and severity of PD (Texas Pierce's Disease Task Force 2004). The insect vectors were not thoroughly identified but glassy-winged sharp-shooters (GWSS), *Homalodisca vitripennis* (Cicadellidae), were suspected. A modest research program was initiated in Texas, funded by the U.S. Department of Agriculture starting in 2002-2003. Within this program, researchers are provided an opportunity to study glassy-winged sharpshooters in their natural habitat. During the past years, intensive studies to identify the vectors and their vectoring potential have been taking place in a growing multi-disciplinary, multi-institutional research program. Exploration for insect species involved, their ecology, host plants used, molecular characterization of *Xf*, vectoring capacity of the Hemiptera captured, natural enemies and population dynamics are well underway.

OBJECTIVES

1. Monitor xylem feeding insect populations in vineyards across Texas. Identify all putative insect vectors of PD. Determine the most common vectors requiring population management, make observations on vector distribution, density and seasonality.
2. Explore for host plants used as breeding sites by insect vectors throughout the year, assess the reproductive state of adult females and determine the age structure composition of the vectors
3. Characterize the proportion of insects carrying *Xf* and the pathotypes involved. Investigate evolution of infection levels throughout the year.

RESULTS

Insect populations in the Hemiptera were monitored on a bi-weekly fashion in 45 vineyards for over 3 years. We placed particular interest in those populations of xylem fluid feeding insects that may play a role in the transmission of *Xf*, causal agent of PD in grapevines. Data indicate the presence of xylem fluid feeding leafhoppers-treehoppers (Membracoidea), spittlebugs (Cercopoidea) and cicadas (Cicadoidea). Of 160 Hemiptera species captured, 25 species have been identified so far as xylem fluid feeding insects present in the vineyards and adjacent natural habitat, all with the potential to carry and transmit *Xf* when feeding on susceptible host plants. Captured insects in the family Cicadellidae (leafhoppers) were the most abundant with a total of 15 species recovered; this family contributed to about 75% of all individuals caught. Predominant species were *Homalodisca vitripennis*, *Graphocephala versuta* and *Clastoptera xanthocephala*, two leafhoppers and a spittlebug. These species together comprised over 90% of all xylem fluid feeding insects identified. *Homalodisca vitripennis* and *C. xanthocephala* were present at each of the surveyed locations. At certain locations, a specific Hemiptera species clearly dominated. Insect species diversity varied from 5 to 15 per location, with an average of 9.18 ± 2.87species per location. Abundance of major xylem fluid feeding Hemiptera species varied greatly throughout time. Monthly variations of the adult populations of xylem fluid feeding Hemiptera well correspond to mean temperatures. These insects' populations increased significantly from April to June when they peaked. After June, insect densities, indicated by trap captures, decreased gradually until they reached their minimal levels between the months of November and April. A similar pattern of

abundance was observed for each of the dominant species recovered. Observations made for *G. versuta* suggest that the host plants found in the habitat outside the vineyards are more suitable than grapevines for feeding and/or reproduction. Initial examination of the three major species by means of Real Time PCR (RT-PCR) indicates that *G. versuta* is more commonly associated with an ornamental strain of *Xf*, rather than the PD strain. *Homalodisca vitripennis* and *C. xanthocephala*, conversely, were both found to be associated with the PD strain of *Xf*. While *C. xanthocephala* is found on sticky traps within the vineyard, there is no evidence yet that it feeds on grape. It may still be important to the epidemiology of Pierce's disease by moving the bacterium from one wild host to another and providing an inoculum source for *H. vitripennis*.

CONCLUSIONS

This study sheds light on the complex system that exists in Texas. These results demonstrated a rich fauna of xylem fluid feeding insects where three species, *H. vitripennis*, *G. versuta* and *C. xanthocephala*, stood out from the others in terms of population densities and made up for over 90% of insects collected. Of these 3 species, one, the glassy-winged sharpshooter, is a known vector of *Xf,* while the other two are not confirmed vectors to date. *Graphocephala atropunctata*, the blue-green sharpshooter is a known vector in the western U.S. and so the *Graphocephala* remain suspects by their relationship to this known western vector. At most locations, xylem fluid feeding Hemiptera population densities remained relatively low throughout the vine's vegetative season, except for June when adult counts were the highest. Important information has been gathered while carrying out this large study in Texas. Although the survey was not exhaustive, i.e., only traps were used and they are not equally attractive to all xylem fluid feeding Hemiptera species, we now have a much clearer idea of several, and certainly of the most common, insects species that can be involved in Pierce's disease of grapevine. Extensive molecular analyses are currently being carried out to confirm which of these insects are associated with grape strains of *Xf*. A number of leafhopper species have been examined by RT-PCR and many are contaminated with *Xf*, indicating an association and a possible vector relationship much broader than first suspected.

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FUNDING AGENCIES

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

ASSOCIATIVE LEARNING OF HOST-PLANT CHEMICAL STIMULI IN IMMATURE GLASSY-WINGED SHARPSHOOTERS

Project Leaders:
Joseph M. Patt
USDA, ARS, KSARC
Beneficial Insects Research Unit
Weslaco, TX 78596
jpatt@weslaco.ars.usda.gov

Mamoudou Sétamou
Texas A & M University, Kingsville
Citrus Center
Weslaco, TX 78596
msetamou@ag.tamu.edu

Research Assistants:
Orlando Zamora
USDA, ARS, KSARC
Beneficial Insects Research Unit
Weslaco, TX 78596

Javier Cavazos
USDA, ARS, KSARC
Beneficial Insects Research Unit
Weslaco, TX 78596

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT
Olfactory conditioning may provide the immature glassy-winged sharpshooter (GWSS) with a mechanism by which they can effectively track their host-plants in space and time. In the presence of vanilla scent, nymphs that ingested vanilla-flavored xylem fluid were significantly more attracted to a neutral visual target than were nymphs that had ingested unflavored xylem fluid. These results are consistent with our previous finding that GWSS response to visual stimuli is enhanced by exposure to host-plant odor.

INTRODUCTION
The nutritional requirements of immature glassy-winged sharpshooters (GWSS) constrain their diet to plants with low amide- and high amino acid concentrations in the xylem fluid (Brodbeck, et al. 1999). To obtain a balanced level of nutrients, nymphs may frequently need to switch host-plants (Brodbeck, et al. 1999, Redak et al. 2004). Locating host-plants that are physiologically-suitable with respect to providing adequate levels of xylem nutrients may require that the nymphs integrate information from several types of host-plant stimuli (Harris and Foster 1995). For example, nymph response to foliar colors (*sensu* Prokopy and Owens 1983, Tipping et al. 2004) is enhanced by the presence of host-plant volatiles (Patt & Sétamou 2006). The ability to learn to recognize stimuli associated with suitable host-plants would facilitate detection and location of host-plants whose distribution varies temporally and spatially within the nymphs' environment (Behmer et al. 2005, Pompilio et al. 2006).

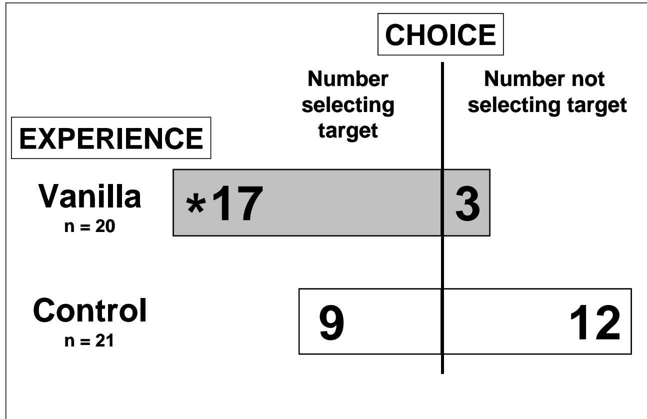
The goals of our ongoing study are to determine whether nymphs can associatively learn to recognize olfactory stimuli produced by host plants, and, if so, to evaluate the relative importance of olfactory conditioning in host-plant recognition.

To provide nymphs for testing, second- to fourth instars were placed on cowpea (*Vicia unguiculata*) sprigs for 1.5 days. The cut-ends of the sprigs were immersed either in hydroponic solution containing a low concentration of vanilla extract, or, as a control, in hydroponic solution alone. After removal from the sprigs, the nymphs' responsiveness to a pale green disk in the presence of vanilla extract odor was tested in an olfactometer (Patt & Sétamou 2006) using no-choice tests. In preliminary tests with blank air, 44% of nymphs from the control group jumped to the pale green target, demonstrating that innate attraction to this color is low. An increased response to the pale green target in the presence of vanilla odor would indicate that the nymphs had developed an attraction to vanilla scent via their previous feeding on vanilla-flavored xylem fluid.

OBJECTIVES
1. Determine whether nymphs can associatively-learn to recognize olfactory stimuli produced by host plants.
2. Evaluate the relative importance of olfactory conditioning in host-plant recognition.

RESULTS
Vanilla extract constituents were detected by gas chromatography-mass spectrometry analysis of ethanolic extractions made from vanilla-treated cowpea sprigs.

Nymphs that fed on plant sprigs with vanilla-flavored xylem fluid were significantly more attracted to the pale green target than nymphs that fed on control sprigs with non-flavored xylem fluid (Figure 1). However, there was no difference between individuals in the experimental and control groups with respect to the amount of time they required to orient- and jump to the visual target (Figure 2).



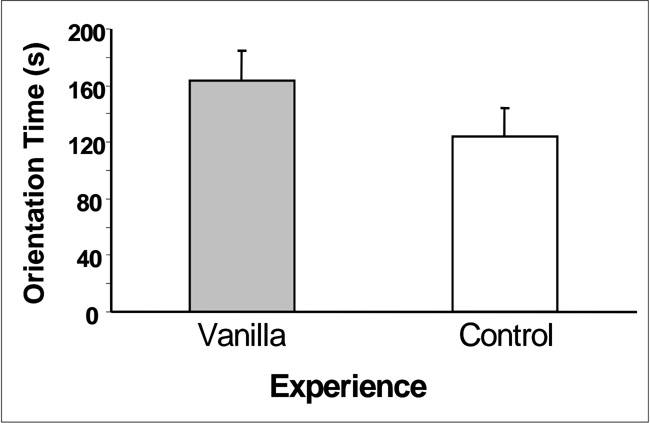


Figure 1. Results of no-choice assays showing the numbers of nymphs that either jumped to a pale green disk ('selecting target') or failed to jump to the disk ('not selecting target'). Nymphs had experience feeding on cowpea sprigs with their cut ends immersed either in hydroponic solution with vanilla extract ('Vanilla') or hydroponic solution alone ('Control'). Significant levels of choice within the same bar are indicated by * = $P \leq 0.01$ (G-test).

Figure 2. Mean time ($\pm$ SE) required by nymphs in each test group to detect and orient to a pale green target in the presence of vanilla scent. N = 17 nymphs in the vanilla group and 9 nymphs in the control group. Means are similar (T-test, P = 0.1933).

CONCLUSIONS

Nymph response to a non-attractive color was enhanced following ingestion of a novel flavor, indicating that immature GWSS are capable of olfactory conditioning. Rapid population growth of GWSS may depend on the close proximity of host plants suitable for successful juvenile development (Redak et al., 2004). Therefore, understanding the mechanisms by which nymphs locate their host-plants is fundamental to developing vegetation management programs aimed at suppressing their population growth and dispersal in complex landscapes. A manuscript describing the design of the behavioral assay in more detail and the results of ongoing experiments will be submitted for publication in the near future.

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FUNDING AGENCIES

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EVALUATION OF BLUE-GREEN SHARPSHOOTER FLIGHT HEIGHT

Project Leader:
Ed Weber
UC Cooperative Extension
Napa, CA 94559
eaweber@ucdavis.edu

Reporting period: The results reported here are from work conducted February 2004 to September 2006.

ABSTRACT
Flight heights of blue-green sharpshooters (BGSS) were monitored in Napa Valley vineyards from March through September for three seasons (2004-2006) using pole towers to position yellow sticky panel traps at heights up to 24 feet. Towers were located adjacent to vineyards at the edge of a riparian zone. Eleven towers were monitored in 2004 and 2006; twelve were monitored in 2005. Trap catches in 2004 were considerably greater than in 2005 and 2006. For the March-May period, 76-99% of the catches were made at 15 feet or lower. These data support the possible use of screen or natural barriers to reduce the number of BGSS entering vineyards in the spring.

INTRODUCTION
Where the blue-green sharpshooter, *Graphocephala atropunctata,* (BGSS) is the primary vector of Pierce's disease (PD), control measures should be aimed at reducing the number of BGSS entering vineyards (Goodwin and Purcell 1992), especially early in the growing season. Early-season infections (March-May) are responsible for most chronic cases of PD vectored by BGSS (Purcell 1975, 1981). Infections resulting from BGSS feeding later in the growing season are not likely to result in PD because most will be eliminated with normal pruning. This is unlike the situation with PD caused by glassy-winged sharpshooter (GWSS) feeding, where chronic infections may occur nearly year-round (Almeida and Purcell 2003).

Vector control measures in the North Coast include the use of insecticides (Goodwin and Purcell 1992) as well as management of riparian plant communities to reduce the number of favorable BGSS breeding host plants (Insley, E., et al. 2000).

Another method of reducing vector numbers is to block their flight into vineyards through the use of physical barriers. This could include the use of tall fences made with insect screening materials, as well as natural barriers created by planting dense stands of conifers or other non-host tree species. Both of these approaches are already being employed in a few vineyards in the North Coast, although there are currently no data to show their impacts. The use of barriers has also been suggested as a management tactic to keep GWSS out of vineyards (Blua and Morgan 2003).

For barriers to be effective, they would need to block the majority of BGSS from entering vineyards, since small numbers of insects can still lead to significant disease development (Purcell 1979). Unfortunately, little is known about the overwintering behavior of BGSS and its preferred winter plant hosts (Purcell 1976). Therefore, it is not clear how tall a barrier would need to be in order to be effective. Most trapping by both researchers and growers has been done from the ground at the 5-6 foot level.

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

OBJECTIVES
1. Evaluate the predominant flight height of BGSS entering vineyards from adjacent riparian habitats through the use of yellow sticky panel traps positioned at heights from 5 to 24 feet.

RESULTS
Eleven pole towers were installed and monitored in the Napa Valley in 2004 and 2006; twelve towers were monitored in 2005. Two of the towers monitored in 2004 were not used in 2005 due to the low number of BGSS trapped at those locations. Three additional towers were installed in 2005. One tower used in 2004 and 2005 was not used in 2006 due to low trap counts. Eight towers were monitored in the same locations in all three seasons. Tower locations covered a distance of approximately 25 miles from the Carneros region in southern Napa County to the outskirts of Calistoga at the north end of Napa Valley. Towers were positioned along riparian zones adjacent to vineyards that had a history of PD.

A diagram of a pole tower is shown in Figure 1. Towers were 25 feet in height, constructed from Schedule 40 PVC pipe with a pulley at the top and a rope running through it. Yellow sticky panel traps were attached to clips on the rope at the following heights: 24 feet, 20 feet, 15 feet and 10 feet. An additional trap at 5 feet was clipped to a metal stake mounted in the ground.

Towers were installed prior to March 9 in 2004 and 2005. In 2006, several towers were damaged by flooding and were not functional until early April. Traps were monitored on a weekly basis through September and numbers of BGSS were recorded. Traps were replaced every two weeks or as needed.

Figure 2 shows the percentage of BGSS trapped at various heights at all towers during the early season period of March-May. This is the critical time period in which most infections leading to chronic cases of PD are likely to occur (Purcell 1975, 1981).

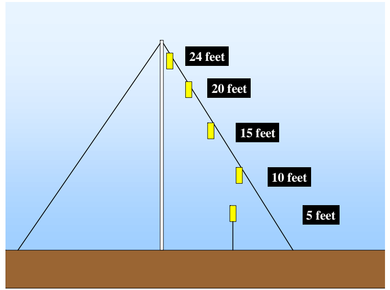

Figure 1: Pole tower diagram.

From March to May, 99% and 86% of BGSS were caught at 15 feet and lower in 2005 and 2006 respectively. During this same time period in 2004, 76% of BGSS were caught in traps 15 feet and lower. With the exclusion of unusual trap catch data from Tower 10 prior to budbreak in March 2004, this figure rises to 88% (Weber 2005). Tower 10 was installed adjacent to a Coast Live Oak tree (*Quercus agrifolia*), an evergreen species that was apparently a preferred host plant prior to budbreak of nearby deciduous species. A record heat wave in early March 2004 (70-85°F) led to significant BGSS flight activity in the vicinity of this tree as evidenced by larger numbers of BGSS caught in the upper traps. This was the only case of greater numbers of BGSS in the upper traps compared to the lower traps during the three years of this study at all towers.

Figure 3 shows the percentage of BGSS trapped at various heights at all towers during the entire trapping period March-September. The data in Figures 2 and 3 show similar trends with most BGSS being caught in traps at 15 feet and lower. In 2004, 83% of BGSS were trapped at 15 feet or below. In 2005 and 2006, 94% and 90% were trapped at 15 feet or below, respectively.

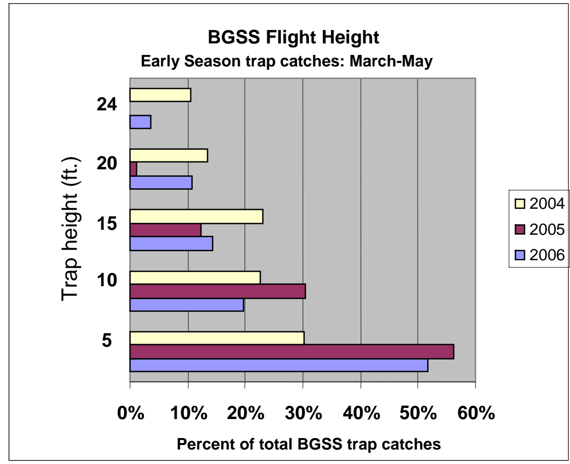

Figure 2. March-May BGSS trap catches by trap height as a percent of total.

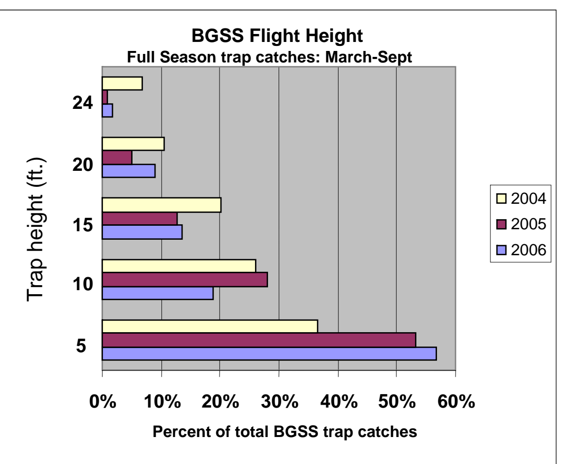

Figure 3. Full season BGSS trap catches by trap height as a percent of total.

Figures 4 and 5 show the total trap catches for the March-May and March-September periods respectively. The data included in these figures are from the eight towers that were monitored at the same locations in all three years. The average cumulative trap catches per tower were 47.6 BGSS in 2004, 11.9 in 2005, and 4.6 in 2006.

The results from this project suggest that a 15-18 foot high barrier could be effective at greatly reducing the number of BGSS entering vineyards. However, previous work with insecticides showed that even with 70-90% reductions in BGSS trap counts, the incidence of PD was not significantly reduced in vineyards planted with highly sensitive varieties (Purcell 1979). Even with a 10-18 foot screen barrier, the number of BGSS flying over the top could still result in significant amounts of PD in an adjacent vineyard.

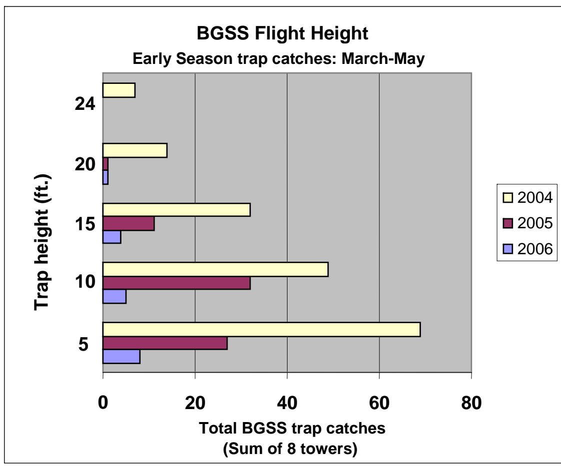

Figure 4. March-May BGSS trap catches. Total counts 2004-2006 from the eight towers used all three years.

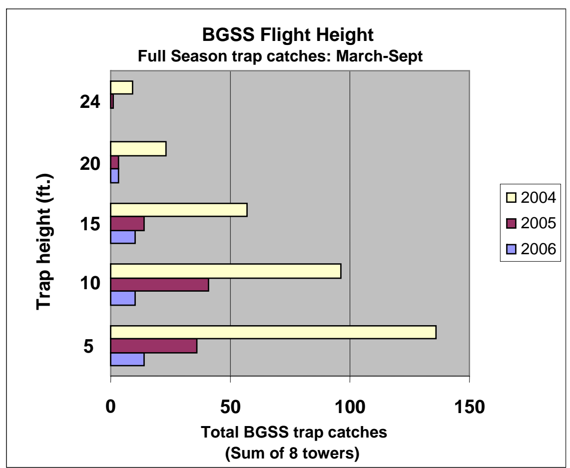

Figure 5. Full season BGSS traps. Total counts 2004-2006 from the eight towers used all there years.

CONCLUSIONS

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

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THE ROLE OF OLFACTORY CUES IN HOST-PLANT SELECTION BY THE GLASSY-WINGED SHARPSHOOTER

Hannah Nadel
USDA – ARS
9611 S. Riverbend Ave.
Parlier, CA 93648

Russell Groves
Department of Entomology
University of Wisconsin
Madison, WI 53706

Ron Seligmann
AgroTalk
6 Agnon St.
Raanana, 43380, Israel

Marshall W. Johnson
University of California
Kearney Agricultural Station
Parlier, CA 93648

ABSTRACT
The glassy-winged sharpshooter (GWSS) is a highly polyphagous and mobile vector of Pierce's disease of grapes. Trap captures in a multi-crop agricultural landscape under constant deficit irrigation suggest that adult GWSS movement is tied to irrigation schedules. To understand the observed patterns of movement, we explored the orientation and feeding responses of adult GWSS toward citrus and avocado plants undergoing various levels of water-deficit and nutritional treatments. Choice and no-choice cage studies indicate that GWSS distinguishes water-stress in hosts and prefers to settle on and feed more on well-hydrated plants. GWSS showed no significant response to a choice of citrus fertilized with ammonium or nitrate forms of nitrogen.

Section 2:
Vector
Management

CURTAILING OVIPOSITION BY THE GLASSY-WINGED SHARPSHOOTER ON NURSERY PLANTS

Project Leaders:

Matthew J. Blua	Richard A. Redak
Department of Entomology	Department of Entomology
University of California	University of California
Riverside, CA 92521	Riverside, CA 92521

Cooperators:

James Bethke	George Gutman	Brad Bowers
Department of Entomology	Bordier's Nursery	Valley Crest Tree Co.
University of California	Irvine, CA 92618	Fillmore, CA. 93015
Riverside, CA 92521		

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

ABSTRACT

The containerized ornamental nursery industry in California has been implicated as the most likely source of new outbreaks of the glassy-winged sharpshooter (GWSS) *Homolodisca vitripennis* (formerly *H. coagulata*) in areas of the state that are not infested. For nurseries in GWSS-infested areas, rigorous quarantine requirements mandate GWSS monitoring, thorough plant inspections at shipping and receiving locations, and insecticide treatments. These requirements assume that one GWSS adult, or one egg mass can initiate a stable population. Thus, plant shipments are frequently rejected at the receiving location on the basis of an egg mass that was not detected and removed during the shipping site inspection. The costs associated with quarantine requirements and shipment rejection has become an important economic problem. Many nurseries in infested areas do not ship their product to areas that are not infested with GWSS, resulting in multi-million dollar losses.

In this study, we examine the impact of selected insecticides on GWSS oviposition on containerized nursery plants. The insecticides we selected for study are currently being used by the nursery industry in California to suppress insect populations. In two trials, examining 4 different plant species and 8 insecticides applied as foliar sprays or soil drenches, we found no suppressive impact on GWSS oviposition.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) *Homolodisca vitripennis* (formerly *H. coagulata*) has arguably become the most important invasive species in California due to its propensity to spread diseases induced by *Xylella fastidiosa* , the most important being Pierce's disease of grapevines (Blua et al 1999). First identified in 1990 from collections in Orange and Ventura counties (Sorensen and Gill 1996), GWSS has spread throughout southern California from San Diego to Santa Barbara counties along the coast, and inland to San Bernardino and Riverside counties. More recently it has become established in Kern County. Over the past several years, local outbreaks of GWSS have been found in central and northern California counties where eradication efforts are underway.

Although no data are available that identify the source of these emerging populations, the ornamental nursery industry of California has taken the brunt of the responsibility on the basis of a most likely scenario involving the movement of the insect on containerized nursery material throughout the state. Additionally, it is widely accepted without evidence that the original establishment of GWSS in southern California occurred by dispersal on commercial nursery stock from the southeastern portion of the U.S. to California. (Sorensen and Gill 1996).

Currently, strict regulations have been imposed on the nursery industry to curtail movement of GWSS via containerized ornamentals transported from infested to non-infested counties. These regulations require thorough inspection by the office of county agricultural commissioners at both the origin of plants destined for transport and their destination. Additionally, local disinfestation protocols require repeated insecticide applications at the majority of nurseries shipping materials out of a quarantine area. Upon detection of GWSS at a destination nursery, costly insecticide treatments of the surrounding area are required as well as destruction of the infested material. Inspections and treatments are labor-intensive, time consuming, and result in substantial extra costs to growers, counties, and ultimately the state. It is important to note that nursery shipments can be rejected, leading to a spraying of the destination location and crop destruction, based on the simple presence of egg masses. In many cases the presence of an old egg mass (i.e. an egg-mass scar on the foliage) has been sufficient to trigger crop destruction, pesticide applications, and additional costly monitoring and surveillance. A determination of the viability of the masses is rarely if ever made. The assumption is that any egg mass detected is viable and capable of establishing a population.

The ornamental nursery industry needs new, cost-effective, solutions to the problem of transporting GWSS, especially as eggs, on nursery stock to non-infested areas of California. We believe that effective solutions can be immediately integrated

into current production systems on the basis of understanding the degree to which registered insecticides curtail oviposition, and knowing the relative susceptibility of various nursery plants to oviposition by GWSS.

OBJECTIVES

1. Determine the impact of selected foliar-applied insecticides applied to drip-irrigated potted plants on GWSS oviposition.
2. Determine the impact of selected soil-applied insecticides applied to overhead sprinkler-irrigated potted plants on GWSS oviposition.

RESULTS

Objective 1

We conducted a randomized block experiment in which *Lagerstroemia indica* and *Tristiana conferta* in 5 gal pots were sprayed to run-off with label rates of Safari 20SG (dinotefuran, 8oz/100gal), Marathon II (imidacloprid, 1.7floz/100gal), Tristar 70 WSP (acetamiprid, 1.2oz/100gal), Tempo SC Ultra (beta-cyfluthrin, 5.4floz/100gal), Deltagard T&O 5SC (deltamethrin, 8floz/100gal), and Sevin SL (carbaryl, 32floz/100gal). An untreated control was the seventh treatment. Plants were exposed to a natural *H. vitripennis* population at the University of California, Riverside, CA, and numbers of GWSS egg masses were counted weekly beginning one week after treatment over a three-week period.

Analysis of variance did not detect significant differences among treatments in the number of GWSS egg masses produced on plants for any of the three weekly counts for both *L. indica* ($F \leq 1.331$, $P \leq 0.269$) and *T. conferta* ($F \leq 0.871$, $P \leq 0.526$) (Figure 1).

Objective 2

We conducted a second randomized block experiment in a greenhouse. In this experiment, *Escalonia fradesii* and *Tecomaria compensis* in 1 gal pots were treated with drench applications of Safari 20 SG (dinotefuran, 4floz of a 24oz/100gal base solution) and Marathon II (imidacloprid, 4floz of a 9.2floz/100gal base solution). An untreated control was the third treatment. Over a 3-week period, 200 *H. vitripennis* adults were released into the greenhouse twice weekly, and egg masses were counted weekly beginning one week after treatment.

Analysis of variance did not detect significant differences among treatments in the number of GWSS egg masses produced on plants for any of the three weekly counts for both *E. fradesii* ($F \leq 1.331$, $P \leq 0.269$) and *T.compensis* ($F \leq 0.871$, $P \leq 0.526$) (Fig. 1). Out of 30 GWSS adults caged on experimental *E. fradesi* for 24 h, 30 survived on control plants, while 0 and 1, respectively, survived on plants treated with Safari and Marathon II three weeks after treatment.

CONCLUSIONS

Thus far, none of the insecticides commonly used in the ornamental nursery industry that we examined has made a direct impact on oviposition by GWSS. However, their indirect impact on oviposition is substantial due to population reduction alone. The need for a means of reducing GWSS oviposition remains strong due to the "0-tolerance" position assumed by current quarantine regulations. Determining a minimum viable population size for GWSS would allow us to refine existing quarantine protocols that mitigate the costs of inspection, monitoring and eradication efforts.

Other than examining additional insecticides and insect repellents, our further research will focus on documenting GWSS behavior to find points that can be exploited to interrupt oviposition.

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FUNDING AGENCIES

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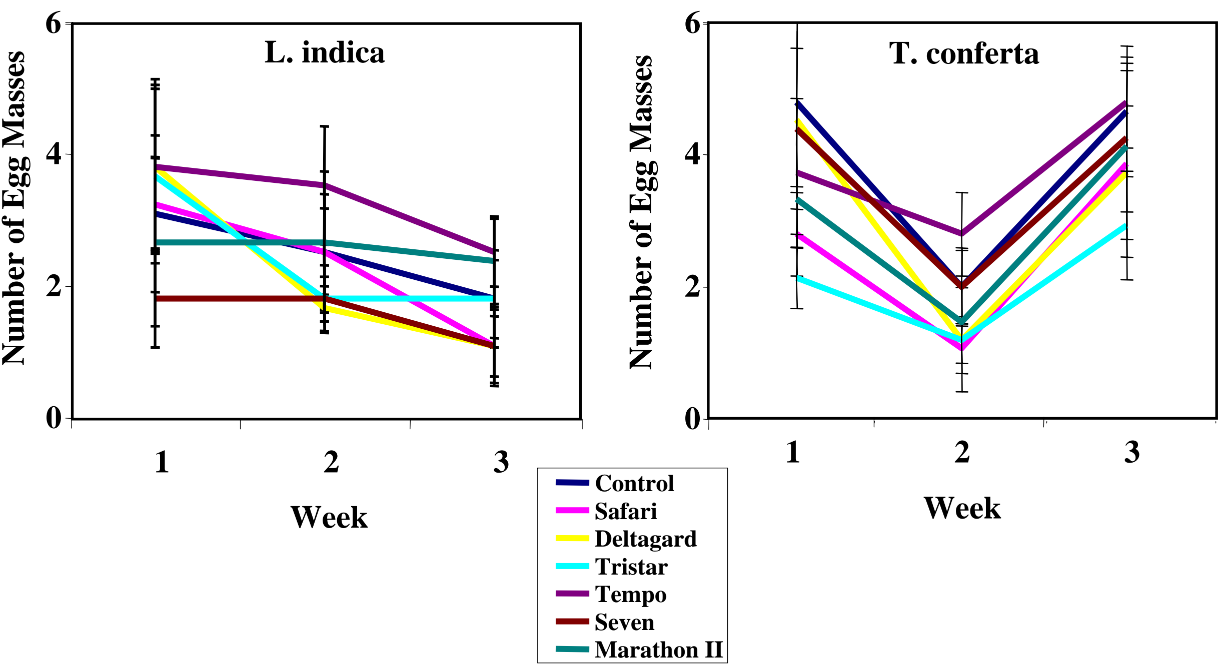

Figure 1. Number of GWSS egg masses on potted *Lagerstroemia indica* and *Tristiana conferta* treated with selected foliar-applied insecticides, and non-treated controls. Insecticides were applied one week before the first week count.

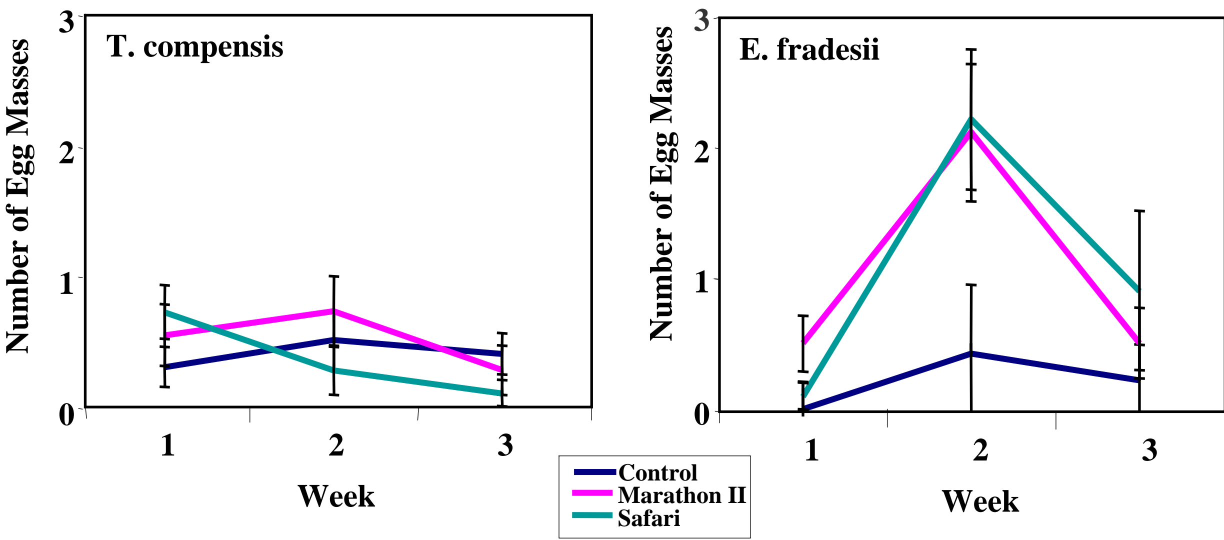

Figure 2. Number of GWSS egg masses on potted *Tecomaria compensis* and *Escalonia fradesii* treated with selected soil-applied insecticides, and non-treated controls. Insecticides were applied one week before the first week count.

POTENTIAL ISOLATES OF THE ENTOMOPATHOGENIC FUNGUS *BEAUVERIA BASSIANA* FOR GLASSY-WINGED SHARPSHOOTER CONTROL

Project Leaders:
Surendra K. Dara
Shafter Research & Extension Center
University of California, Davis
Shafter, CA 93263

Harry K. Kaya
Department of Nematology
University of California
Davis, CA 95616

Cooperator:
Michael R. McGuire
USDA-ARS
Shafter Research & Extension Center
Shafter, CA 93263
(Current address: USDA-ARS, Fort Collins, CO 80526)

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ABSTRACT
Two California isolates and a Texas isolate of the entomopathogenic fungus, *Beauveria bassiana* (Balsamo) Vuillemin, demonstrated their potential as effective pathogens of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (formerly *H. coagulata*). Virulence of these isolates evaluated in the laboratory assays at different conidial concentrations and in small caged tests was similar. Adult GWSS feeding on plants sprayed with fungal inoculum were infected and killed by the fungus in the caged tests. When conidia were exposed to sun light and assessed for their viability, the two California isolates appeared to be more tolerant of solar radiation.

INTRODUCTION
A collaborative project between UC Davis and USDA-ARS is aimed at identifying suitable entomopathogenic fungi for the control of the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (formerly *H. coagulata*), a pest that threatens the grape industry in California as a vector of the Pierce's disease causing bacterium, *Xylella fastidiosa*. Entomopathogenic fungi, which enter the host through the cuticle, are ideal candidates for insects like GWSS with piercing and sucking mouthparts. Entomopatho-genic fungi were isolated from GWSS in the Southeast US (Mizell and Boucias 2002, Kanga et al. 2004). But no fungal pathogen has so far been reported in California GWSS populations. However, we recovered several isolates of two generalist fungi, *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin, from GWSS habitats in California and tested them against GWSS (Dara et al. In Press, Kaya et al. 2004, 2005). We also isolated *B. bassiana* from California harvester ant, *Pogonomyrmex californicus* (Buckley), three-cornered alfalfa hopper, *Spissistilus festinus* (Say) and a darkling beetle from Kern, Fresno and Riverside counties, respectively. These isolates were evaluated, along with a Texas isolate and the commercial isolate, for their virulence to adult GWSS and ability to grow at different temperatures (Dara et al. In Press). Based on the results, two California isolates – recovered from the three-cornered alfalfa hopper and a soil sample from a citrus orchard in Riverside Co – and the Texas isolate were further evaluated for their virulence at different concentrations and efficacy to infect GWSS when sprayed on the plants. Viability of these isolates following exposure to solar radiation and pathogenicity to selected natural enemies were also evaluated.

OBJECTIVES
1. Conduct surveys to find fungal infections in GWSS populations or insects closely related to GWSS.
2. Culture and isolate the fungi and evaluate their pathogenicity against GWSS.
3. Assess environmental effects like temperature and sunlight on conidial survival and germination, fungal growth, and infectivity.
4. Evaluate the host range of fungi that infect GWSS.
5. Conduct small-scale caged tests to evaluate selected pathogens against GWSS.

RESULTS
Natural infections in GWSS populations
We continue to search for natural infections in GWSS populations in southern California. GWSS adults were periodically collected in the urban areas around Bakersfield on Chinese photinia, prostrate acacia, oleander and crepe myrtle. These insects were maintained in the laboratory for the bioassays. No entomopathogenic fungi have been found in these insects.

Virulence of entomopathogenic fungi to GWSS:
Beauveria bassiana
Laboratory-reared GWSS adults supplied by CDFA, Riverside were used for the bioassays. The two California isolates and the Texas isolate of B. bassiana were evaluated against adult GWSS at three fungal concentrations - 105, 107 and 109 conidia/ml. GWSS were anesthetized by exposing them to CO2 for 20 sec and then inoculated by rolling them in a 10 µl drop of conidial suspension. Controls were treated with 0.01% of Silwet, an adjuvant used to prepare conidial suspensions. GWSS were incubated on potted cowpea plants covered with cylindrical cages and their mortality was recorded daily for two weeks. Cadavers were surface sterilized in 3% sodium hypochlorite solution and incubated on water agar for fungal emergence. These assays were repeated twice. There were significant differences ($P < 0.05$) in the infections caused at different concentrations within each isolate (Figs. 1 and 2). But there was no significant difference among the isolates.

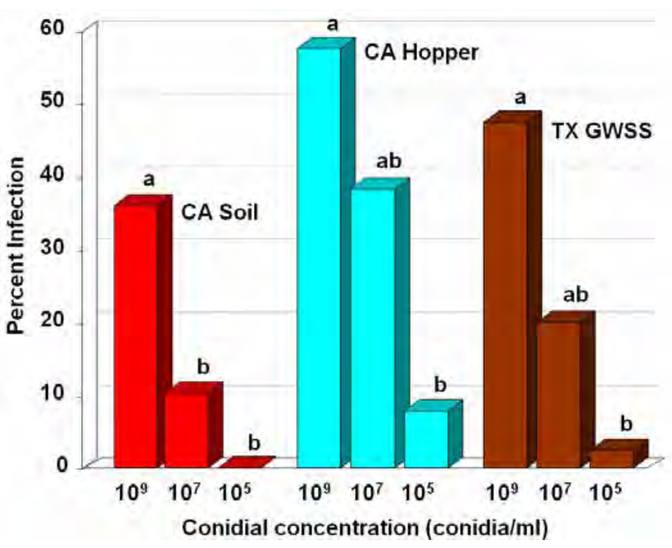

Figure 1. Virulence of selected *B. bassiana* isolates to GWSS at different concentrations

Figure 2. Sporulating cadaver of GWSS infected by a California isolate of *B. bassiana.*

Molecular characterization

Different fungal isolates used against GWSS in our study were characterized using molecular techniques for a better understanding of the isolate identity and the variation in their virulence (Fig. 3). Genetic relatedness of *B. bassiana* isolates from California, Texas and Mississippi were compared with the commercial isolate GHA using single sequence repeat (SSR) markers or microsatellites as described by McGuire et al. (2006). Fungal cultures were grown on Sabouraud dextrose agar enriched with yeast extract, and DNA was extracted using MagAttract 96 DNA Plant kit (Qiagen, Valencia, CA) and a Rectsch MM301 Mixer Mill (Retsch, Germany). Seven PCR primer pairs (Ba01, Ba02, Ba03, Ba05, Ba06, Ba08, and Ba12) which flank SSR markers were used for the molecular characterization of these fungal pathogens. To evaluate the pattern of genetic similarities among the selected isolates of the fungal pathogens, pair-wise genetic similarity coefficient was calculated based on Jaccard's similarity coefficient (Jaccard 1908). A dendrogram was constructed using the neighboring join (N-J) clustering analysis (Saitou and Nei 1987) with midpoint rooting method. All statistical analysis and the construction of the dendrogram were performed using the numerical taxonomy and multivariate analysis system (NTSYS-pc) version 2.1 (Rohlf 2002).

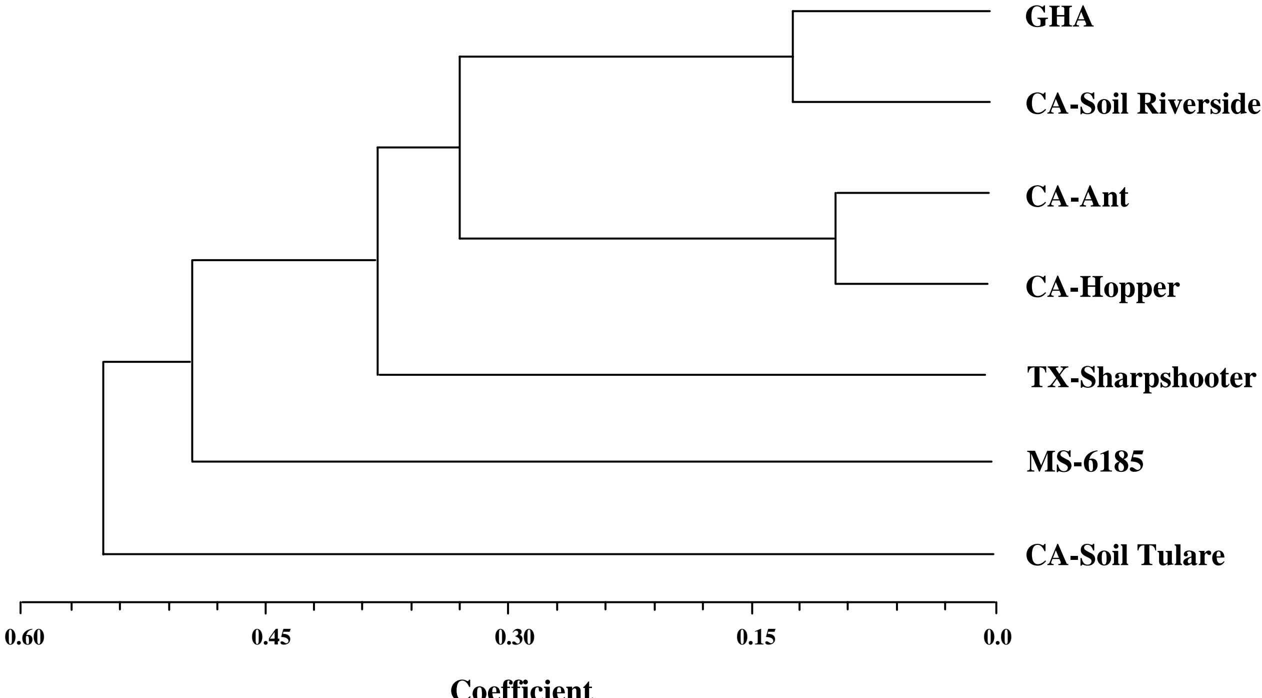

Figure 3. Genetic relatedness of *B. bassiana* isolates based on seven SSR markers

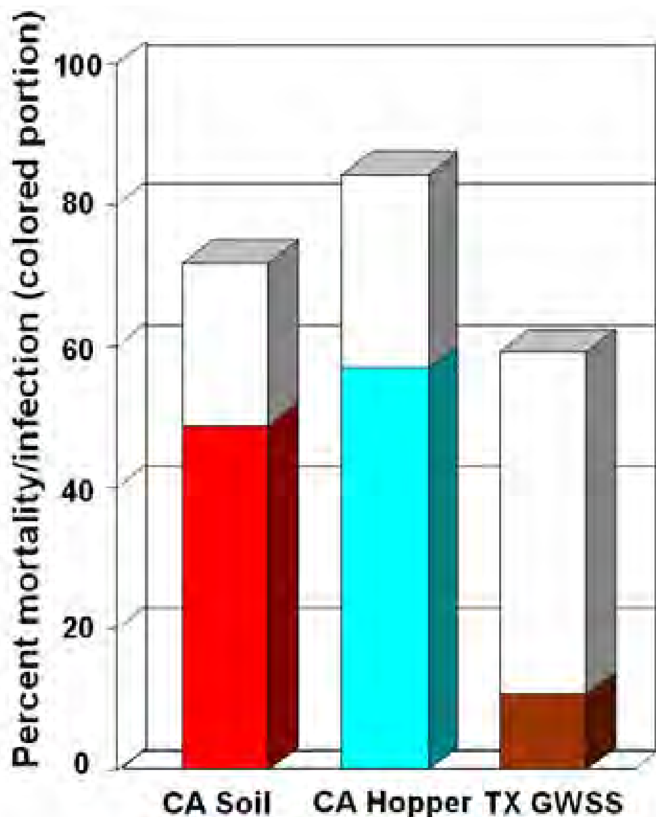

Figure 4. Mortality and infection caused by *B. bassiana* isolates in GWSS feeding on treated plants

Effect of solar radiation on the viability of selected *B. bassiana* isolates
The effect of temperature on the radial growth of different fungal isolates was previously reported (Dara et al. In Press). The effect of exposing the fungal inoculum to solar radiation for 1, 2, 4 and 6 h on the viability of the three selected isolates of *B. bassiana* was evaluated in this study. Treatments included 1X10^9 conidia/ml suspensions of Texas, three-cornered alfalfa hopper and Riverside soil isolates in 0.01% Silwet. A 25 µL conidial suspension was dispensed on a 18X18 mm coverslip as several droplets and allowed to dry under the hood for 30 min. Each treatment had four coverslips placed on a 60 mm filter paper in a 90X15mm Petri plate bottom. Petri plates were arranged on a plastic tray in a radomized complete block design and were partially covered with the lids to prevent heat build up. Tray was placed about 3 m away from the pyranometer at 1 m height on a flat surface. Solar radiation was measured using an Eppley Black and White Pyranometer (645-48) (Eppley Laboratory, Inc., Newport, RI) located at the weather station at the Shafter Research and Extension Center. Pyranometer had a quartz dome which measures the wavelengths from 200 to 4500 nanometers. At designated time intervals, one plate of each treatment was brought back to the laboratory and conidia from one of the coverslips were washed into 1 ml 0.01% Silwet. This conidial suspension was added to 20 ml PDB with gantamicin (100 mg/L) and incubated on a rotary shaker at the room temperature. Germination of conidia was assessed after 24 and 48 h of incubation in PDB to determine the effect of solar radiation on fungal viability. All isolates withstood exposure to solar radiation for up to 4 hours. However, viability was higher for both California isolates compared to the Texas isolate at all time intervals. Between the two California isolates, the isolate from the three-cornered alfalfa hopper advanced more into successive stages of development compared to the soil isolate.

Pathogenicity of the isolates to natural enemies
Pathogenicity of the three isolates of *B. bassiana* to the convergent lady beetle, *Hippodamia convergence* Guérin-Méneville and the egg parasitoid, *Gonatocerus ashmeadi* Girault, was determined in two different assays. Adult lady beetles were purchased from InsectLore (Shafter, CA) and parasitoids were provided by CDFA, Riverside. Treatments included 1X10^9 conidia/ml suspensions of the three selected isolates and Silwet 0.01% as a treated control along with an untreated control. Insects were anesthetized by exposing to CO_2 and inoculated by dipping in conidial suspension. Lady beetles were incubated in Petri plates (90X20 mm) with two screened vents and provided with soaked raisins and strands of paper. Parasitoids were also incubated in similar plates and provided with strips of tissue (Kimwipes) soaked in 50% honey solution. All three isolates were pathogenic to both the lady beetles and the parasitoids.

Efficacy of the selected *B. bassiana* isolates in the cage tests
The three selected isolates of *B. bassiana* were evaluated in cage tests that were repeated thrice. GWSS were collected on prostrate acacia and Chinese photinia in the Bakersfield area and maintained in the laboratory on euonymus plants until used in the test. About a month-old cowpea plants were individually sprayed iwth a 40 ml conidial suspension containing 1x10^{10} viable conidia in 0.01% Silwet, an adjuvant. Plants were dried under shade for 15-20 min before placing them in a cage (BugDorm from BioQuip). Fifty adult GWSS were placed in each cage. Each isolated had only one plant due to the limited availability of GWSS. A plant treated with Silwet was used as a control. Cages were maintained under the laboratory conditions where average temperature was 26.1$\pm$4.0 ^oC, relative humidity fluctuated between 36 and 62% with an average of 42%, and a 16L:8D photoperiod. Mortality of the insects was monitored for two weeks. Cadavers were surface sterilized in 3% sodium hypochlorite solution, followed by rinsing in deionized water, and incubated on 1% water agar at 28 ^oC for fungal emergence. Fungal growth on the cadavers was microscopically examined to determine infection.

Virulence of the three isolates was similar in the caged tests (Fig. 4). However, fungal emergence, in general, occurred more in insects exposed to the three-cornered alfalfa hopper isolate compared to the other isolates. Several of the GWSS feeding

on plants treated with the Texas and the Riverside soil isolate died within one day after the treatment in two tests. It usually takes 3-5 days for the fungus to infect and kill the insects. Suspecting a fungal toxin for this rapid mortality, an assay was conducted using cell free fungal extracts where treated insects did not die ruling out any toxins (results not shown). The exact cause of the rapid mortality in the cage tests was undetermined. However, this would be useful in controlling the insects.

Pseudogibellula formicarum

We previously described the non-pathogenicity of the fungus, *Pseudogibelluala formicarum* (Mains) Samson & Evans, to GWSS in an earlier CDFA report. Because the genus *Pseudogibellula* has species that are pathogenic to invertebrates and *P. formicarum* was isolated from GWSS cadavers in Mississippi, we tested this particular isolate against GWSS in Caifornia. Below, we provide the results of our findings again.

The fungus, *P. formicarum* recovered from GWSS cadavers in Mississippi, was tested in two assays. As this fungus could not be cultured on standard microbial culture media, conidia scraped from the cadavers were added to 0.01% Silwet to prepare the inoculum suspension. In the first assay, CO_2 anesthetized GWSS adults were rolled in 50 µL suspension (~10^6 conidia/ml) and individually incubated in clip cages attached to a euonymus plant. Untreated insects were used as controls. In the second assay, conidial suspensions were injected into adult GWSS at 0.5 µL/insect. Untreated insects and those treated with Silwet were used for comparison. Only five insects per treatment were used in both of these assays due to the limited numbers of insects available. In both assays, *P. formicarum* couldn't infect the treated GWSS. Another assay was also conducted to evaluate the pathogenicity of *P. formicarum* to daddy longlegs spiders (*Holocnemus pluchei*) where the fungus could not infect the spiders.

CONCLUSIONS

The Texas isolate and two California isolates - from the three-cornered alfalfa hopper and the Riverside soil – of *B. bassiana* have the potential to be microbial control agents of GWSS.

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GENETIC CHARACTERIZATION OF *GONATOCERUS TUBERCULIFEMUR* FROM SOUTH AMERICA UNCOVERS DIVERGENT CLADES: PROSPECTIVE EGG PARASITOID CANDIDATE AGENT FOR THE GLASSY-WINGED SHARPSHOOTER IN CALIFORNIA

Project Leaders:
Jesse H. de Leon
USDA, ARS
Beneficial Insects Res. Unit
Weslaco, TX 78596

Guillermo A. Logarzo
USDA, ARS, SABCL
Buenos Aires, Argentina

Serguei V. Triapitsyn
Deparment of Entomology
University of California
Riverside, CA 92521

Cooperator:
David J.W. Morgan
Calif. Dept. of Food & Agric.
Mt. Rubidoux Field Station
Riverside, CA 92501

Reporting Period: The results reported here are from work conducted fiscal year 2005 to fiscal year 2006.

ABSTRACT
In present study we genetically characterized the prospective South American egg parasitoid candidate, *Gonatocerus tuberculifemur* (Ogloblin) of the glassy-winged sharsphooter (GWSS), *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)] for a neoclassical biological control program in California. Two molecular methods, inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting and a phylogeographic approach inferred by the mitochondrial cytochrome oxidase subunit I gene (COI). Five geographic populations from South America were analyzed; in addition, a phylogenetic analysis was performed with several named and two unnamed *Gonatocerus* Nees species. DNA fingerprinting uncovered a fixed geographic banding pattern difference in the population from San Rafael, Mendoza Province, Argentina. The COI analysis uncovered haplotype or geographic structure in *G. tuberculifemur*. A neighbor-joining distance tree clustered the populations into two well-supported distinct clades with very strong bootstrap values (96-100%) with the population from San Rafael clustering into a separate clade than the rest of the South American populations. No haplotype sharing was observed between individuals from the two clades. A phylogenetic analysis performed by the neighbor-joining method of 15 *Gonatocerus* Nees species confirmed species boundaries and again uncovered two distinct clades in *G. tuberculifemur* with very strong bootstrap support (96-100%). The two molecular methods were in accord and the evidence is suggestive of a species level divergence. Because *G. tuberculifemur* is under consideration as a potential biological control agent for the invasive GWSS in California, understanding possible cryptic variation of this species is critical.

INTRODUCTION
Uncertainty exists as to whether egg parasitoids native to California will be as effective against the glassy-winged sharpshooter (GWSS) *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)]as they are in their co-evolved native range (Jones 2001, Logarzo et al. 2003, 2004, Virla et al. 2005). As a consequence, beginning in 2000, egg parasitoids of closely related hosts belonging to the sharsphooter tribe Proconiini [*Tapajosa rubromarginata* (Signoret)] were sought from regions in South America where climate types and habitats were similar to California for a neoclassical biological control program (Jones 2001, Logarzo et al. 2005). In surveys conducted in Argentina and Chile during 2000 through 2005, two prospective egg parasitoid candidate agents were identified among several *Gonatocerus* Nees species reared from *T. rubomarginata* (Jones et al. 2005, Logarzo et al. 2005, Virla et al. 2005). The egg parasitoid candidates from South America were identified by S. V. Triapitsyn (UC-Riverside) as *Gonatocerus tuberculifemur* and *G. metanotalis* (Ogloblin) (Hymenotpera: Mymaridae). *Gonatocerus tuberculifemur* is now being permitted for release in California (CDFA 2005). Mymarid wasps are the best-known egg parasitoids for controlling populations of leafhoppers (Huber 1986, Döbel and Denno 1993). Molecular studies of insects are becoming increasingly important in resolving taxonomic relationships critical to the success of biological control programs. Identifying the correct natural enemy is critical to the success of classical biological control programs. Lack of proper identification procedures has affected several projects (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993).

OBJECTIVE
The aim of the present study was to survey molecular methods useful in egg parasitoid identification and discrimination and investigate the possibility that *G. tuberculifemur* (Ogloblin) could exist as a cryptic species complex. In addition, perform a phylogenetic analysis with several species within the genus *Gonatocerus* Nees to confirm species boundaries and to test the support for the species groups considered.

RESULTS AND CONCLUSIONS
ISSR-PCR DNA fingerprinting. Amplification reactions were performed with geographic populations from Argentina and Chile with 5-9 separate individuals from pooled egg masses per location. Locations included were: Argentina: Rio Colorado (RC) (Rio Negro Province), San Rafael (SR) (Mendoza), San Miguel de Tucumán (SMT) (Tucumán), and Chile: Jalsuri (CH). Previously, we have demonstrated a positive correlation between ISSR-PCR banding patterns and species distinction (de León and Jones 2004, de León et al. 2004a,b, 2006). In addition, we have utilized the method to distinguish about 8 *Gonatocerus* species. As a first approach, we asked whether the ISSR-PCR method was suitable to distinguish geographic populations of *G. tuberculifemur* from Argentina and Chile. The results of this analysis are shown on Figure 1. Three geographic- or population-specific bands were identified within the San Rafael population, as indicated by the arrows. Slight variation was seen within the rest of the populations, but in general, similar banding patterns were observed within these

populations, demonstrating their genetic similarity. The Tunuyán (TU) (Mendoza Province) population was not available at the time of this experiment.

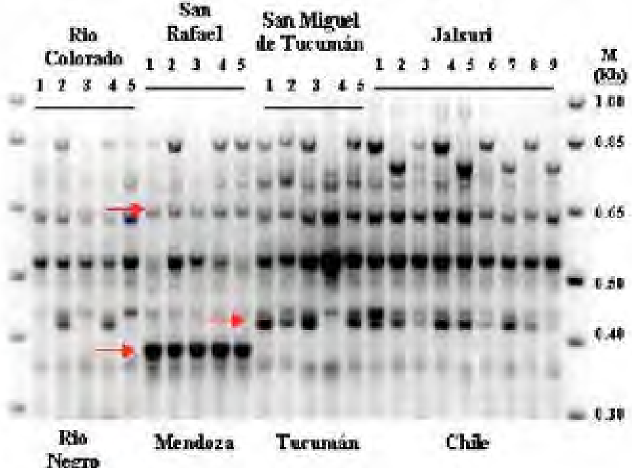

Figure.1. ISSR-PCR DNA fingerprinting of *G. tuberculifemur* populations from Argentina and Chile. Reactions were performed with genomic DNA from 5-9 separate individuals and a 5'-anchored ISSR primer (Zietkiewicz et al. 1994, de León et al. 2004b). Arrows point out the banding patterns differences. M: 1.0 Kb Plus DNA Ladder.

Phylogeographic analysis of populations of *G. tuberculifemur*

Levels of genetic divergence (%D) in the COI partial gene among populations were determined by calculating the pairwise estimates for genetic distance (Table 1). Individuals from each clade were pooled to calculate the pairwise estimates. The intra-populational and -specific variation (0.0-0.6%) was small within each clade and species. The %D within each *G. tuberculifemur* clade was 0.0-0.6, whereas between them was 1.4-2.2. A neighbor-joining distance tree showed that individuals clustered into two well-supported distinct clades with very strong bootstrap values of 96-100%, with all of the San Rafael individuals forming a distinct clade (Figure 2). Though the %D was moderate between the two *G. tuberculifemur* clades, it corroborates with the results seen in Figures 1 and 2 and Table 1, showing a very clear genetic distinction between individuals from the two clades. In addition, haplotype or phylogeographic structure was uncovered in these populations.

Phylogenetic analysis of several named and two unnamed* Gonatocerus *species

Resolution of relationships requires information about variability not only at the level of populations within a species but also between species (Narang et al. 1993); therefore, a molecular systematic approach inferred by the COI gene was undertaken with various named *Gonatocerus* species, along with *G. tuberculifemur* populations from South America. The named *Gonatocerus* species were also included to test the support for the species groups considered. A total of 48 ingroup specimens were analyzed and four specimens from two *Anagrus* Haliday species (also a mymarid genus) were included as outgroups. Each named *Gonatocerus* species formed its own distinct clade or taxonomic unit (Figure 3), confirming the species boundaries of Triapitsyn (2006). For each taxonomic unit, the neighbor-joining distance tree was supported by very strong bootstrap values (96-100%). The specimens of *G. tuberculifemur* again formed two distinct clades among the named *Gonatocerus* species. All specimens from San Rafael clustered into clade 2, whereas the rest of the populations from South America all clustered into clade 1, suggesting that *G. tuberculifemur* contains two distinct lineages. Each of the two unnamed *Gonatocerus* species (*G*. sp. 2 and *G*. sp. 6) from South America also clustered into distinct clades, suggesting that indeed they are separate species.

Two molecular methods were employed to genetically characterize the candidate GWSS egg parasitoid species, *G. tuberculifemur* from South America. ISSR-PCR DNA fingerprinting identified fixed geographic-specific variation in the population from San Rafael (Mendoza). Even though ISSR-PCR markers are scored as dominant, the method is still extremely sensitive and an excellent first approach to detect genetic differences among species, especially haplodipoid species (de León and Jones 2004, de León et al. 2004a,b, 2006). Similarly, the phylogeographic approach inferred by the COI partial gene, detected two well-supported clades in South America. The two molecular methods were in accord and the results are suggestive of a species level divergence. More work is needed to determine whether these two genetically distinct *G. tuberculifemur* clades are actually cryptic or different species. Hybridization and morphological studies are in progress.

Table 1. Pairwise sequence distances (range) of mitochondrial COI partial gene fragments from geographic populations of *G. tuberculifemur* showing percentage divergence. The alignment program ClustalW from DNAStar was utilized for this analysis. To account for intra- and inter-populational variation, several individuals (3-6) were included (15 total). Argentina: Rio Colorado (Rio Negro Province); San Rafael (Mendoza); Tunuyán (Mendoza); San Miguel de Tucumán (Tucumán); and Chile: Jalsuri. Refer to Figure 2 for assignments. *G. annulicornis* (G. ann) (Argentina, South America) and *G. morrilli* (G. mor) (Texas USA, North America) were included as outgroups.

Species/clades	Clade 1	Clade 2	*G. ann*	*G. mor*
Clade 1	0.0-0.6			
Clade 2	1.4-2.2	0.0-0.6		
G. ann	5.0-5.6	5.2-5.8	0.2-0.4	
G. mor	5.6-6.0	6.2-6.7	4.6-4.8	0.0-0.0

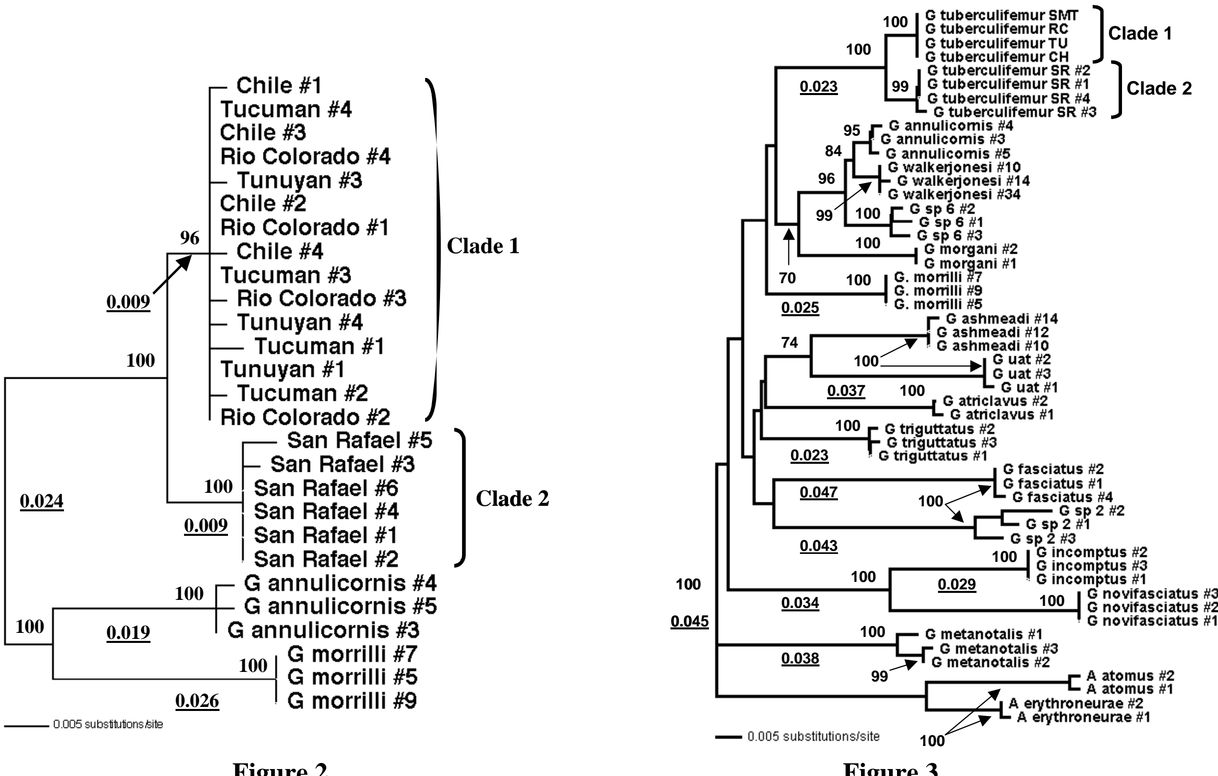

Figure 2. Phylogram of the COI partial gene from geographic populations of *G. tuberculifemur* from Argentina and Chile. Analysis was performed with the alignment program ClustalX and the neighbor-joining distrance tree utilizing the uncorrected 'p' genetic distance was reconstructed with the phylogenetic program PAUP 4.0b10. *G. annulicornis* and *G. morrilli* were included as outgroups. The tree displays branch lengths (below branches, underlined) and bootstrap values (above branches), as percentage of 1000 replications.

Figure 3. Phylogenetic relationships of several named and two unnamed *Gonatocerus* Nees species along with *G. tuberculifemur* geographic populations from South America. Neighbor-joining distance phylogram inferred by the COI partial gene. Anaylsis was performed as described on Figure 2. Two *Anagrus* species (mymarids) were included as outgroups.

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PRELIMINARY EVIDENCE FROM REPRODUCTIVE COMPATIBILITY STUDIES SUGGESTS THAT *GONATOCERUS TUBERCULIFEMUR* EXISTS AS A CRYPTIC SPECIES COMPLEX, OR A NEW SPECIES IS IDENTIFIED: DEVELOPMENT AND UTILITY OF MOLECULAR DIAGNOSTIC MARKERS

Project Leaders:

Jesse H. de Leon
USDA, ARS
Beneficial Insects Res. Unit
Weslaco, TX 78596

Guillermo A. Logarzo
USDA, ARS, SABCL
Buenos Aires, Argentina

Serguei V. Triapitsyn
Deparment of Entomology
University of California
Riverside, CA 92521

Cooperator:

David J.W. Morgan
Calif. Dept. of Food & Agric.
Mt. Rubidoux Field Station
Riverside, CA 92501

Reporting Period: The results reported here are from work conducted fiscal year 2005 to fiscal year 2006.

ABSTRACT
Recent work uncovered divergent clades or distinct lineages in populations of *Gonatocerus tuberculifemur* from South America. *G. tuberculifemur* is a prospective egg parasitoid candidate agent for a neoclassical biological control program in California against the invasive glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)]. In the present study, we developed molecular diagnostic markers by two approaches to distinguish field-collected populations of *G. tuberculifemur* for reproductive compatibility studies. The two diagnostic assays were: polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the mitochrondrial cytochrome oxidase subunit I gene (COI) and inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting. Clade-specific restriction enzymes generated bands of the correct size with high specificity. Analysis of two isofemales lines created from freshly field-collected populations belonging to clade 1 (Tunuyán) and clade 2 (San Rafael) showed that both of our developed molecular diagnostic markers correctly genotyped these isofemale lines, confirming the utility of our diagnostic markers. Based on our molecular work, we predicted that *G. tuberculifemur* individuals belonging to the two distinct clades would not hybridize. Preliminary mating compatibility studies between these two isofemale lines demonstrated that our prediction was indeed correct. Interspecific crosses produced only male offspring, whereas, the intraspecific control crosses produced both males and females or fertile offspring. Taken together, both our molecular work and the preliminary reproductive compatibility studies strongly suggest that *G. tuberulifemur* either exists as a cryptic species complex or a new species is identified. Since *G. tuberculifemur* is under consideration as a biological control agent against the invasive GWSS in California, understanding cryptic variation of this species is critical.

INTRODUCTION
Egg parasitoids of closely related hosts belonging to the sharsphooter tribe Proconiini [*Tapajosa rubromarginata* (Signoret)] were sought from regions in South America where climate types and habitats were similar to California for a neoclassical biological control program (Jones 2001, Logarzo et al. 2003, 2004, 2005). In surveys conducted in South America during 2000 through 2005, prospective egg parasitoid candidate agents were identified among several *Gonatocerus* Nees species (Hymenotpera: Mymaridae) reared from *T. rubomarginata* (Jones et al. 2005, Logarzo et al. 2005, Virla et al. 2005). One candidate was identified by S. Triapitsyn (UC-Riverside) as *Gonatocerus tuberculifemur* (Ogloblin) (Hymenotpera: Mymaridae) and is now being permitted for release in California (CDFA 2005). Identifying the correct natural enemy is critical to the success of classical biological control programs. Lack of proper identification procedures has affected several projects (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993).

OBJECTIVES
1. Develop molecular diagnostic markers by two methods, ISSR-PCR DNA fingerprinting and PCR-RFLP of the COI gene to distinguish *G. tuberculifemur* isofemale lines belonging to the two distinct well-supported clades [accompanying report and de Leon et al. (2006)]
2. After freshly collecting and creating isofemale lines, genotype them to determine which clade they belong to, and initiate reproductive compatibility studies with individuals from the distinct two clades.

RESULTS AND CONCLUSIONS
Polymerase Chain Reaction-Restriction Fragment Length Polymorphism diagnostic assays
Restriction enzyme maps of the partial COI gene were generated from individuals belonging to the two clades of *G. tuberculifumer* [accompanying report and de León et al. (2006)] and clade-specific restriction enzymes were identified that distinguished individuals from the two clades. The results of this experiment are shown on Figure 1. Digestion with the clade 1- and clade 2-specific restriction enzymes generated bands of the expected sizes (Figures 1A and 1B) with high specificity. After the completion of all molecular work, including the development of the diagnostic markers, the next step was to determine the utility of the markers. Based on our molecular work, we predicted that *G. tuberculifemur* individuals from clade 1 and clade 2 were not reproductively compatible. To initiate these studies, fresh field collections of *G. tuberculifemur* were made in both Tunuyán and San Rafael**,** populations belonging to clade 1 and clade 2, respectively and isofemales lines were created. PCR-RFLP diagnostic assays confirmed that the isofemale lines carried the correct genotypes (Figure 1C), that is, as predicted from our molecular work the Tunuyán isofemale line belongs to clade 1 and the San Rafael

isofemale line belongs to clade 2. Analysis of the isofemale lines with the second diagnostic assay (ISSR-PCR) confirmed the above findings (Figure 2). The current results confirm the utility of our developed molecular diagnostic markers.

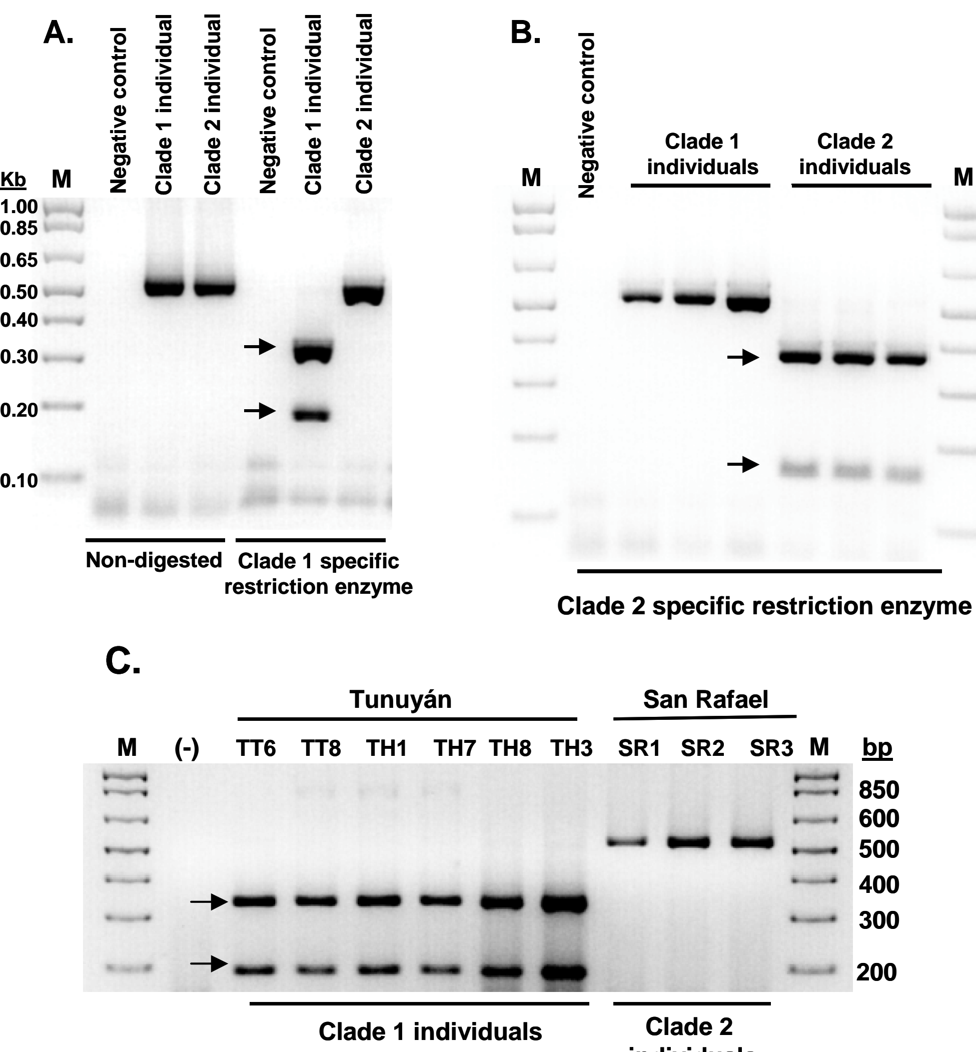

Figure 1. Representative example of the developed polymerase chain reaction-restriction fragment length polymporphism (PCR-RFLP) diagnostic assay. A. Digestion with the clade 1-specific restriction enzyme produces two bands: 193- and 325-bp, **B.** Digestion with the clade 2-specific restriction enzyme also generates two bands with the following sizes: 157- and 361-bp., and **C.** PCR-RFLP diagnostic assays of **'isofemale lines'** using the clade 1- specific restriction enzyme. TT, individuals from Tunuyán emerging from *T. rubromarginata* (Proconiini tribe); TH, individuals from Tunuyán emerging from *Hortensia similis* (Cicadellini tribe); and SR, individuals from San Rafael emerging from *T. rubommarginata*. **M**, 1.0 Kb Plus DNA Ladder.

Preliminary Reproductive compatibility studies

Field host range tests indicated that *G. tuberculifemur* has a limited development on some Cicadellini hosts showing a broader host range in the field than in the laboratory (where *G. tuberculifemur* only developed on Proconiini sharpshooters). The conflicting host ranged results could be due to the existence of sympatric cryptic species of *G. tuberculifemur* in the test area. Genotyped isofemale lines belonging to clade 1 (Tunuyán) and clade 2 (San Rafael), both of Mendoza Province, were carried out to evaluate mating compatibility. Direct and reciprocal crosses were performed: ♀San Rafael x ♂Tunuyán and ♀Tunuyán x ♂San Rafael and their controls (at least 3 replicates each and between 13-20 eggs per cross). Results are shown as mean percentage of individuals emerging from eggs parasitized by *G. tuberculifemur* females (Figure 3). Interspecific crosses produced only male offspring, whereas the intraspecific control crosses produced both males and females or fertile offspring, indicating that the populations from San Rafael and Tunuyán, which are about 100 km apart from each other, were reproductively incompatible and therefore reproductively isolated. More work is needed to complete these studies, which are in progress. Together, both our molecular data (de León et al. 2006) and the preliminary crossing studies strongly suggest that *G. tuberculifemur* either exists as a cryptic species complex or a new species has been identified. In addition, preliminary morphological work of individuals from the two clades suggests some slight differences (unpublished data, S.

Triapitsyn). Genotyping of *G. tuberculifemur* colonies reared and maintained at both Riverside, CA (UC-Riverside) and Edinburg, TX (USDA, APHIS) confirmed that both colonies or isofemale lines belong to clade 1.

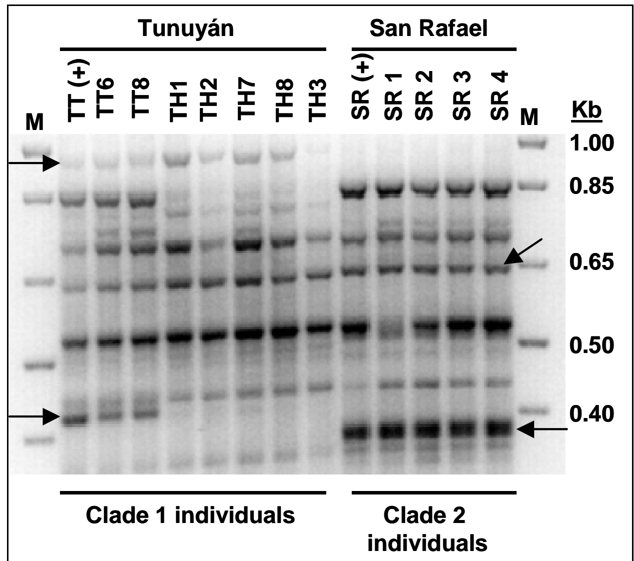

Figure 2. ISSR-PCR DNA fingerprinting using a 5'-anchored ISSR primer (Zietkiewicz et al. 1994) of 'isofemale lines' created after molecular characterization of *G. tuberculifemur* was complete. TT, individuals from Tunuyán emerging from *T. rubromarginata* (Proconiini tribe); TH, individuals from Tunuyán emerging from *Hortensia similis* (Cicadellini tribe); and SR, individuals from San Rafael emerging from *T. rubommarginata*. **M**, 1.0 Kb Plus DNA Ladder. Arrows point to banding pattern differences.

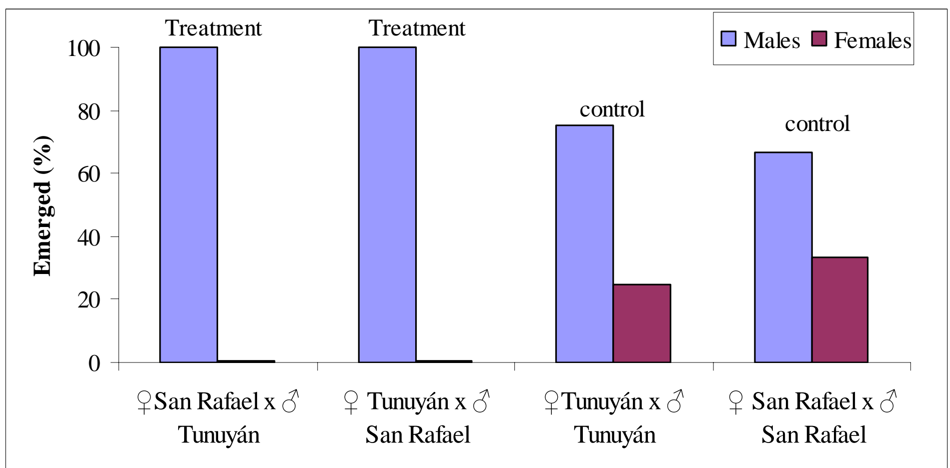

Figure 3. Preliminary hybridization studies (above). Mean percentage of female and male offspring emerging from eggs parasitized by G. tuberculifemur females obtained from different crosses with isofemale lines created from two populations from South America, Tunuyán and San Rafeal, belonging to two well-supported distinct clades (de León et al. 2006).

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ISSR-PCR DNA FINGERPRINTING UNCOVERS DISTINCT BANDING PATTERNS IN *GONATOCERUS* SPECIES 3 (*G*. sp. 3) INDIVIDUALS EMERGING FROM DIFFERENT HOST TRIBES: A PROSPECTIVE EGG PARASITOID CANDIDATE AGENT FOR THE GLASSY-WINGED SHARPSHOOTER IN CALIFORNIA

Project Leaders:
Jesse H. de Leon
USDA, ARS
Beneficial Insects Res. Unit
Weslaco, TX 78596

Guillermo A. Logarzo
USDA, ARS, SABCL
Buenos Aires, Argentina

Serguei V. Triapitsyn
Department of Entomology
University of California
Riverside, CA 92521

Cooperator:
David J.W. Morgan
Calif. Dept, of Food & Agric.
Mt. Rubidoux Field Station
Riverside, CA 92501

Reporting Period: The results reported here are from work conducted fiscal year 2005 to fiscal year 2006.

ABSTRACT
We started work to genetically characterize a prospective glassy-winged sharsphooter (GWSS), *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)] egg parasitoid biological control candidate agent from South America known as *Gonatocerus* species 3 (*G*. sp. 3). This species is morphologically very similar to *G. tuberculifemur*, another prospective agent from South America. We asked two questions, 1) are *G*. sp. 3 and *G. tuberculifemur* the same species and 2) are two collections of *G*. sp. 3 individuals emerging from different host tribes (Proconiini and Cicadellini) genetically distinct. Or, in both cases, are we seeing genetic variation of the same species. Two molecular methods were utilized to begin to study these species, the very sensitive inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting and mitochondrial cytochrome oxidase subunit I gene (COI) variation. ISSR-PCR analysis performed together on both *G*. sp. 3 and *G. tuberculifemur* uncovered the following: 1) as previously shown, *G. tuberculifemur* geographic populations were genetically distinct, 2) *G*. sp. 3 and *G. tuberculifemur* were very clearly distinct, and 3) banding patterns differences (about four bands) distinguished the two collections of *G*. sp. 3. A single most parsimonious tree clustered the current specimens in the following fashion: 1) as previously shown, the geographic populations of *G. tuberculifemur* clustered into two well-supported distinct clades with very strong bootstrap values (90-99%), and 2) the *G*. sp 3 collections clustered along with clade 2 (San Rafael population) of the *G. tuberculifemur* populations, though one *G*. sp. 3 collection (Jan 05; Proconiini host) forms a unique clade with moderate bootstrap support (63%). Even though, the divergence between the two *G*. sp. 3 collections was very small, the two shared no haplotypes. The current results confirm that ISSR-PCR DNA fingerprinting using a 5'-anchored ISSR primer is an excellent molecular diagnostic tool for distinguishing *G*. sp. 3 from both clades of *G. tuberculifemur*. COI sequence variation effectively distinguished *G*. sp. 3 from *G. tuberculifemur* individuals from clade 1, though it did not effectively separate *G*. sp. 3 from *G. tuberculifemur* individuals from clade 2 (San Rafael population). We conclude that based on ISSR-PCR analysis, *G*. sp. 3 and *G. tuberculifemur* and both collections of *G*. sp. 3 are clearly genetically distinct. The only way to confirm whether these specimens are actually cryptic or different species is by performing hybridization studies. These molecular results are important to the biological control program in California.

INTRODUCTION
Beginning in 2000, egg parasitoids of closely related hosts belonging to the sharsphooter tribe Proconiini [*Tapajosa rubromarginata* (Signoret)] were sought from regions in South America where climate types and habitats were similar to California for a neoclassical biological control program (Jones 2001, Logarzo et al. 2003, 2004, 2005). In surveys conducted in Argentina and Chile during 2000 through 2005, prospective egg parasitoid candidate agents were identified among several *Gonatocerus* Nees species (Hymenotpera: Mymaridae) reared from *T. rubomarginata* (Jones et al. 2005, Logarzo et al. 2005, Virla et al. 2005). Several unnamed egg parasitoid candidate agents within the genus *Gonatocerus* from South America were identified by S. Triapitsyn (UC-Riverside). Phylogenetic analysis inferred by COI sequencing on two of the unnamed species (*G*. sp. 2 and *G*. sp. 6) are reported in an accompanying report and elsewhere (de León et al. 2006b). The data suggests that these unnamed species are valid species or taxonomic units. A third unnamed species known as *G*. sp. 3 was also identified that is morphologically very similar or almost identical to another South America species, *G. tuberculifemur* (Ogloblin) (S. Triapitsyn, unpublished data). Since this and other South American species are prospective biological control agents, molecular studies are critical to help resolve the taxonomic status of this and other species. Identifying the correct natural enemy is critical to the success of classical biological control programs, since lack of proper identification procedures has affected several projects (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993).

OBJECTIVES
The aim of the present study was to survey molecular methods to study *G*. sp. 3. Morphologically, this species is almost identical or very similar to *G. tuberculifemur* (S. Triapitsyn, unpublished data). The first objective was to begin to gain insights as to whether *G*. sp. 3 and *G. tuberculifemur* are distinct species or whether variation of the same species exists, and the second objective was to determine whether collections *G*. sp. 3 emerging from different host tribes (Proconiini and Cicadellini) are genetically distinct.

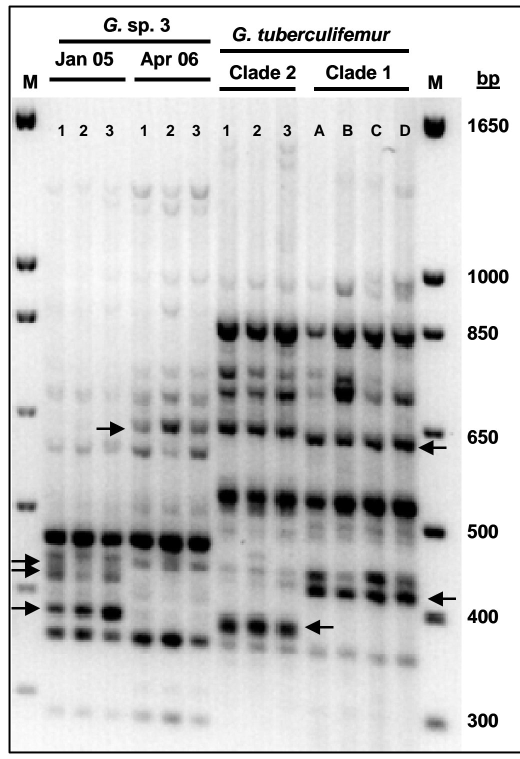

Figure 1. ISSR-PCR DNA fingerprinting of two *G*. sp. 3 populations from San Miguel de Tucumán, Tucumán Province, Argentina. Reactions were performed with genomic DNA from 3-4 separate individuals and a 5'-anchored ISSR primer (Zietkiewicz et al. 1994, de León et al. 2004b). *G*. sp. 3 are from two different collection dates, January 2005 [emerged from *T. rubromarginata* (Proconiini leafhopper) and April 2006 emerged from *Plesiommata mollicella* (Cicadellini leafhopper)]. *G. tuberculifemur* collections are as follows- Clade 2 are from San Rafael (SR) (Mendoza Province) Argentina and Clade 1 are: A, Rio Colorado (RC) (Rio Negro); B, San Miguel de Tucumán (SMT) (Tucumán); C, Chile (CH); and D, Tunuyán (TU) (Mendoza). Arrows point out the banding patterns differences. M: 1.0 Kb Plus DNA Ladder.

RESULTS AND CONCLUSIONS

ISSR-PCR DNA fingerprinting

Previously (de León et al. 2006b) and in an accompanying report, we demonstrated molecular differences in populations of *G. tuberculifemur* from South America. ISSR-PCR DNA fingerprinting uncovered fixed geographic variation and COI variation uncovered divergent clades in *G. tuberculifemur*. Since it is thought that *G*. sp. 3 could actually be *G. tuberculifemur*, we asked two questions: 1) based on ISSR-PCR, are *G*. sp. 3 and *G. tuberculifemur* genetically distinct, and 2) based on ISSR-PCR, are there distinct differences in *G*. sp. 3 individuals emerging from different hosts tribes [San Miguel de Tucumán (Tucumán)]. The results of this experiment are shown on Figure 1 and we make three observations: 1) as previously shown (de León et al. 2006b) the two clades of *G. tuberculifemur* are distinguished, see arrows that point to different bands; 2) very clear banding pattern differences between *G*. sp. 3 and *G. tuberculifemur*, and 3) banding pattern differences between the two collections of *G*. sp. 3, about four bands distinguish the two collections. These results are very reproducible.

Phylogeographic analysis between the two collections of G. sp. 3 and among geographic populations of *G. tuberculife*mur inferred by the mitochondrial COI partial gene

The single most parsimonious tree is supported by strong bootstrap values, 63-99% for the ingroups, and 100% for the outgroups (*G. annulicornis* and *G. morrilli*) (Figure 2). As shown previously (de León et al. 2006b) and in an accompanying report, populations of *G. tuberculifemur* clustered into two well-supported clades (90-99%). In general, the two collections of *G*. sp. 3 clustered with the *G. tuberculifemur* clade from San Rafael, though *G*. sp. 3 from the Jan 05 collection emerging from the Proconiini host appears to be slightly diverged as seen by a distinct clade with moderate bootstrap support (63%). It is interesting to note that even though, based on COI analysis, the divergence between the two collections of *G*. sp. 3 is very small, they did not share haplotypes.

Two molecular methods were employed to genetically study a GWSS candidate agent, G. sp. 3 from South America. ISSR-PCR DNA fingerprinting identified fixed geographic-specific variation among the G. tuberculifemur populations (de León et al. 2006b) and fixed genetic variation between the two collections of G. sp. 3 emerging from different hosts. Even though ISSR-PCR markers are scored as dominant, the method is still extremely sensitive and an excellent first approach to detect genetic differences among species, especially haplodipoid species (de León and Jones 2004, de León et al. 2004a,b, 2006a,b). A phylogenetic approach inferred by the COI partial gene, detected two well-supported clades in G. tuberculifemur (de León et al. 2006b). However, in the present situation with G. sp. 3, the COI gene was less sensitive than ISSR-PCR. A possible explanation for the COI sequence analysis being less sensitive than ISSR-PCR could be that G. sp. 3 diverged a very short time ago from G. tuberculifemur and the COI gene is not yet variant enough at this time to properly distinguish them. Time since divergence is a very significant factor as reviewed in Roderick and Navajas (2003). Another explanation is that the two collections of G. sp. 3 emerged from different hosts. More work and an increased number of specimens are needed to determine whether G. sp. 3 and G. tuberculifemur are actually different species and whether the two collections of G. sp. 3 are different strains or whether what we are seeing is just genetic variation of the same species. To resolve these issues, crossing studies should answer our questions. Hybridization and morphological studies are presently being planned. For now, we can positively state that G. sp. 3 and G. tuberculifemur and the two collections of G. sp. 3 are clearly genetically distinct by ISSR-PCR DNA fingerprinting. COI variation effectively distinguished G. sp. 3 from G. tuberculifemur individuals from clade 1, but not clade 2. So, the following questions arise from these studies: Is ISSR-PCR using a 5'-anchored ISSR primer more sensitive than DNA sequencing and can ISSR-PCR detect differences in individuals of the same species emerging from different hosts, or rather do the different hosts change their genetic structure? The ISSR-PCR technique targets random simple sequence repeats or microsatellites targeting the whole genome, thus revealing highly polymorphic banding patterns (Zietkiewicz et al. 1994). Therefore, increased power to resolve genetic relationships comes with information from many loci within the nuclear DNA (de León and Jones 2004, de León et al. 2004a,b, 2006,

Zietkiewicz et al. 1994). The results of the present study are important to the biological control program in California against the invasive GWSS.

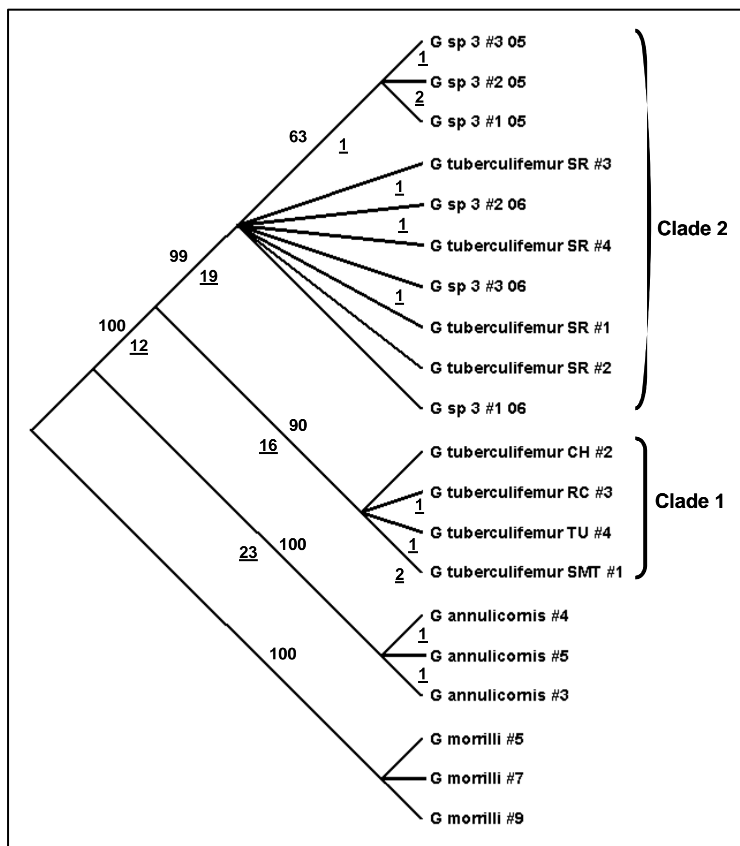

Figure 2. Slanted cladogram inferred by the COI partial gene from two collections of *G.* sp. 3 from Argentina (San Miguel de Tucumán, Tucumán Province). Analysis was performed with the alignment program ClustalX and the single most parsimonious tree was reconstructed with the phylogenetic program PAUP 4.0b10. *G. annulicornis* and *G. morrilli* were included as outgroups. The tree displays branch lengths (below branches, underlined) and bootstrap values (above branches), as percentage of 1000 replications. Tree length = 57 steps; consistency index (CI) = 0.982; and retention index (RI) = 0.994. Included for comparison are populations of *G. tuberculifemur* from South America: SR, San Rafael; CH, Chile; RC, Rio Colorado; TU, Tunuyán; and SMT, San Miguel de Tucumán.

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GENETIC STUDIES OF *GONATOCERUS METANOTALIS* POPULATIONS FROM ARGENTINA UNCOVER DIVERGENT CLADES: A PROSPECTIVE EGG PARASITOID CANDIDATE AGENT FOR THE GLASSY-WINGED SHARPSHOOTER IN CALIFORNIA

Project Leaders:
Jesse H. de Leon
USDA, ARS
Beneficial Insects Res. Unit
Weslaco, TX 78596

Guillermo A. Logarzo
USDA, ARS, SABCL
Buenos Aires, Argentina

Serguei V. Triapitsyn
Deparment of Entomology
University of California
Riverside, CA 92521

Cooperator:
David J.W. Morgan
Calif. Dept. of Food & Agric.
Mt. Rubidoux Field Station
Riverside, CA 92501

Reporting period: The results reported here are from work conducted fiscal year 2005 to fiscal year 2006.

ABSTRACT
Two molecular methods were utilized to genetically distinguish geographic populations of *Gonatocerus metanotalis* (Ogloblin) (Hymenoptera: Mymaridae) from Argentina and to begin to test the possibility that this South American species could exist as a cryptic species complex. *Gonatocerus metanotalis* is a prospective egg parasitoid candidate agent for a neoclassical biological control program in California against the invasive glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)]. Six populations were analyzed: Campo Grande (Misiones Province), Tartagal (Salta), Tafí Viejo (Tucumán), near PROIMI (Tucumán), Santa Clara (Jujuy), Clorinda (Formosa). As a first approach, inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting was performed with a 5'-anchored ISSR primer. Several distinct banding patterns were identified among the populations with some band sharing and in certain populations (Tartagal and Santa Clara) there was extensive variation. Next, a phylogeographic analysis inferred by the mitochondrial cytochrome oxidase subunit I (COI) gene was performed. A neighbor-joining distance tree clustered the *G. metanotalis* populations into three main distinct clades supported by very strong bootstrap values (100%), uncovering haplotype or phylogeographic structure. With the exception of one population (Campo Grande), all individuals from the populations fell into one of the three clades. Individuals from Campo Grande clustered into the three clades, suggesting that three sympatric strains may be present in this location. A phylogenetic analysis performed by the neighbor-joining algorithmic method along with other named and two unnamed *Gonatocerus* Nees species (15) confirmed species boundaries and again uncovered three main distinct clades in *G. metanotalis*. Very strong bootstrap support (100%) was seen for both the *G. metanotalis* clades and for all of the *Gonatocerus* species. Understanding possible cryptic variation in this prospective GWSS egg parasitoid candidate agent is critical to the biological control program in California.

INTRODUCTION
A biological control program is currently in progress in California against the glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) [=*H. coagulata* (Say)] (Hemiptera: Cicadellidae) (CDFA 2003). Biological control is an important component of the management of the GWSS in California (Morgan et al. 2000, Jones 2001). Uncertainty exists as to whether egg parasitoids native to California will be as effective against the GWSS as they are in their co-evolved native range (Jones 2001, Logarzo et al. 2003, 2004, Virla et al. 2005). Beginning in 2000, egg parasitoids of closely related hosts belonging to the sharsphooter tribe Proconiini [*Tapajosa rubromarginata* (Signoret)] were sought from regions in South America where climate types and habitats were similar to California for a neoclassical biological control program (Jones 2001, Logarzo et al. 2005). In surveys conducted in South America during 2000 through 2005, prospective egg parasitoid candidates were identified among several *Gonatocerus* Nees species reared from *T. rubomarginata* (Logarzo et al. 2005, Virla et al. 2005). One egg parasitoid candidate was identified by S. Triapitsyn (UC-Riverside) as *G. metanotalis* (Ogloblin) (Hymenotpera: Mymaridae). Molecular studies of insects are becoming increasingly important in resolving taxonomic relationships critical to the success of biological control programs. Identifying the correct natural enemy is critical to the success of classical biological control programs (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993, Unruh and Woolley 1999).

OBJECTIVES
The aim of the present study was to survey molecular methods useful in egg parasitoid identification and discrimination and to begin to investigate the possibility that *G. metanotalis* could exist as a cryptic species complex in South America. In addition, perform a phylogenetic analysis with several named and two unnamed species within the genus *Gonatocerus* Nees to confirm species boundaries and to test the support for the species groups considered.

RESULTS AND CONCLUSIONS
ISSR-PCR DNA fingerprinting
Amplification reactions were performed with geographic populations from Argentina with 3-5 separate individuals from pooled egg masses per location. Populations included: Campo Grande (Misiones Province); Tartagal (Salta); Tafí Viejo (Tucumán); near PROIMI (Tucumán); Santa Clara (Jujuy); and Clorinda (Formosa). Previously, we demonstrated a positive correlation between ISSR-PCR banding patterns and species distinction (de León et al. 2004, 2006a,b). In addition, we have utilized the method to distinguish about 8 *Gonatocerus* species (de León et al. 2006b). As a first approach, we asked whether the ISSR-PCR method was suitable to distinguish geographic populations of *G. metanotalis* from Argentina. The results of

this analysis are shown on Figure 1. Several banding patterns were identified among the populations with some band sharing and in certain populations (Tartagal and Santa Clara) there was extensive variation. These ISSR-PCR results are clearly the first indication that genetic differences exist among the *G. metanotalis* populations from Argentina.

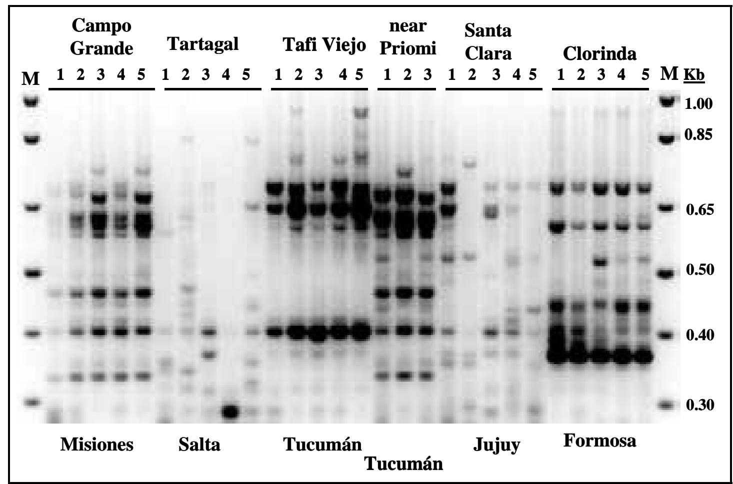

Figure 1. ISSR-PCR DNA fingerprinting of *G. metanotalis* populations from Argentina. Reactions were performed with genomic DNA from 3-5 separate individuals and a 5'-anchored ISSR primer (Zietkiewicz et al. 1994, de León et al. 2004). **M:** 1.0 Kb Plus DNA Ladder.

Phylogeographic analysis of populations of *G. metanotalis*

Levels of divergence in the COI partial gene among populations from Argentina were determined by calculating the pairwise estimates for genetic distance (Table 1). Individuals clustered into three main distinct clades or groups (see Figure 2 for assignments) and individuals from each clade were pooled to calculate the pairwise estimates. The intra-clade divergence (%D) ranged from 0.0-1.0, whereas the inter-clade %D ranged from 2.4-4.6. The present results clearly indicate a deep divergence among the three main groups.

Table 1. Pairwise sequence distances (range) of the mitochondrial COI partial gene from geographic populations of *G. metanotalis* from Argentina showing percentage divergence (%D). The alignment program ClutstalW from DNAStar was utilized for this analysis. To account for intra- and inter-populational variation, several individuals (3-4) were included. Populations from Argentina included: Campo Grande (Misiones Province); Tartagal (Salta); Tafí Viejo (Tucumán); near PROIMI (Tucumán); Santa Clara (Jujuy); and Clorinda (Formosa). Individuals clustered into three clades, see Figure 2 for assignments. *Gontacerus ashmeadi* (G. ash) (CA) were utilized as an outgroup.

Species/clades	Clade 1	Clade 2	Clade 3	*G. ash*
Clade 1	0.0-1.0			
Clade 2	2.4-3.6	0.0-0.8		
Clade 3	3.6-4.6	3.8-4.6	0.0-0.6	
G. ash	9.0-9.9	8.6-9.2	8.2-8.6	0.0-0.2

A neighbor-joining distance tree showed that individuals clustered into three well-supported distinct clades with very strong bootstrap values (100%) (Figure 2), uncovering haplotype or phylogeographic structure. With the exception of one population (Campo Grande), all of the individuals clustered into one of the three clades. These results suggest that three sympatric strains of *G. metanotalis* may be present in Campo Grande, although collection of more specimens are needed to confirm this observation. It is interesting to note that within clade 1, there appears to be a further subdivision with the appearance of three subclades. This observation would agree with the results of the ISSR-PCR experiment (Figure 1) that shows about five total banding patterns within the *G. metanotalis* populations.

Phylogenetic relationships among *Gonatocerus* Nees species

Resolution of relationships requires information about variability not only at the level of populations within a species but also between species (Narang et al. 1993, Unruh and Woolley 1999); therefore, a molecular systematic approach was undertaken with various named and two unnamed *Gonatocerus* species, along with the six *G. metanotalis* populations from Argentina

(Figure 3). These species were also included to test the support for the species groups considered. The topology of the neighbor-joining distance tree was supported by very strong bootstrap values, 100% support was seen for all species or taxonomic units, confirming species boundaries (Triapitsyn 2006, Triapitsyn et al. 2006). The phylogenetic analysis again clustered *G. metanotalis* individuals from Argentina into three main distinct clades as seen in Figure 2. Because each *Gonatocerus* species formed its own unique clade or taxonomic unit, the possibility exists that each *G. metanotalis* clade represents a different strain. This observation is also supported by the results of the ISSR-PCR experiment (Figure 1). In addition, because of the deep divergence among *G. metanotalis* clades, the possibility that a cryptic species complex was identified within *G. metanotalis* in Argentina is high. Another possibility is that we may be uncovering different species**.** Phylogenetic analyses of *Gonatocerus* species inferred by COI sequence data have been reported elsewhere (PD reports 2006 and de León et al. 2006b), but are expanded here.

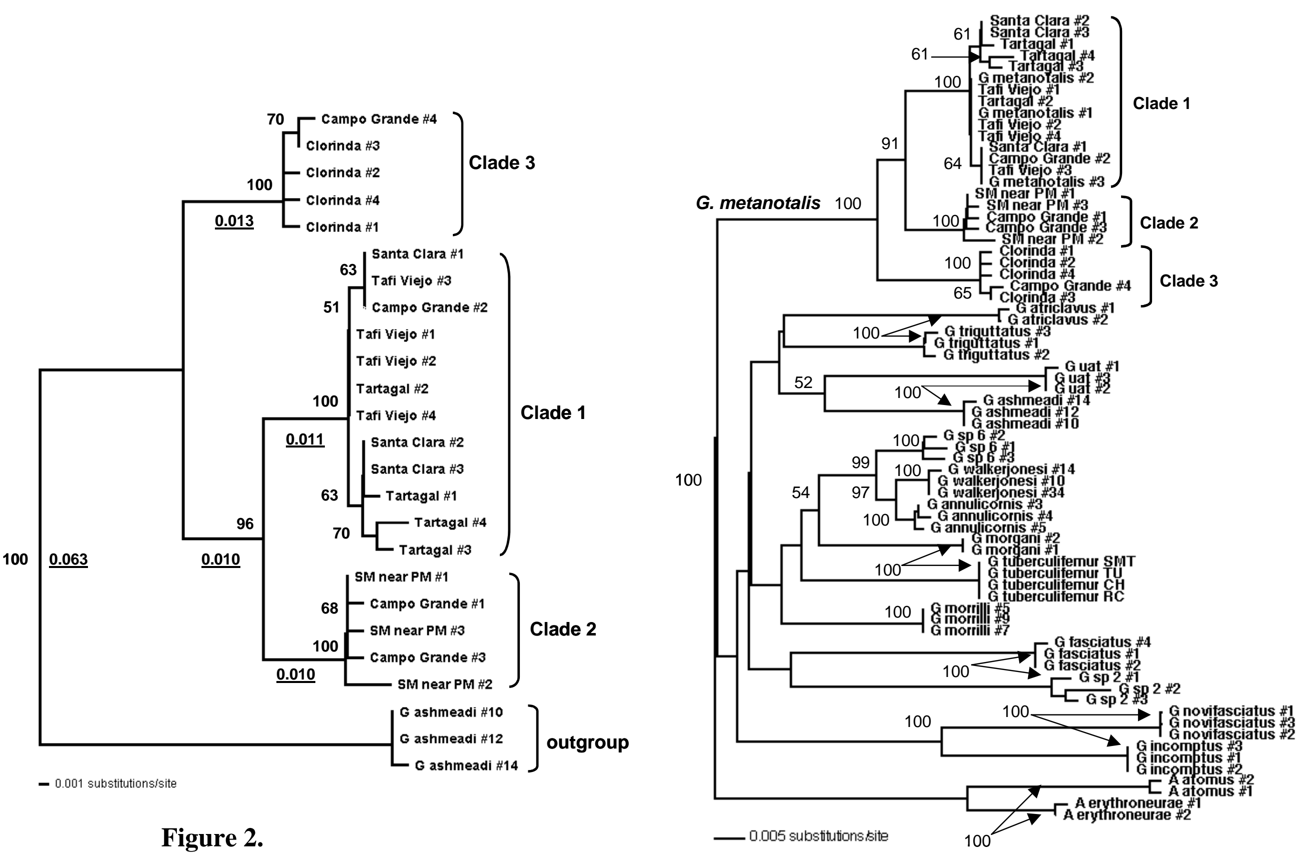

Figure 2.

Figure 3.

Figure 2. Phylogram inferred by the COI partial gene from geographic populations of *G. metanotalis* from Argentina. Analysis was performed with the alignment program ClustalX and the neighbor-joining distance tree utilizing the uncorrected 'p' genetic distance was reconstructed with the phylogenetic program PAUP 4.0b10. To account for intra- and inter-populational variation, several individuals (3-4) were included. *G. ashmeadi* (CA) were utilized as an outgroup. The tree displays branch lengths (below branches, underlined) and bootstrap values (above branches), as percentage of 1000 replications.

Figure 3. Phylogenetic relationships of several named and two unnamed *Gonatocerus* Nees species along with *G. metanotalis* populations from Argentina. Neighbor-joining distance phylogram inferred by the COI partial gene. Anaylsis was performed as described on Figure 2. Two *Anagrus* species (mymarid genus) are included as outgroups.

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Additional note: We acknowledge Marissa González and Lisa A. Ledezma (current address: USDA, APHIS, PPQ-Edinburg, TX) and Laura Varone and Vanina Varni (USDA, SABCL-Argentina) for their excellent technical assistance.

THE UTILITY OF THE INTERNAL TRANSCRIBED SPACER REGION 2 (ITS2) IN CONFIRMING SPECIES BOUNDARIES IN THE GENUS *GONATOCERUS*: COMPARISON TO THE CYTOCHROME OXIDASE SUBUNIT I (COI) GENE AND TAXONOMIC DATA: MOLECULAR KEY BASED ON ITS2 SIZES

Project Leaders:
Jesse H. de Leon
USDA, ARS
Beneficial Insects Res. Unit
Weslaco, TX 78596

Guillermo A. Logarzo
USDA, ARS, SABCL
Buenos Aires, Argentina

Serguei V. Triapitsyn
Deparment of Entomology
University of California
Riverside, CA 92521

Cooperator:
David J.W. Morgan
Calif. Dept. of Food & Agric.
Mt. Rubidoux Field Station
Riverside, CA 92501

Reporting Period: The results reported here are from work conducted fiscal year 2005 to fiscal year 2006.

ABSTRACT
We sequenced the nuclear ribosomal internal transcribed spacer region 2 (ITS2) from several glassy-winged sharpshooter (GWSS) [*Homalodisca vitripennis* Germar (=*H. coagulata* Say)] egg parasitoid species (Hymenoptera: Mymaridae) belonging to the genus *Gonatocerus* Nees to test the utility of this fragment to confirm species boundaries and to define phylogenetic relationships. A total of 35 specimens belonging to 10 named species, one unnamed species, and two specimens from another mymarid genus (*Anagrus erythroneurae*) (outgroup) were analyzed. A phylogenetic tree generated using the neighbor-joining algorithmic method showed that each named *Gonatocerus* species formed its own unique taxonomic unit or clade with very strong bootstrap support (100%), confirming species boundaries. The ITS2 fragment confirmed species boundaries as well as cytochrome oxidase subunit I (COI) sequence data. Furthermore, the phylogenetic relationships among species generated by the ITS2 fragment were in excellent agreement with those delineated by taxonomic data. The current results clearly confirm the utility of the ITS2 fragment in confirming species boundaries of egg parasitoids beloning to the genus *Gonatocerus*. The results showed that the ITS2 fragment appears to be phylogenetically more informative or valuable than that inferred by COI sequence data. Since several important *Gonatocerus* species were analyzed, a molecular key based on ITS2 sizes was developed. In the event two species (e. g., *G. ashmeadi* and *G. metanotalis* and *G. walkerjonesi* and *G. annulicornis*) were found with similarly sized ITS2 fragments, inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting was performed to distinguish them. ISSR-PCR very clearly distinguished the aforementioned species, demonstrating that it is an excellent molecular diagnostic tool. The current results are important to the biological control program in California.

INTRODUCTION
Accurately identifying natural enemies is critical to the success of classical biological control programs and lack of proper identification procedures has affected several projects (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993). Among others, DNA markers such as the nuclear ribosomal internal transcribed spacer regions (e. g., ITS2) are used to characterize parasitoid taxa because these DNA regions usually evolve relatively rapidly (Hillis and Dixon 1991, Narang et al. 1993). The ITS regions have been used extensively in the examination of the taxonomic status of species and for diagnostic purposes (Collins and Paskewitz 1996, Stouthamer et al. 1999). Many examples of phylogenetic studies inferred by the nuclear ITS regions or fragments, including different sized fragments, exists in the literature (Marinucci et al. 1999, Förster et al. 2000, Pryor and Gilbertson 2000, Alvarez and Hoy 2002, Thomson et al. 2003, de León et al. 2006a, Wagener et al. 2006), including those by Stouthamer et al. (1999).

OBJECTIVES
Sequence the nuclear ribosomal internal transcribed spacer region 2 (ITS2) from several GWSS egg parasitoid species (11) belonging to the genus *Gonatocerus* to test the utility of this rDNA fragment to: 1) confirm species boundaries and 2) define phylogenetic relationships.

RESULTS AND CONCLUSIONS
Species boundaries inferred by the ribosomal internal transcribed spacer region 2 (ITS2)
We obtained 8 of the 13 named *Gonatocerus* Nees species delineated by Triapitsyn (2006) and Triapitsyn et al. (2006) and several named and one unnamed species from South America for a total of 11 species. A total of 35 ingroup specimens were analyzed and two specimens from *Anagrus erythroneurae* Trjapitzin & Chiappini (also a mymarid species) were included as an outgroup. Each named *Gonatocerus* species formed its own taxonomic unit or distinct clade (Figure 1), corroborating the species boundaries of Triapitsyn (2006) and Triapitsyn et al. (2006). A neighbor-joining distance tree showed that each taxonomic unit was supported by very strong bootstrap values, in fact, each received 100% support. In addition, the unnamed *Gonatocerus* species (*G.* sp. 6) from Argentina also clustered into its distinct clade, suggesting that it is a separate or valid species. Analysis of several other *Gonatocerus* species inferred by the ITS2 DNA fragment are in progress to complete this project. As previously demonstrated by Vickerman et al. (2004) and Triapitsyn et al. (2006) no divergence or differences were seen in the five geographic populations [California, Texas (Weslaco and San Antonio), Florida, and Louisiana] of *G. ashmeadi*, as they all formed their unique clade.

Comparsion of ITS2 DNA sequences to those of the COI partial gene sequences
To confirm the utility of using the ITS2 DNA fragment in these types of studies, we matched the mitochrondrial cytochrome oxidase subunit I (COI) gene sequences to the same *Gonatocerus* species. A phylogenetic analysis of several *Gonatocerus* species inferred by the COI gene has been reported elsewhere [accompanying report and de León et al. (2006b)]. Comparison of the neighbor-joining distance tree generated by ITS2 fragments to that generated by the COI gene (Figure 2), confirms that the ITS2 fragment can be used to determine species boundaries of egg parasitoids belonging to the genus *Gonatocerus*. As with the ITS2 fragments, very strong bootstrap support (95-100%) was seen with each taxonomic unit or distinct clade generated by the COI gene (de León et al. 2006b).

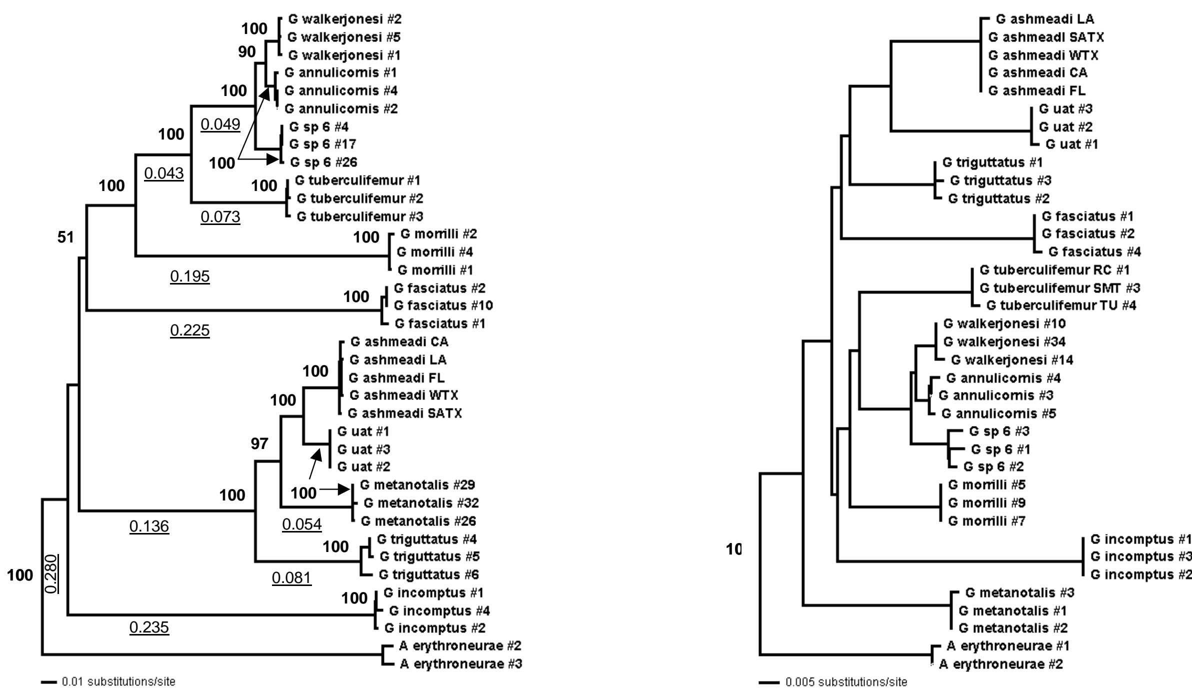

Figure 1. ITS2 **Figure 2. COI**

Figure 1. (ITS2) and Figure 2. (COI) Phylograms of GWSS egg parasitoid species beloning to the genus *Gonatocerus* inferred by ITS2 and COI sequence data. Analyses were performed with the alignment program ClustalX and the neighbor-joining distance trees utilizing the uncorrected 'p' genetic distance were reconstructed with the phylogenetic program PAUP 4.0b10. *Anagrus erythroneurae* (a mymarid species) was included as an outgroup. The tree displays branch lengths (below branches, underlined) and bootstrap values (above branches), as percentage of 1000 replications.

Comparsion to taxonomic data
To confirm the utility of the ITS2 fragment in defining phylogenetic relationships among egg parasitoid species within the genus *Gonatocerus*, a comparison of the topology of the phylogenetic tree was made to the morphological work delineated by Triapitsyn (2006) and Triapitsyn et al. (2006). Morphologically, almost every species in the USA has its 'look-a-like' species in the Neotropics, particularly in Argentina (S. Triapitsyn, unpublished data). The following South and North American species pair-up morphologically: respectively *G*. sp. 2 and *G. fasciatus*; *G. annulicornis* and *G. walkerjonesi*; *G. metanotalis* and *G. triguttatus*; and *G. uat* and *G. ashmeadi*. *Gonatocerus morrrilli* belongs to the *morrilli* subgroup of the *ater* species group of *Gonatocerus* along with the following species: *G. annulicornis*, *G. walkerjonesi*, *G. sp. 6*, and *G. morgani* (S. Triapitsyn, unpublished data and Triapitsyn 2006). In addition, *G. novifasciatus* and *G. incomptus* are also related (Triapitsyn 2006). Not all mentioned *Gonatocerus* species were analyzed at the time of this report. The phylogenetic relationships inferred by ITS2 fragment sequence data analyzed by the neighbor-joining method of the South and North American *Gonatocerus* species in the current study are all in accord with the morphological evaluations mentioned above (Triapitsyn 2006, Triapitsyn et al. 2006). Based on the phylogenetic tree, the following species paired-up in the following fashion: *G. ashmeadi* and *G. uat* (Triapitsyn et al. 2006, de León et al. 2006b); *G. walkerjonesi* and *G. annulicornis* (de León et al. 2006a); *G. morrilli* and *morrilli* group (de León et al. 2006a); *G*. sp. 6 and *morrilli* group (de León et al. 2006b); and to some extent *G. metanotalis* and *G. triguttatus* (except for the color and several other differences, *G. ashmeadi*, *G. uat*, *G. triguttatus*, and *G. metanotalis* are all quite similar morphologically, and the above molecular analysis supports their close relationship. We noticed in our phylogenetic analyses inferred from both COI and ITS2 that *G. tuberculifemur* clustered with the *morrilli* group (current study and de León et al. 2006b). The topology of the phylogenetic tree generated by the COI sequence data differed slightly from that generated by the ITS2 fragment with the placement of three species: *G. fasciatus*, *G. metanotalis*, and *G. fasciatus*, thus differing from the morphological work of Triapitsyn (2006) and Triapitsyn et al. (2006).

The current results showed that the nuclear ribosomal ITS2 fragment appears to be phylogenetically more informative or valuable than that inferred by COI sequence data. A similar observation has been seen in phylogenetic studies of *Diadegma* species (Hymenoptera: Ichneumonidae) (Wagener et al. 2006).

Molecular key based on ITS2 sizes

Several of the most important egg parasitoid species were analyzed in the current study and therefore allowed for the development of a molecular key based on the specific sizes of the various ITS2 fragments. Many species have unique ITS2 sizes, but others (e. g., *G. ashmeadi* and *G. metanotalis* and *G. walkerjonesi* and *G. annulicornis*) have similar sizes (Table 1). The only concern in these cases would be if any of the South America species (*G. metanotalis* and *G. annulicornis*) were to be released in California.

Table 1. Molecular key based on ITS2 sizes. *Includes populations from California, Texas (Weslaco and San Antonio), Louisiana, and Florida; [a]Very close in size; [b]Overlap in size; [c]Prospective GWSS egg parasitoid candidate agents from South America.

***Gonatocerus* species**	***n***	**ITS2 range (bp)**
G. ashmeadi	29*	939-948[a]
G. triguttatus	3	995-1006
G. fasciatus	3	1071-1077
G. morrilli	8	1063-1066
G. walkerjonesi	6	851-853[b]
G. tuberculifemur[c]	3	823-825
G. metanotalis[c]	3	929-932 [a]
G. annulicornis	4	850-854 [b]
G. uat	4	914-915
G. sp. 6[c]	3	867-868
G. incomptus	4	870-876
G. novifasciatus	1	762

ISSR-PCR DNA fingerprinting

Although there exist several approaches [e. g., PCR-RFLP (Stouthamer et al. 1999)] to distinguish the aforementioned species containing similarly sized ITS2 fragments, a sensitive diagnositc approach is by inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting using a 5'-anchored ISSR primer (Zietkiewicz et al. 1994). Therefore, we submitted the following species: *G. ashmeadi* and *G. metanotalis* and *G. walkerjonesi* and *G. annulicornis* to ISSR-PCR DNA fingerprinting and the results are shown on Figure 3. As seen from the figure, a very clear distinction is made between the species-pairs, including all four species. Unique ISSR-PCR banding patterns were obtained per species. Even though variation is seen in *G. annulicornis*, the total banding pattern is still different from the rest of the species. The current results demonstrated that ISSR-PCR is an excellent molecular diagnostic tool with haplodiploid species, an observation seen recently with several *Gonatocerus* species (de León et al. 2005, de León et al. 2006a, b, de León and Morgan 2006).

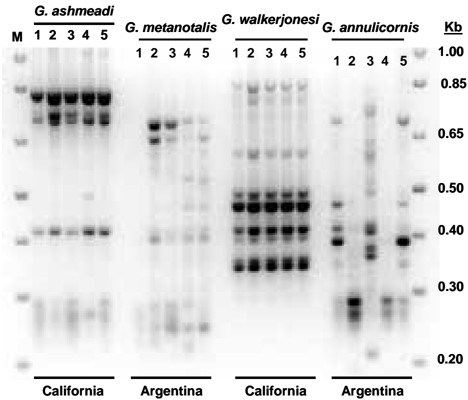

Figure 3. ISSR-PCR DNA fingerprinting of *Gonatocerus* species with similarly sized ITS2 fragments. Reactions were performed with genomic DNA from five separate individuals and a 5'-anchored ISSR primer. M, 1.0 Kb plus DNA Ladder.

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DEVELOPMENT AND UTILITY OF A 'ONE-STEP' SPECIES-SPECIFIC MOLECULAR DIAGNOSTIC MARKER FOR *GONATOCERUS MORRILLI* DESIGNED TOWARD THE INTERNAL TRANSCRIBED SPACER REGION 2 (ITS2) TO MONITOR ESTABLISHMENT IN CALIFORNIA

Project Leaders:		**Cooperators:**	
Jesse H. de Leon	David J.W. Morgan	Serguei V. Triapitsyn	Guillermo A. Logarzo
USDA, ARS	Calif. Dept. of Food & Agric.	Deparment of Entomology	USDA, ARS, SABCL
Beneficial Insects Res. Unit	Mt. Rubidoux Field Station	University of California	Buenos, Aires, Argentina
Weslaco, TX 78596	Riverside, CA 92501	Riverside, CA 92521	

Reporting Period: The results reported here are from work conducted fiscal year 2005 to fiscal year 2006.

ABSTRACT

In addition to the 'one-step' species-specific molecular diagnostic ISSR-PCR DNA fingerprinting method, we developed an additional 'one-step' molecular diagnostic marker 'gmtx' toward *Gonatocerus morrilli* (Howard) designed toward the ribosomal internal transcribed spacer region 2 (ITS2) to aid in monitoring establishment in California. *Gonatocerus morrilli*, the imported natural enemy from Texas, is very closely related to *G. walkerjonesi*, the native California species. Specificity assays with this newly developed marker and a total of 16 *Gonatocerus* Nees species demonstrated that it was highly specific toward the species that it was designed for (*G. morrilli*), as cross-reactivity was not seen with any of the tested species, including all species belonging to the *morrilli* subgroup of the *ater* species group of *Gonatocerus*. Anaysis of the 'release' 'TX/MX' colony used before the summer of 2005 with this species-specific diagnostic marker confirmed previous results that the 'release' 'TX/MX' colony was not the imported *G. morrilli*, but the native species *G. walkerjonesi*, confirming a colony contamination. Analysis of post-released *G. morrilli* collections with this diagnostic marker detected *G. morrilli* in a site where it was previously released, in accordance with our recent finding using two other diagnostic markers used in combination, ITS2 fragment size and ISSR-PCR DNA fingerprinting. The current results confirm the utility of the newly developed species-specific ITS2 molecular diagnostic marker as an excellent tool to aid in monitoring the establishment of the imported natural enemy of the glassy-winged sharpshooter, *G. morrilli*. These results and molecular tools are critical to the biological control program in California. We now have in our hands the molecular technology to evaluate the *G. morrilli* biological control program in California from start to finish, that is, monitor establishment, dispersal, and efficacy of natural enemies and improve mass rearing.

INTRODUCTION

Accurately identifying natural enemies is critical to the success of classical biological control programs and lack of proper identification procedures has affected several projects (Messing and Aliniazee 1988, Löhr et al. 1990, Narang et al. 1993). In addition, reliable methods or molecular markers are needed for distinguishing various exotic strains of these biological control agents from those indigenous to the U. S., including parasitoids from different states within the U. S. [e. g., *G. morrilli* (Howard) and *G. walkerjonesi* (Triapitsyn) (Hymenoptera: Mymaridae)]. Molecular markers are also needed to monitor the establishment of released populations and for the detection of cross-contamination between cultures of cryptic or closely related species (Powell and Walton 1989, Menken and Ulenberg 1987, Narang *et al*. 1993, Hopper *et al*. 1993, Unruh and Woolley 1999). The ribosomal internal transcribed spacer (ITS) regions have been used extensively in the examination of the taxonomic status of species and for diagnostic purposes (reviewed in Collins and Paskewitz 1996). Stouthamer *et al*. (1999) have used ITS2 rDNA fragment sizes as taxonomic characters to develop a precise identification key for sibling species of the genus *Trichogramma*. In cases where species were observed with similar sized ITS fragments the authors suggested a two-step procedure, amplification followed by restriction digestion called polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). If considering this procedure, having several species with similarly or overlapping ITS2 fragment sizes (accompanying PD report) would require development of several species-specific 'two-step' PCR-RFLP assays. The amount of steps can increase further if more than one restriction enzyme is used. Several *Gonatocerus* Nees species have similarly or overlapping ITS2 fragments (accompanying PD report). Having a couple of 'one-step' procedures, along with a rapid crude DNA extraction method, would, of course, be desirable. In addition, confirming results with more than one molecular diagnostic marker adds confidence and accuracy to the results. Recently, we demonstrated that inter-simple sequence repeat-polymerase chain reaction (ISSR-PCR) DNA fingerprinting using a 5'-anchored ISSR primer is an excellent 'one-step' molecular diagnostic tool with haplodipoid glassy-winged sharpshooter (GWSS) [*Homalodisca vitripennis* (Germar)] egg parasitoids, such as, *G. morrilli*, *G. tuberculifemur* (Ogloblin), *G. metanolatis* (Ogloblin), and *G*. sp. 3 (accompanying PD reports and de León et al. 2004a, 2006a,b). We have been using a combination of two methods to aid in detecting, distinguishing, and monitoring released populations of *G. morrilli* imported from Texas, specifically, ITS2 fragment size and ISSR-PCR DNA fingerprinting with excellent success (de León and Morgan 2005, 2006). Recent molecular genetic studies by de León et al. (2004a) and Smith (2005) demonstrated that the origin of the GWSS that invaded California was Texas and that GWSS geographic populations clustered into two distinct clades. Furthermore, scientific evidence from both of these studies also demonstrated that the GWSS that recently invaded the Pacific Island of French Polynesia (Secretariat of the Pacific Community, 2002) clustered along with the Texas and California or the 'western and southwestern' clade, strongly suggesting that GWSS invaded French Polynesia via California.

OBJECTIVE

In addition to ISSR-PCR DNA fingerprinting, develop an additional 'one-step' molecular diagnostic marker toward *Gonatocerus morrilli* based on ITS2 species-specific primers. Confirming results with two 'one-step' molecular diagnostic markers adds confidence and accuracy to the results.

RESULTS AND CONCLUSIONS

Development and specificity of the species-specific ITS2 diagnostic marker 'gmtx' for *Gonatocerus morrilli*

A species-specific marker 'gmtx' (gm = *G. morrilli* and tx = Texas) was designed toward the ITS2 rDNA fragment of *G. morrilli*, the imported egg parasitoid species from Texas. *G. morrilli* is very closely related to the native species from California, *G. walkerjonesi* (León et al. 2004a, 2006a, Triapitsyn 2006). For several years it was difficult to distinguish these two species (S. V. Triapitsyn, personal communication), therefore, making it impossible to monitor the *G. morrilli* biological control program in California. To determine the specificity of the 'gmtx' diagnostic marker (expected size = 204-bp), we tested specific amplification assay conditions, a rapid crude DNA extraction procedure, and screened a total of 16 *Gonatocerus* Nees GWSS egg parasitoid species for cross-reactivity. Figure 1, which shows 11 *Gonatocerus* species, shows that the diagnostic marker 'gmtx' was highly specific toward the species (*G. morrilli*) that it was designed for. We also screened the following species: *G. tuberculifemur* (clade 2), *G. morgani*, *G*. sp. 2, *G. atriclavus*, and *G. novifasciatus* (data not shown). Cross-reactivity with our specific amplification assay conditions was not seen with any of the *Gonatocerus* species tested, including all species belonging to the *morrilli* subgroup of the *ater* species group of *Gonatocerus*. The results demonstrated that the 'gmtx' molecular diagnostic marker was highly specific toward *G. morrilli*, making it highly suitable to use as a monitoring tool for *G. morrilli* post-released populations in California.

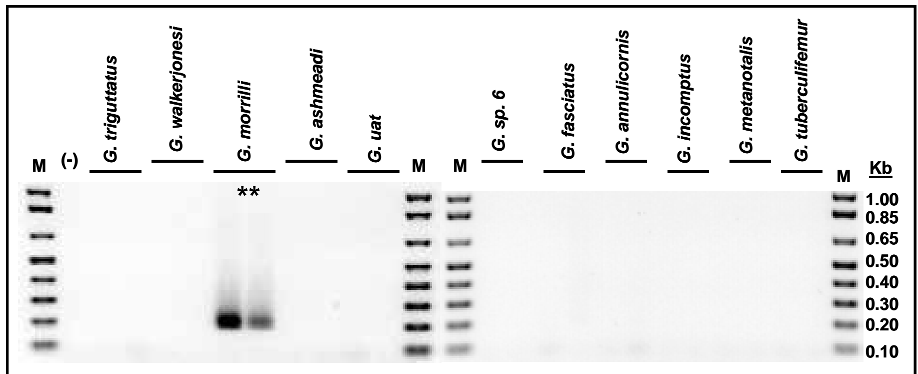

Figure 1. Specificity assays using the species-specific ITS2 molecular diagnostic marker 'gmtx'. Genomic DNA from two individuals per species was used in amplification assays to test for cross-reactivity of the 'gmtx' marker (204-bp) with specific assay conditions. The *Gonatocerus* species are listed on the figure. The following species were also tested, but not shown here: *G. tuberculifemur* (clade 2), *G. morgani*, *G*. sp. 2, *G. atriclavus*, and *G. novifasciatus*. (-), negative control, no template DNA. **M**, 1.0 Kb Plus DNA Ladder.

Molecular diagnostic analysis of the 'release' 'TX/MX' colony used before the summer of 2005 by the ITS2 rDNA species-specific marker, 'gmtx'

Previously, we demonstrated the utility of using two diagnostic marker sets in combination to genotype the 'release' 'TX/MX' colony and to detect post-released *G. morrilli* populations in California (de León and Morgan 2005, 2006). Those results demonstrated that the 'release' 'TX/MX' colony was not the imported species *G. morrilli*, but the native California species, *G. walkerjonesi*; indicating a contamination of the colony. In the current study, we again tested the same 'release' 'TX/MX' colony, along with *G. morrilli* (TX) and *G. walkerjonesi* (CA) as control species, with our newly developed 'gmtx' species-specific molecular diagnostic marker. Amplification with the 'gmtx' marker showed positive banding in only in the control *G. morrilli* (TX) (Figure 2), but not in the other control *G. walkerjonesi*. Amplification with this marker of the 'release' 'TX/MX' colony also produced negative banding. The current results with our diagnostic species-specific ITS2 marker are in accordance with our previous findings (de León and Morgan 2005, 2006), that is, the 'release' 'TX/MX' colony was not *G. morrilli*, but the native *G. walkerjonesi*, again confirming a colony contamination.

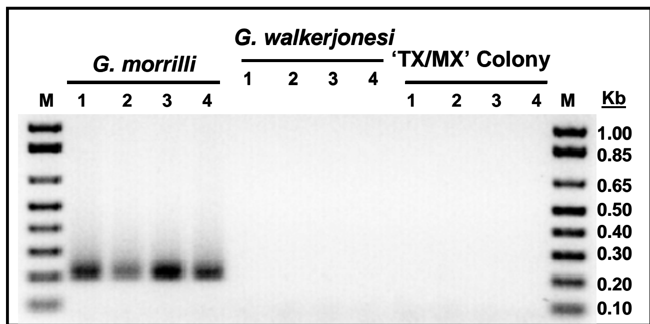

Figure 2. Evaluation of the 'release' 'TX/MX' colony that was used before the summer of 2005. *G. morrilli* (TX) and *G. walkerjonesi* (CA) are included as control species. The ITS2 species-specific diagnostic marker 'gmtx' was used in diagnostic amplification assays. Four individuals per species were included. **M**, 1.0 Kb Plus DNA Ladder.

The utility of the ITS2 species-specific molecular diagnostic marker as a tool to evaluate post-released *G. morrilli* populations in California

In the summer of 2005, after the confirmation of the colony contamination, *G. morrilli* from Texas was imported to California to restart the biological control program and shortly thereafter releases were started in California. We screened a few post-released populations from San Diego and Riverside Counties with our newly developed diagnostic marker, 'gmtx'. Amplification with 'gmtx' produced positive banding in one location (Figure 3, lane E) where *G. morrilli* (TX) was previously released, whereas, amplification was not seen in the rest of the post-released collection sites (Figure 3, lanes A, B, C, D, and F); indicating that *G. morrilli* was detected. The current results are in accordance with our previous results using other diagnostic markers (de León and Morgan 2005, 2006). The current results confirm the utility of our newly developed ITS2 species-specific molecular diagnostic marker as an excellent tool to aid in monitoring the establishment of *G. morrilli* in California. These results and molecular tools are critical to the biological control program in California. We now have the molecular technology to evaluate the *G. morrilli* biological control program in California from start to finish, that is, monitor establishment, dispersal, and efficacy of natural enemies and improve mass rearing.

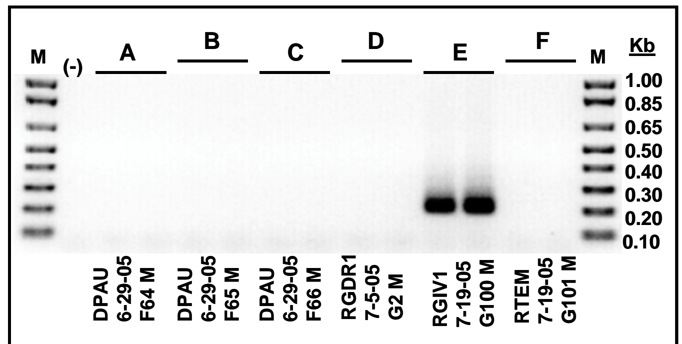

Figure 3. An example of the utility of the species-specific ITS2 diagnostic marker 'gmtx' in evaluating post-released *G. morrilli* collections. Two individuals per collection site were tested with the diagnostic marker. Collection sites: DPAU1, Pauma, San Diego County; RGDR1, Meyers St, Riverside County; RGlV1, Glen Ivy, Riverside County; RTEM2, Temecula, Riverside County. **M**, 1.0 Kb Plus DNA Ladder.

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IDENTIFYING KEY PREDATORS OF THE GLASSY-WINGED SHARPSHOOTER IN A CITRUS ORCHARD

Project Leaders:

Valerie Fournier
Division of Insect Biology
University of California
Berkeley, CA 94720

James Hagler
Arid Land Agricultural
Research Center
USDA-ARS
Maricopa, AZ 85040
JHagler@wcrl.ars.usda.gov

Kent Daane
Division of Insect Biology
University of California
Berkeley, CA 94720
DAANE@uckac.edu

Jesse de Leon
Beneficial Insects
Research Unit
USDA-ARS
Weslaco, TX 78596

Cooperators:

David H. Akey
Arid Land Agric. Res. Center
USDA-ARS
Maricopa, AZ 85040

Matthew J. Blua
Department of Entomology
University of California
Riverside, CA 92521

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

Reporting Period: The results reported here are from work conducted October 1, 2005 to October 1, 2006.

ABSTRACT
Over 1,500 predators were screened for glassy-winged sharpshooter (GWSS) remains using a GWSS egg-specific monoclonal antibody (MAb) and several GWSS-specific genetic markers. Specimens were collected in 2002 and 2003 from a citrus orchard (Riverside, CA) harboring high densities of GWSS. We found that 6.2% of all specimens examined tested positive for GWSS remains. The most frequent predators to test positive included the assassin bug, *Zelus renardii* (Kolenati) (Hemiptera: Reduviidae) and the spiders *Trachelas pacificus* Chamberlin and Ivie (Araneae: Corinnidae) and *Olios* sp. (Araneae: Sparassidae) with 41, 22, and 19% of the specimens testing positive with either ELISA and/or PCR, respectively.

INTRODUCTION
Effective control of GWSS will require an areawide integrated pest management approach (AW-IPM). A major component of AW-IPM is the exploitation of the pest's natural enemies, which, when utilized to their greatest potential, can increase the effectiveness of other control tactics. Very little information exists on GWSS's predaceous natural enemies. Identifying the impact of predators can be challenging as they are usually small, elusive, nocturnal or cryptic. Direct visual field observations of predation are rare and often difficult to obtain. While predation studies using enclosures can provide some indication of predator impact, it fails to recreate natural conditions and can result in an overestimation of predation. A more valid method to qualitatively identify predators of key pests in nature is by the molecular analysis of predator gut contents for pest remains (reviewed in Sheppard and Harwood 2005). The state-of-the-art predator stomach content analyses include both MAb-based enzyme-linked immunosorbant assays (ELISA), which detect prey-specific proteins (Hagler et al. 1994ab, Schenk and Bacher 2004), and polymerase chain reaction (PCR)-based assays, which detect prey-specific DNA (Zaidi et al. 1999, Agustí et al. 2003). While DNA-based approaches reveal the prey identity at the species-level, they are unable to indicate which prey life stage is consumed. In contrast, pest-specific and life stage-specific MAbs can target a particular life stage of a given species, providing a higher level of precision to document predation (Hagler and Naranjo 1996). Combining both assays can provide a powerful tool to study predation on the GWSS.

To this end, genetic markers were designed using the cytochrome oxidase gene subunit I (COI) to detect and amplify a GWSS-specific fragment (de León et al. 2006), and a GWSS-egg specific MAb was developed to detect GWSS egg-specific protein (Hagler et al. 2002, Fournier et al. 2006).

OBJECTIVE
The main objective of this research is to identify the key predators of the different life stages of GWSS. More specifically, our aim is to determine the proportion of predators feeding on the various GWSS life stages in a citrus orchard. Using GWSS-specific ELISA and PCR assays, we examined the guts of 1,507 field-collected generalist predators. Results obtained from this research will aid in evaluating the efficacy of generalist predators for conservation biological control program.

RESULTS
Generalist arthropod predators were collected during 2002 and 2003 from a citrus orchard located at the Agricultural Operations Farm at the University of California, Riverside, CA. Collections were performed by beating the foliage or fogging the citrus trees with pyrethrum insecticide. Densities of GWSS were recorded as well (Blua and Akey, unpublished data). For each group of predators, we conducted lab trials to generate negative controls (i.e. individuals with no GWSS remains in their guts) and positive controls (i.e. individuals fed GWSS). Predators were frozen, sorted and then screened for GWSS remains with GWSS egg-specific sandwich ELISA and GWSS-specific PCR assays. Materials and methods employed were similar to the ones described in Fournier et al. (2006) and de León et al. (2006). Predators were scored positive for prey remains if the 197-bp specific GWSS DNA fragment was successfully amplified. With ELISA, specimens

were scored positive if they yielded an optical density response five standard deviations above that of their respective negative control mean (Sutula et al. 1986).

Table 1 reports the PCR and ELISA results for all the predator specimens collected from the citrus orchard (N=1,507). Our study showed that 6.2% of all specimens were found positive for GWSS remains. True bugs and spiders were the two groups with the highest percentages of positives, with respectively 28 and 18% of the specimens testing positive with ELISA and/or PCR. Among these groups, *Zelus renardii* (Hemiptera: Reduviidae), *Trachelas pacificus*, (Araneae: Corinnidae), and *Olios* sp. (Araneae: Sparassidae) were the most common predators of GWSS, with respectively 41, 22, and 19% of the specimens testing positive. Earwigs (N=661) and beetles (N=465), the two groups comprising the greatest number of specimens collected, only yielded 5.0 and 2.6% positive reactions, respectively.

Table 1. Results from predator gut content analyses using GWSS-specific PCR and ELISA. Predators were collected from GWSS-infested citrus trees in Riverside, CA.

Predator Group	N	% ELISA positive (a)	% PCR positive (b)	% overall positive (c)
True bugs (Hemiptera)	25	20%	23%	28%
Ants (Hymenoptera)	121	2.5%	1%	3.3%
Spiders (Araneae)	198	12%	12%	18%
Beetles (Coleoptera)	465	1%	2%	2.6%
Earwigs (Dermaptera)	661	2%	3.5%	5%
Others (various orders)	37	2.7%	0%	2.7%
Total	**1,507**	**3.2%**	**3.8%**	**6.2%**

(a) an individual was determined "positive" if GWSS egg-specific MAb detected egg protein in its gut.
(b) an individual was determined "positive" if GWSS-specific fragment was successfully amplified from its gut.
(c) % of specimens that tested positive for GWSS remains with either one, or both types of gut assay.

CONCLUSIONS

In contrast to our previous study (Fournier et al. 2005; Fournier et al., in preparation), in which predators (N=1,235) were collected from various ornamental plants in the urban areas of Bakersfield, CA and assayed with identical ELISA and PCR probes presented here, the current analyses with citrus-collected predators revealed a much lower percentage of overall specimens yielding positive response for GWSS remains (6.2% compared to 14.8%). Among other things, this observation is likely to be due to the differences in GWSS populations and predator complexes between the two systems. For instance, the host plants from which specimens were collected in the urban settings harbored higher abundance of spiders from the families Clubionidae, Salticidae and Agelenidae, which commonly prey upon GWSS. Similarly, lacewings and praying mantis were much more abundant in the urban settings than in the citrus orchard and commonly tested positive for GWSS remains.

Here we successfully implemented a GWSS-specific ELISA and PCR assay to analyze the guts of field-collected predators. Once the key predators of the various life stages of GWSS are identified, this information can be used to develop more ecologically-based management programs to control GWSS in California.

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EXPLORATION FOR BIOLOGICAL CONTROL AGENTS IN THE NATIVE RANGE OF THE GLASSY-WINGED SHARPSHOOTER

Project Leader:
John Goolsby
USDA-ARS Beneficial Insects Research Unit
Weslaco, TX 78596

Researchers:
Blake Bextine
Department of Biology
University of Texas at Tyler
Tyler, TX 75799

Jeff Skevington
Agriculture and Agri-Food Canada
Ottawa, ON, KIA OC6, Canada

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ABSTRACT

Surveys in the native range of the glassy-winged sharpshooter (GWSS) *Homalodisca vitripennis* are continuing to discover nymphal parasitoids and to determine the ecology and phenology of GWSS in undisturbed natural areas. Fifteen sites with stands of native *Vitis* spp. in southeastern Texas have been surveyed monthly from October 2005 to present. The focus is on big-headed flies (Pipunculidae), which are known to be nymphal parasitoids of sharpshooters. Several methods have been used to survey for the parasitic flies, including yellow sticky cards, malaise traps, sweeping, hand collection, and tethered nymphal sentinels. Larval pipuculids have been dissected from hand collected *Oncometopia orbona* feeding on mustang grapes. Numerous adult *Eudorylas* spp. have been collected by sticky traps, sweeping, and malaise traps that may be associated with GWSS. Peak populations of Pipunculidae, including *Eudorylas* and *Tomosvaryella* spp., occurred in February and October. Populations of GWSS began to increase in March and peaked in July. GWSS adults collected in March from survey locations were all positive for the presence of *Xylella fastidiosa* in their foreguts.

INTRODUCTION

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis,* is native to Northeastern Mexico and the Southeastern U.S., and the origin of the invasive California populations is reported by de León et al. (2004) to be Texas. Most of the entomological and epidemiological information regarding this pest is derived from its status as a vector of Pierce's disease, *Xyllela fastidosa (Xf),* in cultivated hosts. Much less is known about the field ecology and phenology of GWSS and its natural enemies in its native habitat in the Southeastern U.S. Recent surveys in the native range and research on biological control agents has focused on egg parasitoids of GWSS (Mizzell and Andersen 2003, Hoddle and Tripitsyn 2004, Luck et al. 2004, Irwin and Hoddle 2005, Jones et al. unpublished data). *Gonatocerus* spp. egg parasitoids have been collected from the native range of Texas, Florida and Northeastern Mexico, and released in California where several species are now established (CDFA 2004). Nymphal parasitoids of GWSS, including Pipunculidae, have not been evaluated as biological control agents. Skevington and Marshall (1997) review the natural history and rearing of Pipinculidae. They indicate that many pipunculids are oligophagous and show specificity at the genus level. Five new pipunculid-sharpshooter host associations have been documented by Skevington et al. 2006 (submitted). The focus of our research is to discover, identify and evaluate the pipunculid parasitoids of GWSS and other sympatric sharpshooters. We will also use this survey of sharpshooters to determine the seasonal percentage of adults infected *Xf* in native habitats for comparison to agricultural settings in California where GWSS is invasive.

OBJECTIVES

1. Conduct monthly surveys in the native range of GWSS.
2. Determine the phenology and ecology of GWSS and other sharpshooters
3. Determine the species composition of GWSS natural enemies in their native habitat.
4. Develop methods for collection of parasitized GWSS nymphs and adult parasitoids.
5. Investigate the biology and biological control potential of GWSS nymphal parasitoid species.

RESULTS

Fifteen field sites have been established in southeastern Texas (Goolsby and Setamou 2005). The sites are located in eight different biogeographic zones. The transect starts at the southern tip of Texas in the Lower Rio Grande Valley in Weslaco, extending northwest to the Texas Hill Country near New Braunfels, northeast to the Piney Woods near Houston, and south along the coastal plain. Each site has natural stands of native *Vitis* spp. Five yellow sticky cards were placed monthly at each location starting in October 2005.

The mean number of GWSS and *Oncometopia orbona* (F.) adults in yellow sticky card traps for Giddings, TX are shown in Figure 1. The numbers of sharpshooters at this site are consistently high, which may be due to large stands of mustang grape, *Vitis mustangensis* and close proximity to Yegua Creek. *Oncometopia orbona* populations peak in early spring followed by GWSS. This phenology results in nymphal sharpshooter populations throughout the spring and summer which may be exploited by pipunculid parasitoids.

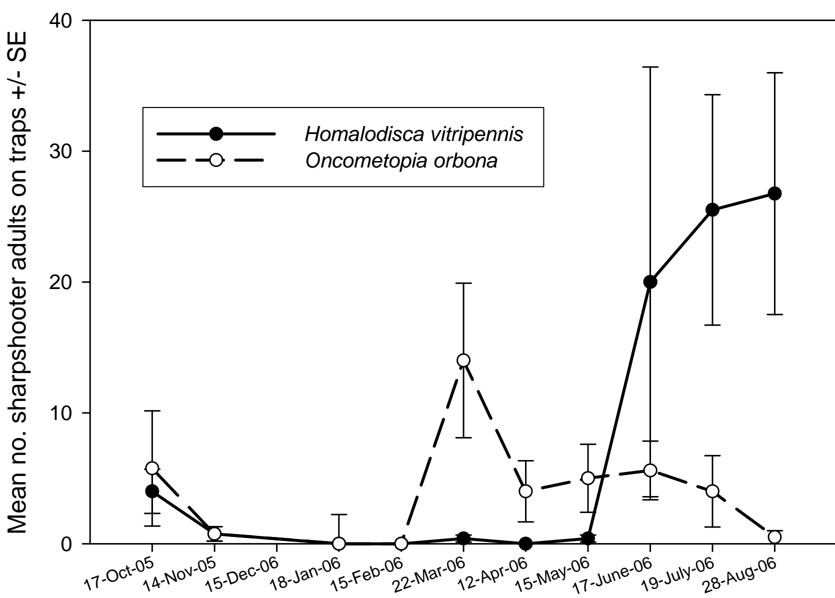

Figure 1. Yellow sticky trap catches of sharpshooter adults adjacent to mustang grape stands near Giddings, TX (Oct. 05- Aug 06).

Pipunculidae have been collected in yellow sticky traps from each survey location in Southeast Texas. Most individuals thus far have been recovered from October to February. Individuals collected in the yellow sticky traps fall into four different genera (Table 1). *Eudorylas* is the most likely parasitoid of GWSS based on its known biology and host associations. Further, an unknown species of *Eudorylas* was collected from *Oncometopia orbona* (F.) near Giddings, TX in October 2005.

Several methods have been investigated for recovery of Pipunculidae in addition to yellow sticky traps, including Malaise traps, hand collecting of adults for dissection and to hold for emergence of parasitic flies, sweeping, and tethering of nymphs. Malaise traps near Weslaco were placed next to naturalized grapes adjacent to a large natural area with diverse vegetation. Several *Eudorylas* sp. adults have been collected from the traps and held for identification. Once the species associated with GWSS has been identified, and the ideal time and location are determined, Malaise traps fitted with dry collection cones could be useful in collecting large numbers of adult pipunculids. Through dissections, pipunculid larvae have been recovered in *Oncometopia orbona* adults (Skevington et al. submitted). Several pipunculid flies have been recovered from sweeping but their host associations are not known. Adults in flight move in a characteristic slow hover, occasionally lighting on plants to feed on honeydew. Hundreds of GWSS adults have been hand collected from the survey locations and held in cages under greenhouse conditions, however without success in rearing out pipunculid adults. It is possible that the parasitic flies emerge from late instar nymphs (which are rare in the field), or the behavior of the parasitized individuals is altered, making them inaccessible for collection. Methods have been developed for tethering of sentinel nymphs to overcome the difficulties of collecting nymphs in the field. Current methods involve tethering of nymphs on silk threads glued to the thorax. The end of the tether can be placed on foliage in the areas frequented by the pipunculid adults. The optimal period for Pipunculidae appears to be late Fall through early Spring. Plans have been made to intensively survey for Pipunculidae during this time and to employ tethered nymphs in the most promising locations.

Table 1. Numbers of Pipunculidae adults collected from March to June 2006 in the yellow sticky traps.

Location	*Eudorylas*	*Tomosvaryella*	*Chalarus*	*Cephalops*
Weslaco	5	6		
George West	11	25		
Pleasanton	0	5	2	
New Braunfels	3	9		
Giddings	1	1		
Hempstead				1
Refugio				1
King Ranch	1			1

Sharpshooters collected in March 2006 were assayed for the presence of *Xf* using molecular techniques developed by Bextine et al. (2005). This date is significant because this is when 'red-winged' GWSS first appear in the field, which is indicative of a new generation of adults. High levels of *Xf* were detected in GWSS (13/13), *O. nigricans* (8/21), *O. orbona* (39/51). The assay of *Xf* in sharpshooter adults will continue for a full annual cycle.

CONCLUSIONS

Adult *Oncometopia* spp. reach a peak in late Spring, followed by GWSS which peaks in mid-Summer. Nymphal populations therefore must be common one to two months prior, which should correspond to peak pipunculid activity. Future efforts will be focused on collecting and evaluating pipunculids using sticky traps, Malaise traps, dissection of hand collected sharpshooters and tethered sentinel nymphs. Pipunculid immatures collected from GWSS and other sympatric sharpshooters will be sequenced and compared with sequence data from known adult populations. This molecular tool may help to determine the identity of the GWSS pipunculid and further refine the search areas and methods. Our goal for the next year is to evaluate these nymphal parasitoids of GWSS as biological control agents and export them to CDFA for mass rearing and release in California, to minimize the impact of GWSS on agricultural producers.

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AN IMMUNOLOGICAL APPROACH FOR QUANTIFYING PREDATION RATES ON THE GLASSY-WINGED SHARPSHOOTER

Project Leaders:

James Hagler
USDA-ARS
U.S. Arid Land Agric.Res. Center
Maricopa, AZ 85239

Russell Groves
Department of Entomology
University of Wisconsin
Madison, WI 53706

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

David Morgan
CDFA, PDCP
Mount Rubidoux Field Station
Riverside, CA 92501

Reporting Period: The results reported here are from work conducted September 1, 2005 to September 1, 2006.

ABSTRACT

The gut contents of 376 individual predators were assayed for glassy-winged sharpshooter (GWSS) remains using a multitude of ELISAs designed to detect predation on various GWSS life stages. We found that almost 10% of the predators examined contained GWSS remains in their guts. We recorded 10, 17, and 20 predation events on the GWSS egg, nymph, and adult stages, respectively. Of the predators examined in this study, *Collops vittatus* (20.6%) and *Hippodamia convergens* (16.7%) had the highest percentage of individuals positive for GWSS remains. Approximately 10% of five of the other predator species and none of the earwigs tested contained GWSS remains.

INTRODUCTION

Predaceous natural enemies can be important regulators of arthropod populations (Luff, 1983). However, accurately identifying key predators of most pests is very difficult because predators and their prey can often be small, elusive, and cryptic. Hence, visual field observations of predation are extraordinarily difficult to obtain. Perhaps the most frequently used experimental approach for evaluating predaceous natural enemies in the field are through studies conducted in field cages (Luck *et al.*, 1988). Such studies require manipulation of either the natural enemy or the targeted prey population(s) within the cage. Mortality of the pest can be estimated based on the presence or absence of the pest over time (Smith & De Bach, 1942; Luck *et al.*, 1988). Such studies have documented the qualitative impact of manipulated predator assemblages on many types of pests, but they do not provide quantitative information on predation rates or evidence of which predator in the assemblage is exerting the greatest biological control. Often the only direct evidence of arthropod predation can be found in the stomach contents of predators. Currently, the state-of-the-art predator stomach content assays include immunoassays (typically ELISA) for the detection of pest-specific proteins (Hagler & Naranjo, 1996) and PCR assays for the detection of pest-specific DNA (de León *et al.*, 2006).

ELISAs using pest-specific monoclonal antibodies (MAbs) have been widely used to identify key predators of certain pests, including the glassy-winged sharpshooter (GWSS) (Fournier *et al.*, 2006). The simplicity and low cost of ELISA lends itself to the efficient screening of hundreds of field-collected predators per day (Hagler & Naranjo, 2005). However, MAb development is technically difficult, costly, and time consuming for wide scale appeal (Greenstone, 1996). Moreover, pest-specific ELISAs share the same limitation as the other predator evaluation methods; the quantification of predation rates is impossible (see Hagler & Naranjo, 1996 for a review). PCR assays using pest-specific DNA probes might be less expensive to develop (Greenstone & Shufran, 2003), but PCR assays are also not quantifiable and they are more costly, technical, tedious, and time consuming than ELISAs (pers. obs.). These difficulties have resulted in a dearth of information on the quantitative impact that generalist predators have on suppressing pest populations.

The many shortcomings of each method of predator assessment described above were the impetus for us to develop a technique to more efficiently quantify predator activity. The technique combines our previous research using pest-specific MAb-based ELISAs to detect predation (Fournier *et al*., 2006) with protein marking ELISAs developed to study arthropod dispersal (Hagler *et al*., 2002; Jones *et al*., 2006). In this study we erected 40, 1-m long field cages on selected citrus branches. We then placed (using a paper clip) a sentinel GWSS egg mass (ca. 6 to 12 eggs per mass) on the underside of a randomly selected leaf in each cage along with two individuals each of *Hippodamia convergens*, *Collops vittatus*, *Chrysoperla carnea*, *Labidura riparia*, *Geocoris punctipes*; and one individual each of *Sinea confusa* and *Zelus renardii*. One hour later, we released two GWSS adults and two nymphs into each cage. The four mobile GWSSs were each marked with a different protein before they were introduced into each cage. After 6 hours, each citrus branch was cut at its base, just below each cage, and immediately frozen on dry ice. Each predator was then analyzed by four protein-specific ELISAs to determine if they contained marked GWSSs in their guts. Additionally, the gut contents of each predator was examined by a GWSS egg-specific sandwich ELISA (Fournier *et al*., 2006) to determine the frequency of predation on GWSS eggs.

OBJECTIVES

We are in the final phase of a multi-year research project dedicated to:

1. Quantifying predation rates on GWSS nymphs and adults
2. Qualifying predation on GWSS eggs. Using a novel multiple prey marking technique (Hagler, 2006) and a GWSS egg-specific MAb (Fournier *et al*., 2006) we simultaneously examined the gut contents of a total of 376 predators from seven predator species for the presence of five GWSS prey items (e.g., GWSS egg protein, 2 protein marked nymphs and 2 protein marked adults).

RESULTS

The recovery rate of the predators ranged from 55% for earwigs and lacewings to 97.5% for the assassin bugs and lady bugs (Table 1). The low recovery rate of earwigs and lacewings is likely due to their ability to escape from the cages (e.g., earwigs), they were the victims of interguild predation (e.g., lacewings) (Hagler, 2006), or they were overlooked in the sorting process (e.g., lacewings).

Of the 376 predators examined by ELISA, a total of 37 predatory events (9.8% of all the predators examined) were recorded in this study. Of these, 2.7% (n=10) were the result of predation on the GWSS egg stage. Unfortunately, due to the limitations of using a pest-specific ELISA (as mentioned above), we cannot determine if the positive reactions were due to predation on one or more eggs. The number of predation events recorded for the GWSS nymph and adult stages were 17 (4.5% of the population) and 20 (5.3% of the population), respectively. Since these predation events were each detected with a specific protein ELISA, we are confident that these results represent the first quantified results of predation using molecular gut content methods (e.g., immunological or DNA based). Interestingly, of the 376 predators assayed, only three predators yielded more that one positive ELISA reaction. Specifically, one lady beetle yielded a positive reaction for at least one GWSS egg and an adult, one assassin bug (*S. confusa*) yielded a positive reaction for both of the marked GWSS nymphs released into its cage, and one flower beetle yielded a positive reaction for both marked GWSS adults released into its cage.

Table 1. Predator gut content ELISA results yielded by the 376 individuals assayed for the presence of GWSS eggs, nymphs, and adults.

Species (common name)	Total Number Released	Total Number Recovered (%) After 6 h	# Positive (%) for GWSS Egg Stage	# Positive (%) for GWSS Nymph Stage	# Positive (%) for GWSS Adult Stage	Total (%) by Species
Hippodamia convergens (lady beetle)	80	78 (97.5%)	4 (5.1%)	4 (5.1%)	5 (6.4%)	**13 (16.7%)**
Collops vittatus (flower beetle)	80	68 (85.0%)	2 (2.9%)	7 (10.3%)	5 (7.4%)	**14 (20.6%)**
Chrysoperla carnea (lacewing)	80	44 (55.0%)	1 (2.3%)	0 (NA)	2 (4.5%)	**3 (6.8%)**
Labidura riparia (earwig)	80	44 (55.0%)	0 (NA)	0 (NA)	0 (NA)	**0 (NA)**
Geocoris punctipes (big-eyed bug)	80	64 (80.0%)	3 (4.7%)	3 (4.7%)	2 (3.1%)	**8 (12.5%)**
Sinea confusa (assassin bug)	40	39 (97.5%)	0 (NA)	2 (5.1%)	3 (7.7%)	**5 (12.8%)**
Zelus renardii (assassin bug)	40	39 (97.5%)	0 (NA)	1 (2.6%)	3 (7.7%)	**4 (10.3%)**
Total (%) by GWSS Life Stage	**480**	**376 (78.3%)**	**10 (2.7%)**	**17 (4.5%)**	**20 (5.3%)**	**37 (9.8%)**

CONCLUSIONS

Although it is widely accepted that predators play a role in pest regulation, we still have an inadequate understanding of and ability to predict their impact in cropping systems. The impact that predators have on suppressing GWSS populations goes unrealized due to the difficulties of assessing arthropod predation. The prey marking technique (Hagler, 2006) combined with a GWSS egg-specific gut content ELISA (Fournier *et al*., 2006) circumvented many of the shortcomings of the current methods used to study predation. Here, we quantified predation on GWSS nymphs and adults and qualified predation on GWSS eggs. This information and the data presented by Fournier *et al*. (in this volume) will be used to develop a comprehensive biological control program that better conserves the populations of those predators exerting the greatest control on the various GWSS life stages.

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ARE GLASSY-WINGED SHARPSHOOTER POPULATIONS REGULATED IN CALIFORNIA? LONG-TERM PHENOLOGICAL STUDIES & CONSTRUCTION OF MULTI-COHORT LIFE TABLES FOR THE GLASSY-WINGED SHARPSHOOTER IN CITRUS ORCHARDS

Project Leader:
Mark S. Hoddle
Department of Entomology
University of California
Riverside, CA 92521
mhoddle@ucr.edu

Reporting Period: The results reported here are from work conducted March 2002 to August 2006.

ABSTRACT

Glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*; formerly *H. coagulata*) population densities have been steadily declining over a 4.5 year period in organic lemons grown in an experimental study plot at University of California, Riverside Ag. Ops. Peak adult GWSS populations in August 2006 were just 32% of those observed around August 2002. It is uncertain if egg parasitism, which has consistently averaged ~ 20% per year of GWSS egg masses, is responsible for the observed decline. Density dependent analyses of time series data are planned once data sets are large enough. Phenological observations are being complimented with recently initiated multi-cohort stage frequency life tables to provide greater insight into GWSS population dynamics and identification of life stages most susceptible to mortality factors.

INTRODUCTION

In California, there is a guild of natural enemies attacking the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*; formerly *H. coagulata*). The dominant parasitoid attacking GWSS in California is *Gonatocerus ashmeadi* followed by *G. morrilli*. *G. triguttatus* from Texas and *G. fasciatus* from Louisiana have been released in California, but widespread establishment is uncertain. Completion of recent studies investigating the effect of varying temperatures on development, survivorship, and reproductive output coupled with GIS modeling, strongly suggests that climate is a major limiting factor for *G. triguttaus* in California, while *G. ashmeadi* is more robust to California's varying climatic conditions (see article in this Proceedings). Other minor parasitoid species include *G. novofasciatus*, *Ufens* sp., and *Zagella* sp. Together, this guild of parasitoids provides an average of ~15% parasitism of GWSS eggs laid during the spring generation and ~20-25% of GWSS eggs laid during the summer generation in commercial citrus orchards. The average year round parasitism level of ~20% falls short of the 33-36% level determined necessary for successful classical biological control (Hawkins and Cornell, 1994). However, data collected from bi-weekly monitoring over the last five years from an organic commercially-managed citrus orchard in Riverside indicates that GWSS populations are declining steadily each year (Figure 1). It is uncertain what the significance is of parasitism of GWSS eggs by mymarid parasitoids to this downward population trend (Figure 2). There are at least four possible reasons for low seasonal parasitism levels in California: (1) competitive exclusion amongst members of the GWSS parasitoid guild is reducing effective biological control. (2) An extremely aggressive and efficacious natural enemy that can dominate the system to the almost total exclusion of all current parasitoids has not been established in California and is needed for successful biological control of GWSS. (3) The absence of resource subsidies such as nectar provided by flowering plants in agroecosystems may limit parasitoid efficacy because longevity and fecundity is significantly reduced when parasitoids can not access carbohydrates. Understorey management may be an important cultural strategy to benefit parasitoids if it can be demonstrated not to enhance GWSS and *Xylella* populations. (4) Climate, in particular, prolonged cool periods over winter when GWSS eggs are unavailable probably has a severe affect on parasitoid reproductive success. There are two general approaches to investigating population phenomena in the field: (1) long-term phenology studies which can be used to tease out density-dependent and density-independent factors affecting population dynamics, and (2) life tables that dissect populations by life stage to determine the magnitude of change between developmental stages, and if possible elucidation of factors impacting survivorship within life stages. Both approaches need to be conducted concurrently in the same field plots using standardized methods to better understand mechanisms underlying long-term population fluctuations for GWSS in California.

OBJECTIVES

1. Construct multi-cohort life tables for glassy-winged sharpshooter nymphs and adults in citrus orchards.
2. Continue the 3 years of bi-weekly surveys of GWSS eggs, nymphs, and adults, and associated rearing of parasitoids from harvested egg masses in citrus at Ag Ops, UC Riverside.

RESULTS

The population monitoring study and measures of percentage parasitism clearly indicate that GWSS densities have continued to decline steadily at the long-term monitoring plot (Figure 1) and percentage parasitism have remained relatively constant over this time period (Figure 2). Detection of density-dependent mortality from sequential census data such as that presented here is notoriously difficult and the results of analytical models differ in outcomes depending on assumptions made even when dummy data sets have been constructed to show density dependent mortality. One of the major problems with these

types of analyses is serial correlation, where densities at N_t directly influence the population at N_{t+1}. Recent developments in analyses of time series data, such as those we are collecting for GWSS are now providing much more robust tests that overcome autocorrelation problems. The Partial Rate Correlation Function is a relatively new statistical procedure specifically designed for time series analysis of biological populations to detect density dependent feed back. Literature searches so far indicate that PRCF is the best of the extant techniques for analyzing long-term population counts. Consequently, census data collected from GWSS monitoring will be subjected to PRCF once we have data for a minimum of 10 consecutive years to determine if density dependent or density independent feed back is responsible for observed fluctuations from generation to generation. Detection of density dependent mortality will indicate that populations are being regulated, and could suggest that natural enemy populations are responsible. Currently, our data set is too short to determine if parasitoid activity is providing density dependent mortality and is subsequently responsible for decreasing GWSS densities at the study site. There are several techniques that are amenable to constructing life tables for GWSS using stage frequency data from field surveys. GWSS motiles are relatively easy to classify based on developmental stage. Therefore the numbers in each developmental stage can be determined and the data used to estimate numbers entering a life stage, survival rates in a stage, life stage duration, and numbers successfully entering the next stage. The Kiritani-Nakasuji-Manly (K-N-M) method has been used to construct life tables for insects with discrete generations from regularly collected census data of field populations. The ideas behind this model are simple and robust. The area under a life stage frequency curve is determined by: (1) the number of individuals entering that life stage through time; (2) a survival parameter that determines the numbers exiting the life stage to enter the next developmental stage, and (3) the length of time that particular life stages last for. The K-N-M model can be applied to the spring and summer generations of GWSS as the amount of over lap between life stages across generations are minimal. Work is ongoing for the K-N-M life tables and too few data have been collected this summer for any meaningful analyses.

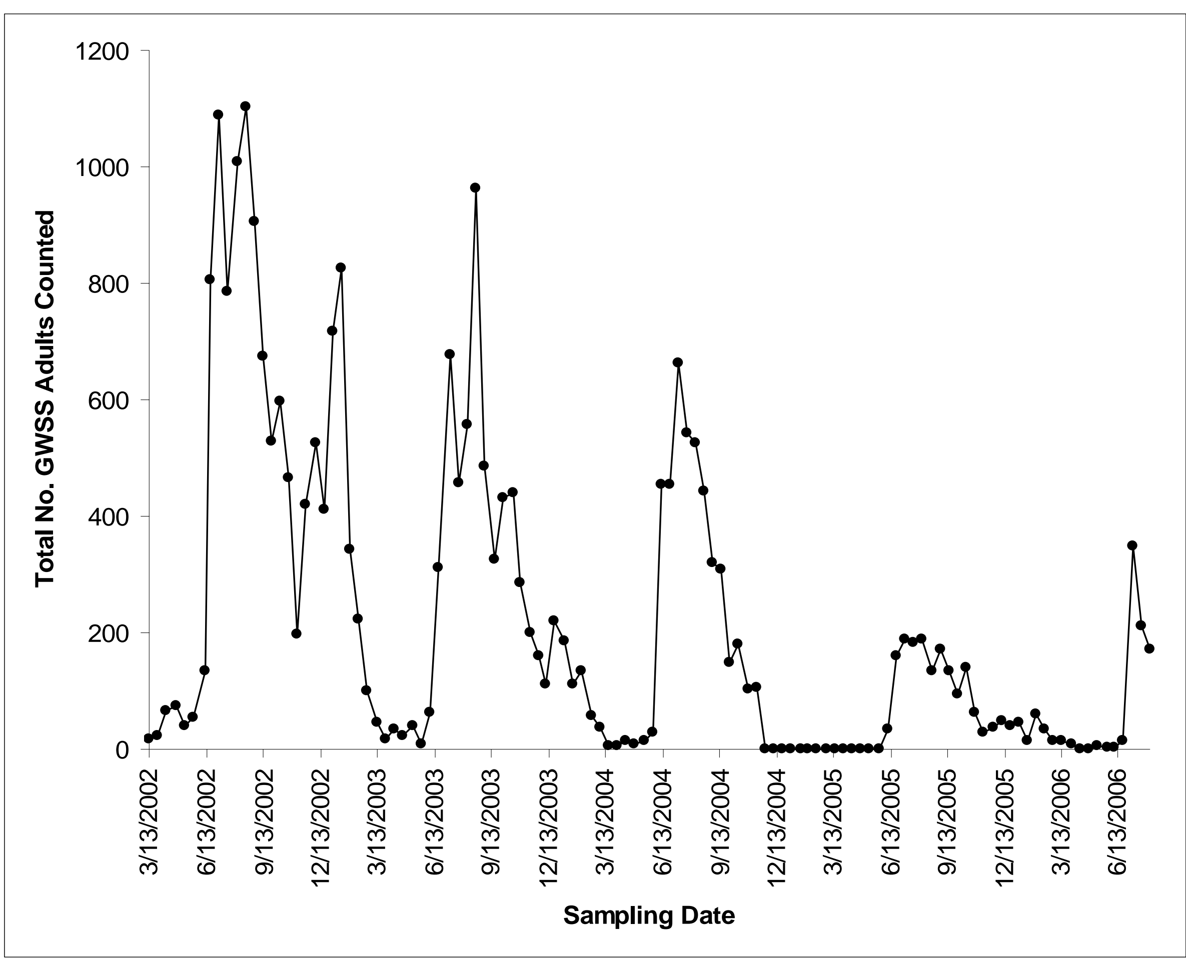

Figure 1. Phenology of adult GWSS in Eureka lemons. Data are total counts from timed five minute surveys made every two weeks of 10 mature lemon trees at Ag. Ops. University of California, Riverside.

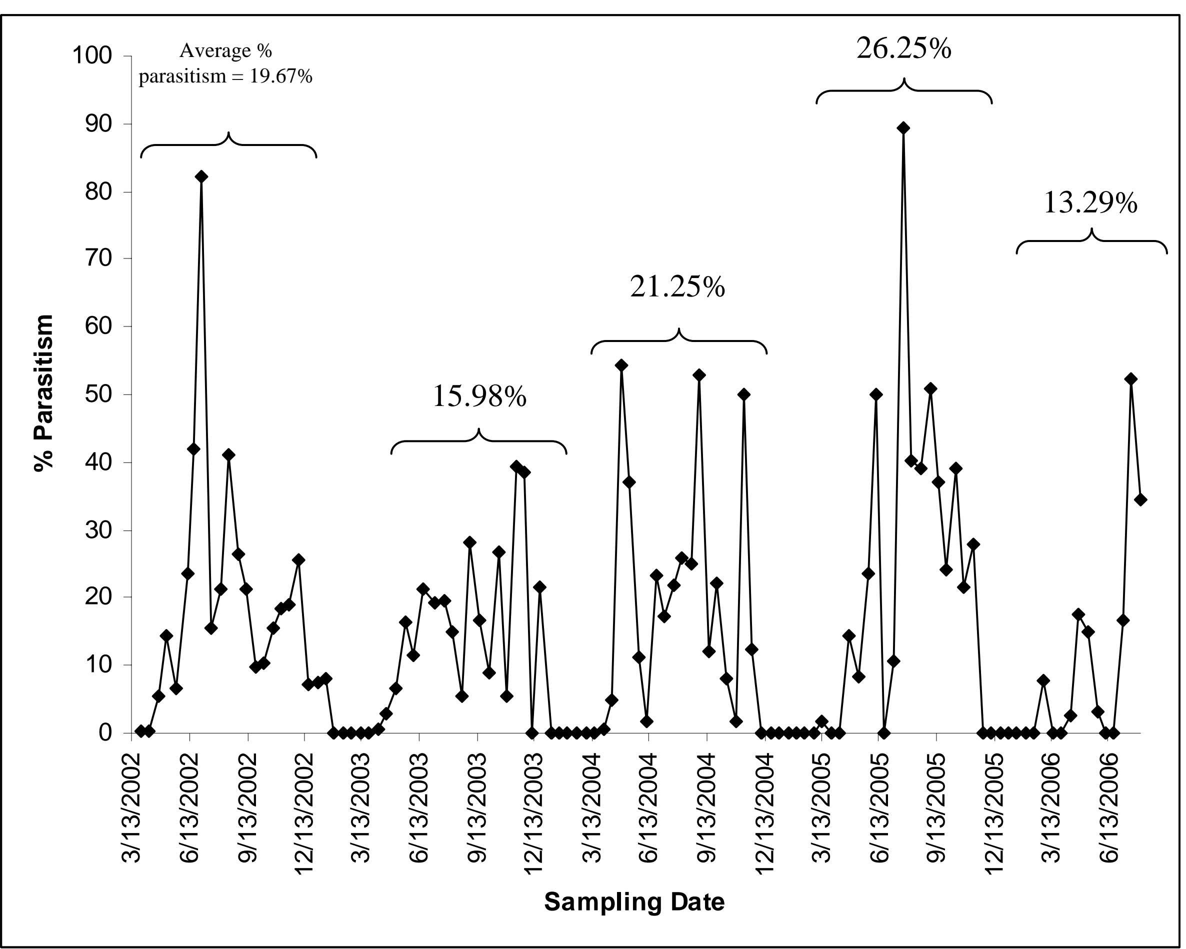

Figure 2. Percentage parasitism estimates of GWSS eggs in Eureka lemons. GWSS egg masses are collected from timed five minute surveys made every two weeks of 10 mature lemon trees at Ag. Ops. University of California, Riverside. Harvested leaves are returned to the laboratory, the number of eggs per egg mass are counted and parasitoid emergence and species identity per egg is determined. Overall average egg parasitism = 19.34%

CONCLUSIONS

GWSS populations appear to be showing a steady annual decrease in numbers in an organic lemon orchard at the University of California, Riverside. Percentage parasitism of GWSS eggs by mymarid parasitoids, in particular, *G. ashmeadi*, has remained relatively constant from year to year at ~20%. It is unknown if this level of parasitism is sufficient to have caused the steady decline in GWSS numbers observed over the past five years. The construction of the K-N-M life table from multi-cohort sampling data is incomplete as just one summer's worth of data has been collected. The life table aspect of the project is ongoing.

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DETERMINING THE DAY-DEGREE REQUIREMENTS FOR GLASSY-WINGED SHARPSHOOTER DEVELOPMENT AND QUANTIFICATION OF DEMOGRAPHIC STATISTICS AT FIVE TEMPERATURES

Project Leader:
Mark S. Hoddle
Department of Entomology
University of California
Riverside, CA 92521
mhoddle@ucr.edu

Reporting Period: The results reported here are from work conducted July 2006 to August 2006.

ABSTRACT
Glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*; formerly *H. coagulata*) developmental and reproductive biology has received very little attention from researchers investigating management strategies for this pest. This is a major impediment to rearing this insect for experimental work, developing management plans, understanding interactions with natural enemies, predicting incursion risk into new areas, and spread in recently inoculated areas. Field-oriented management plans for GWSS, if they are to be effective, need solid data on day-degree accumulations to predict pest developmental times, number of expected generations per year, and estimates of expected longevity and fecundity. The purpose of this grant is to generate these fundamental biological data for GWSS to assist pest management programs, biological control efforts, and incursion risk management. Work investigating the developmental and reproductive biology of GWSS at 20°C and 30°C is underway and should be completed by the end of the year (i.e., December 2006).

INTRODUCTION
Completed studies have comprehensively quantified the day-degree requirements of *Gonatocerus ashmeadi* and its demographic statistics across five temperatures (Pilkington & Hoddle, 2006a). These temperature derived data were modeled and equations generated were put into a GIS model built from 381 weather stations in California (CA). Geographic Information System (GIS) output using temperature data and relationships between *G. ashmeadi* development and population growth predicted the "intensity" of generational turnover and population growth throughout CA for this parasitoid. These results may indicate where *G. ashmeadi* can be expected to invade in California should its host, the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*; formerly *H. coagulata*), invade these areas too (Pilkington & Hoddle, 2006b). Similar work has been completed and submitted for publication on *G. trigutattus* (Pilkington & Hoddle, 2006c, d). However, these analyses for parasitoids and GIS application are moot unless they can be overlaid and compared with similar predictions for GWSS from comparably generated and analyzed data on its developmental and reproductive biology. Consequently, the intent of this project is to develop estimates of reproductive output at five different temperatures, and time to complete development at these experimental temperatures. Together these data will enable GIS modeling to predict incursion risk and intensity of population growth of GWSS in different areas of California and these models can be compared to similar data and GIS models for *G. ashmeadi* and *G. triguttatus*.

OBJECTIVES
1. Develop day-degree models for GWSS by quantifying the developmental biology at 5 different temperatures (15, 20, 25, 30, & 33°C).
2. Quantify reproductive biology and generate demographic statistics from $l_x m_x$ life tables at five experimental temperatures.
3. Use day-degree data (Objective 1) and demographic estimates (Objective 2) in GIS to predict the geographic range of GWSS within California, and intensity of population turnover in areas vulnerable to incursion. These predictions will be compared to those generated for two egg parasitoids of GWSS, *G. ashmeadi* and *G. triguttatus*.

RESULTS
This work is ongoing and results for 20°C and 30°C are not yet available but work should be completed by the end of 2006 for these two temperatures.

CONCLUSIONS
This work is ongoing and will be completed by the next PD Symposium in 2007.

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

Project Leader:
Mark S. Hoddle
Department of Entomology
University of California
Riverside, CA 92521
mhoddle@ucr.edu

Reporting Period: The results reported here are from work conducted July 2006 to August 2006.

ABSTRACT

Gonatocerus tuberculifemur and *G*. sp. 6. *morrilli* complex are two sharpshooter parasitoids from Argentina that have been held at the UC Riverside I & Q facility since September 2002 and reared on glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*; formerly *H. coagulata*) egg masses. There is substantial uncertainty about the safety of releasing these agents and whether they would provide additional control of GWSS in California or disrupt the efficacy of the existing parasitoid complex which has been constructed with natural enemies that have evolved to exploit GWSS. The purpose of this grant is to ascertain in Quarantine whether these two neoclassical biological control agents from Argentina can outperform the dominant GWSS parasitoid in California, *G. ashmeadi*. These data will help guide the decision to release the Argentinean parasitoids from quarantine for liberation and establishment in California. Work has not commenced on this project as the quarantine colony was recently infused with "new blood" from specimens collected in Argentina in August 2006.

INTRODUCTION

Neoclassical or new association biological control is the deliberate establishment of natural enemies against a target pest with which these natural enemies have no evolutionary history. The intent of this form of inoculative biological control is to suppress target pest populations by creating novel pest-natural enemy associations. The rationale for this strategy is the development of new exploiter-victim relationships which are hypothesized to be more effective at controlling pests. Greater impact can occur because new association avoids using old association co-evolved natural enemies that have developed population stabilizing mechanisms with the pest. It is proposed that old associations potentially result in higher population equilibrium densities compared to what would be observed if a novel efficacious natural enemy was attacking the pest with which there is no evolutionary history (Hokkanen and Pimental, 1984; 1989). Neoclassical biological control is considered to be the least ethically defensible course of action when considering use of natural enemies for pest control because of: (i) uncertainty over adverse effects of novel associations on pest population dynamics, and (ii) potential loss of ecological functions of native species because of non-target attacks (Ehler, 2000). However, these potential concerns should be addressed on a case by case basis, rather than relying on broad generalizations that ranks the ethical desirability of employing new associations lower than old associations, and the environmental risk factor substantially higher. Survey work by the USDA in Argentina has revealed a complex of parasitoid species attacking Proconiini [this is the same tribe that the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*; formerly *H. coagulata*) belongs to] sharpshooters in South America. Some of these discovered species are new to science, and two species, *Gonatocerus tuberculifemur* and *G*. sp. 6, from Argentina have been in quarantine at UC Riverside since September 2002. These two parasitoids dominated the natural enemy fauna attacking Proconiini cicadellids in arid areas of Argentina (i.e., provinces of Mendoza and Rio Colorado) and it is thought that they may be well suited to California's climate and could provide substantial control of GWSS. Limited work on host specificity testing conducted by the CDFA suggests that native California non-Proconiini sharpshooters are not at substantial risk from non-target attacks by *G. tuberculifemur* (Pickett pers. comm. 2005). However, all native U.S. Proconiini sharpshooters are considered to be at high risk of attack by these Argentinean parasitoids (Goolsby pers. comm. 2005). Consequently, concerns have been raised about non-target impacts on native USA Proconiini sharpshooters that could result from establishing these Argentinean parasitoids in California. The most salient risk scenario is the successful incursion of native GWSS habitat in the southeast USA and northeast Mexico by these neoclassical biological control agents from Argentina. This could readily occur via the transportation of plant material from California to Florida that carries GWSS egg masses parasitized by *G. tuberculifemur* or *G*. sp. 6. Should this occur, potential impact on native southeastern USA Proconiini sharpshooters is almost certain to occur, but the magnitude of the severity of successful infiltration is impossible to predict *a priori*. Consequently, the purpose of this research project is to determine if the neoclassical biological control agent, *G. tuberculifemur*, is competitively superior to the omnipresent *G. ashmeadi*, and exhibits the potential to be an extremely aggressive and efficacious natural enemy that can dominate the system to the almost total exclusion of all current parasitoids thus providing higher levels of biological control of GWSS than is currently observed.

OBJECTIVES

1. Ascertain oviposition preferences of *G. ashmeadi*, *G. tuberculifemur*, & *G*. sp. 6 for GWSS egg masses of different ages.
2. Determine the competitiveness of these parasitoids simultaneously foraging for GWSS egg masses in simple and complex environments.
3. Statistically compare the functional response of each species attacking GWSS egg masses of different sizes.
4. Compare the mean daily and lifetime reproductive output for each species at 20, 25, and 30°C.
5. Determine mean developmental times for each species at 20, 25, and 30°C.

RESULTS

No results have been achieved on this project so far. Fast progress is expected next season when GWSS colonies begin to produce abundant egg masses for experimentation.

CONCLUSIONS

Work is yet to commence on this project. Rapid progress is expected once the project starts in spring 2007.

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FUNDING AGENCIES

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REALIZED LIFETIME PARASITISM OF GLASSY-WINGED SHARPSHOOTER EGG MASSES BY *GONATOCERUS ASHMEADI*

Project Leader:
Mark S. Hoddle
Department of Entomology
University of California
Riverside, CA 92521
mhoddle@ucr.edu

Collaborators:
Nic Irvin
Department of Entomology
University of California
Riverside, CA 92521

Robert Luck
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT
Oosorption and egg maturation results suggested that *Gonatocerus ashmeadi* is a pro-synovigenic species and females mature more eggs during their lifetime. In the absence of hosts, oosorption was initiated on day 7, where the number of reabsorbed eggs increased at a rate of 1-4 eggs per day. In the presence of hosts female *G. ashmeadi* matured 3-27 eggs per day.

INRODUCTION
The self-introduced *Gonatocerus ashmeadi* (Vickerman et al. 2004) is the key natural enemy of glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*; formerly *H. coagulata*) egg masses in CA at present (Pilkington et al. 2005). Over summer, parasitism levels of GWSS egg masses and individual eggs in masses by *G. ashmeadi* can approach 100% but parasitism levels of the spring generation of GWSS are substantially lower, and parasitism generally averages ~19-20% (Pilkington et al., 2005; Triapitsyn and Phillips 2000). Naturally occurring populations of *G. ashmeadi* in CA have been augmented with mass reared individuals from populations found in the southeastern U.S.A. and northeastern Mexico which encompasses the home range of GWSS (CDFA 2003).

Substantial laboratory work with *G. ashmeadi* has been conducted in an attempt to understand and parameterize basic aspects of this parasitoid's reproductive biology, and host selection behaviors. Irvin and Hoddle (2005a) have evaluated oviposition preferences of *G. ashmeadi* when presented GWSS eggs of various ages. Interspecific competition between *G. ashmeadi* with *G. triguttatus* and *G. fasciatus* for GWSS egg masses of different ages has been assessed (Irvin and Hoddle 2005b; Irvin et al. 2005) along with factors influencing the sex ratio of offspring (Irvin and Hoddle 2006a). The effect of resource provisioning and nutrient procurement on the longevity of *G. ashmeadi* has also been determined (Irvin and Hoddle 2006b). Furthermore, Pilkington and Hoddle (2006) have assessed laboratory-level fecundity rates of *G. ashmeadi* under different constant temperature regimens.

The GWSS-*Gonatocerus* system has benefited from this intensive laboratory study to generate a basic understanding of factors influencing host selection and parasitism success. The next step that is now required is to test hypotheses generated from lab studies in the field. Field level assessments will help determine the most important aspect of the GWSS biological control program: "How big an impact do individual female *G. ashmeadi* parasitoids have on GWSS population growth via parasitization of eggs?" Addressing this question will allow us to form a much better understanding of the levels of control we can expect from *G. ashmeadi* individually and collectively on GWSS population growth in the field during the spring and summer generations.

OBJECTIVES
To measure real lifetime contributions of individual female *G. ashmeadi* to the parasitism of GWSS egg masses in citrus orchards. Before field assessments can be conducted, laboratory studies will be run to ascertain and verify four critical factors outlined below. Answers to these four critical factors will allow us to develop a composite index that describes the correlative relationship of these four factors that will predict parasitoid age and egg load in the field and to assess the contribution of individual female parasitoids to GWSS suppression under field conditions. The four critical factors are:

1. Determine the relationship between adult female *G. ashmeadi* size as measured by right hind tibia length (HTL) and 24-hr egg load for spring and summer generations (this work was completed and reported in Hoddle et al. 2005).
2. Ascertain the extent to which oosorption occurs, and the length of time without ovipositing that is required to initiate this physiological response if it does occur.
3. Determine whether female parasitoids can mature eggs in excess of those they are born with.
4. Estimate parasitoid age using near infrared spectroscopy (NIRS) (Perez-Mendoza et al. 2002) and develop an alternative measure for comparison by developing a wing deterioration index that estimates parasitoid "age" through visually grading the severity of 'wear and tear' (i.e., numbers of broken setae) of setae on wings (this work was completed and reported in Hoddle et al. 2005).

RESULTS AND DISCUSSION

Hoddle et al. (2005) reported the relationship between *G. ashmeadi* size (tibia length) and <24 hr egg load and described two methods of identifying female age through a wing wear index and using NIRS. Research reported here details oosportion and egg maturation rates for *G. ashmeadi.* These results are preliminary as we are still working on more thorough statistical analyses. With this information we aim to develop a composite index that describes the correlative relationship of the four factors listed above that will predict parasitoid age and egg load in the field and this will allow us to assess the contribution of individual female parasitoids to GWSS suppression under field conditions at time of death.

Oosorption

Gonatocerus parasitoids are generally classified as strictly pro-ovigenic (Jervis and Copland, 1996) where females emerge with a full load of mature eggs and do not mature more eggs as they age (Quicke, 1997). Results obtained so far suggest that *G. ashmeadi* may be partially syn-ovigenic. Completed studies suggest that females emerge with ~30 mature eggs and can mature more over the course of their life time (Figure 1). *G. ashmeadi* females that have access to 50% honey-water but not GWSS eggs developed 4-8 mature eggs per day up to around seven days of age before egg load in females began to decline at a rate of 1-8 eggs per day because of oosorption (Figure 1). In the absence of hosts, females appear to reabsorb mature eggs theoretically enabling them to redirect energy into host seeking and survival, a characteristic of syn-ovigenic species (Jervis et al., 1996). Figure one demonstrates that female *G. ashmeadi* oosorption was initiated on day 7, and the number of reabsorbed eggs increased at a rate of 1-4 eggs per day, to 12 eggs on day 13. In this species, oosorption is obligatory because egg maturing continues in the absence of hosts (Quicke, 1997). However, results also show that the total number of eggs present in *G. ashmeadi* ovaries (potential fecundity) over their lifetime was similar to the predicted <24 hr potential fecundity as estimated from hind tibia length (using data from objective A above). This demonstrates that in the absence of hosts, female *G. ashmeadi* do not mature more eggs than what they emerge with, a characteristic of pro-ovigenic species. In this study, parasitoid age can be converted to physiological age using day-degree estimates (Pilkington and Hoddle, 2006).

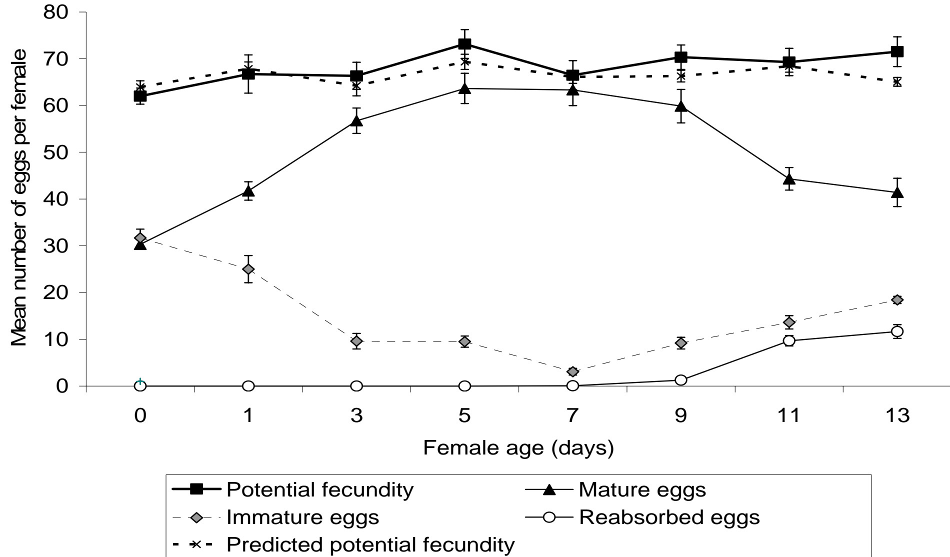

Figure 1. The effect of age on the mean number of mature eggs, immature eggs, reabsorbed eggs, potential fecundity and predicted potential fecundity (as predicted from hind tibia length) in ovaries of female *G. ashmeadi* presented with no hosts for 13 days.

Egg Maturation

Figure 2 shows that on day 13, potential fecundity was 77 eggs higher than the predicted <24 hr potential fecundity as estimated from hind tibia length. This suggests that *G. ashmeadi* mature more eggs as they parasitize hosts during their lifetime and indicates that this species is partially syn-ovigenic. Potential fecundity (realized fecundity + eggs present in ovaries) data demonstrates that in the presence of hosts female *G. ashmeadi* matured 3-27 new eggs per day. It is possible that potential fecundity was underestimated in this study because some eggs oviposited by females may be unaccounted for due to superparasitism or early larval death. The potential fecundity of females given hosts for one day after emergence was 26 eggs lower than the predicted <24 hr potential fecundity. This may indicate that 26 eggs were lost due to superparasitism or early larval death.

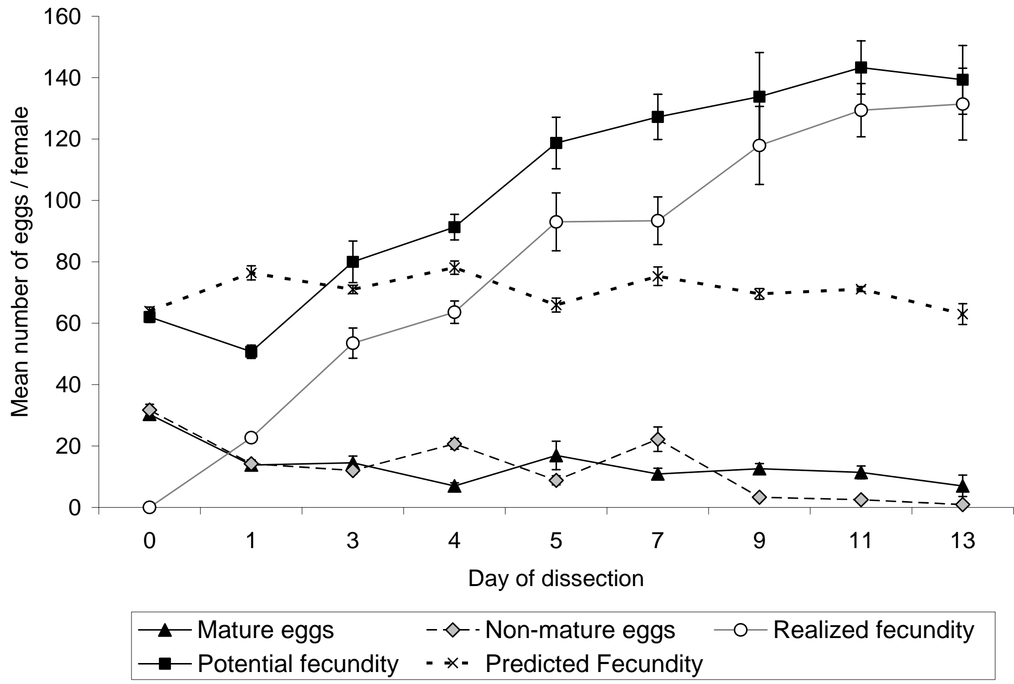

Figure 2. The number of mature and non-mature eggs present in ovaries, realized fecundity (successful parasitism), potential fecundity (realized fecundity + eggs present in ovaries) and predicted fecundity (estimated from hind tibia length) of female *G. ashmeadi* offered hosts daily for 0-13 days after female emergence.

CONCLUSIONS

Oosorption and egg maturation results suggest that *G. ashmeadi* is a pro-synovigenic species. Females have the ability to mature eggs in excess of those they emerge with over their lifetime. In the absence of hosts, oosorption was initiated on day 7, where the number of reabsorbed eggs increased at a rate of 1-4 eggs per day. In the presence of hosts, female *G. ashmeadi* matured 3-27 new eggs per day. Together with previous data (the relationship between adult female *G. ashmeadi* size and 24-hr egg load, and a wing deterioration index that estimates parasitoid age), these components will be used to develop a composite index that will predict parasitoid age and egg load in the field and help determine how many eggs individual female *G. ashmeadi* parasitize in the field up to the time of death. In 2006 we collected ~20 dead female *G. ashmeadi* from the field using funnel traps loaded with dry ice. Females will be aged and egg load at time of emergence will be estimated from hind tibia length. The egg load at time of death (when oosorption and egg maturation are figured into the model) will allow us to estimate the average number of GWSS eggs females parasitize before dying. These estimates of realized field fecundity will allow us to form a much better understanding of what levels of control individual *G. ashmeadi* in the field are achieving.

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FUNDING AGENCIES

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DEVELOPING DAY-DEGREE MODELS TO PREDICT SPREAD WITHIN CALIFORNIA OF *GONATOCERUS TRIGUTTATUS* RELEASED FOR GLASSY-WINGED SHARPSHOOTER CONTROL, AND USING DEMOGRAPHIC DATA TO INVESTIGATE PARASITOID SPREAD IN CALIFORNIA

Project Leader:
Mark S. Hoddle
Department of Entomology
University of California
Riverside, CA 92521
mhoddle@ucr.edu

Researcher:
Leigh Pilkington
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted March 2005 to April 2006.

ABSTRACT

The reproductive and developmental biology of *Gonatocerus triguttatus* Girault, a parasitoid of the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*; formerly *H. coagulata*), was determined at five constant temperatures in the laboratory; 15; 20; 25; 30; and 33°C. At 25°C, *G. triguttatus* maintained the highest successful parasitism rates with 25.1% of parasitoid larvae surviving to adulthood and lowest parasitism was observed at 15°C with 7.3% of parasitoid larvae surviving to adulthood. Lifetime fecundity was greatest at 25°C and fell sharply as temperature either increased or decreased around 25°C. Temperature had no effect on sex ratio of parasitoid offspring. Mean adult longevity was inversely related to temperature with a maximum of 20.6 days at 15°C to a minimum of four days at 33°C. Developmental rates increased nonlinearly with increasing temperatures. Developmental rate data was fitted with the modified Logan model for oviposition to adult development times across each of the five experimental temperatures. Optimal, lower, and upper lethal, temperature thresholds for *G. triguttatus* were, 30.7°C, 10.4°C and 38.8°C, respectively. The lower developmental threshold estimated with linear regression was 10.57°C, and is very close to the lower temperature threshold estimated by the modified Logan model. Linear regression of developmental rate across all five experimental temperatures indicated that 204 degree-days were required above the minimum threshold of 10.57°C to complete development. Demographic parameters were calculated and pseudo replicates for intrinsic rate of increase (r_m), net reproductive rates (R_o), generation time (T_c), population doubling time (T_d), and finite rate of increase (□) were generated using the bootstrap method. Mean bootstrap estimates of demographic parameters were compared across temperatures using ANOVA and nonlinear regression.

INTRODUCTION

Gonatocerus triguttatus Girault, (Hymenoptera: Mymaridae) is a solitary endoparasitoid that attacks eggs of sharpshooters in the cicadellid tribe Proconiini. This parasitoid was originally described from specimens reared from an unidentified leafhopper egg mass collected in Trinidad, and has been subsequently reared from glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*; formerly *H. coagulata*; Hemiptera: Cicadellidae) egg masses collected in Texas and Mexico. This natural enemy has a natural range that includes southeastern U.S.A. and northeastern Mexico where it is associated with GWSS. *Gonatocerus triguttatus* was deliberately imported from Texas U.S.A. and introduced into California U.S.A. in 2002 as part of a classical biological control program against GWSS. Some recoveries from release areas have been made tentatively suggesting *G. triguttatus* may have established perennial populations in California (Pilkington et al., 2005). A thorough understanding and characterization of biological attributes of natural enemies such as degree-day requirements, and intrinsic rates of increase can have multiple practical applications, such as: (1) quantification of the reproductive and developmental biology of candidate natural enemies can assist with predicting potential establishment and population growth of natural enemies introduced into a new area, (2) can aid preliminary evaluation of natural enemies for use potential use in classical biological control, (3) assist with interpretation of natural enemy impact and spread in the field, and (4) provide realistic values for parameters of models investigating incursion risks pertaining to movement of natural enemies into environments beyond those intended for permanent inhabitation. Improved understanding of the basic biology of *G. triguttatus*, a recently released and established natural enemy of GWSS in California, will assist mass-rearing efforts of this parasitoid; optimize timing of inoculative field releases; facilitate better understanding of parasitoid spread and impact on GWSS in various climatic zones in California; and will assist with targeted collecting for biotypes of *G. triguttatus* in the home range of GWSS that may exhibit unique climatic adaptations that current parasitoid populations in California lack.

OBJECTIVES

1. Develop day-degree models for mymarid parasitoids by quantifying the developmental and reproductive biology of *G. triguttatus* at 5 different temperatures (this work has been completed for the principal egg parasitoid of GWSS, *G. ashmeadi*).
2. Use day-degree data from Objective 1 in a Geographic Information Systems approach to predict the geographic range of parasitoids within California and use GIS to map these predictions to known and potential GWSS distributions.

RESULTS

The reproductive and developmental biology of *G. triguttatus* was determined at five constant temperatures in the laboratory; 15; 20; 25; 30; and 33°C. At 25°C, *G. triguttatus* maintained the highest successful parasitism rates with 25.1% of parasitoid larvae surviving to adulthood and lowest parasitism was observed at 15°C with 7.3% of parasitoid larvae surviving to adulthood. Lifetime fecundity was greatest at 25°C and fell sharply as temperature either increased or decreased around 25°C. Temperature had no effect on sex ratio of parasitoid offspring. Mean adult longevity was inversely related to temperature with a maximum of 20.6 days at 15°C to a minimum of four days at 33°C. Developmental rates increased nonlinearly with increasing temperatures. Developmental rate data was fitted with the modified Logan model for oviposition to adult development times across each of the five experimental temperatures. Optimal, lower, and upper lethal, temperature thresholds for *G. triguttatus* were, 30.7°C, 10.4°C and 38.8°C, respectively. The lower developmental threshold estimated with linear regression was 10.57°C, and is very close to the lower temperature threshold estimated by the modified Logan model. Linear regression of developmental rate across all five experimental temperatures indicated that 204 degree-days were required above the minimum threshold of 10.57°C to complete development. Demographic parameters were calculated and pseudo replicates for intrinsic rate of increase (r_m), net reproductive rates (R_o), generation time (T_c), population doubling time (T_d), and finite rate of increase (λ) were generated using the bootstrap method. Mean bootstrap estimates of demographic parameters were compared across temperatures using ANOVA and nonlinear regression (Figure 1).

The number of expected generations of *G. triguttatus* was estimated using life table statistics and degree-day requirements from Objective 1 above. Between zero to 18.9 and zero to 25.3 generations per year were estimated across different climatic regions in California, using life table and degree-day models, respectively. Temperature-based values for net reproductive rate, R_o, were estimated in California using a laboratory-derived equation and ranged from zero to approximately 29.4 and analyses indicate that a minimum of seven to 7.8 generations (calculated using life table and degree-day models) are required each year to sustain a population of *G. triguttatus* in a given area. Long-term weather data from 381 weather stations across California were used with an Inverse-Distance Weighting algorithm to map various temperature-based demographic estimates for *G. triguttatus* across the entire state of California. This Geographic Information Systems model was used to determine number of *G. triguttatus* generations based on day-degree accumulation, generation time, T_c, and R_o. GIS mapping indicated that the only areas in California that may have climatic conditions favorable for supporting the permanent establishment of invading populations of *G. triguttatus,* should *H. vitripennis* successfully establish year-round populations, are Imperial, San Diego, Riverside, Orange and the southern areas of Santa Barbara, Ventura, Los Angeles and San Bernardino counties. Northern counties in California that experience cooler average year round temperatures do not appear to be conducive to the establishment of permanent populations of *G. triguttatus* (Figure 2).

CONCLUSIONS

G. triguttatus has been mass released in southern California since 2002, and small localized populations appear to have established, but have failed to become robust, abundant, and widespread (D. Morgan pers. comm. 2006). Two potential reasons may exist for these localized low density populations of *G. triguttatus*: (1) not enough time has elapsed since release and establishment for *G. triguttatus* to have reached its full potential, (2) in the field this parasitoid is an ineffective competitor with self-introduced and omnipresent *G.ashmeadi*. Laboratory studies suggest interspecific competition with *G. ashmeadi* may be severely limiting to *G. triguttatus*, in comparison to *G. ashmeadi*, *G. triguttatus* has reduced longevity, parasitizes fewer GWSS eggs, spends more time resting and grooming, and in some instances devotes little time to defending host patches from competitors. The reduced impact of *G. triguttatus* as a regulating factor of populations of GWSS in southern California may also be influenced by climatic conditions in the invaded areas. Low temperatures over winter appear reduce or prevent oviposition by GWSS for extended periods which results in a shortage of hosts for *G. triguttatus*, and other mymarid parasitoids attacking GWSS. Further, *G. triguttatus* has not been recorded from an alternative host in California, *H. liturat*a, whereas the common *G. ashmeadi* is often associated with this common native sharpshooter. Temperature can have a significant impact on R_o estimates for *G. triguttatus*. The fitted quadratic model for R_o, a measure of a population's growth rate, indicated that at approximately 14.9°C the value of R_o falls below 1.0, indicating that parasitoid population growth will cease and begin to contract. During this 10 year span, the weather station at the University of California, Riverside Agricultural Operations Facility recorded the average daily temperature fall below 14.9°C 127 times or 35% of the year. The ten-year average daily temperature in Riverside falls below 14.9°C in a single, discrete block of 100 days typically over the period November to March. During this three month time span, temperatures fluctuate from a minimum of 3.8°C to a maximum high temperature of 22.89°C. Although temperatures rise above the development threshold required by *G. triguttatus*, calculations indicate that the population would accumulate enough degree-days to complete 2.5 generations in this time. Temperatures may rise enough to prompt sporadic oviposition by parasitoids if host eggs are available but persistent low temperatures over winter will retard parasitoid population growth. Host availability notwithstanding, this suggests populations of *G. triguttatus* in Riverside California would contract markedly over the period November-March each year because of impaired reproductive performance at temperatures below 14.9°C periods for prolonged periods. Consequently, demographic data from studies completed here, coupled with long-term weather data sets for southern California, may explain why populations of *G. triguttatus* are not more common, widespread or have been particularly successful in attacking abundant GWSS egg masses in California.

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FUNDING AGENCIES

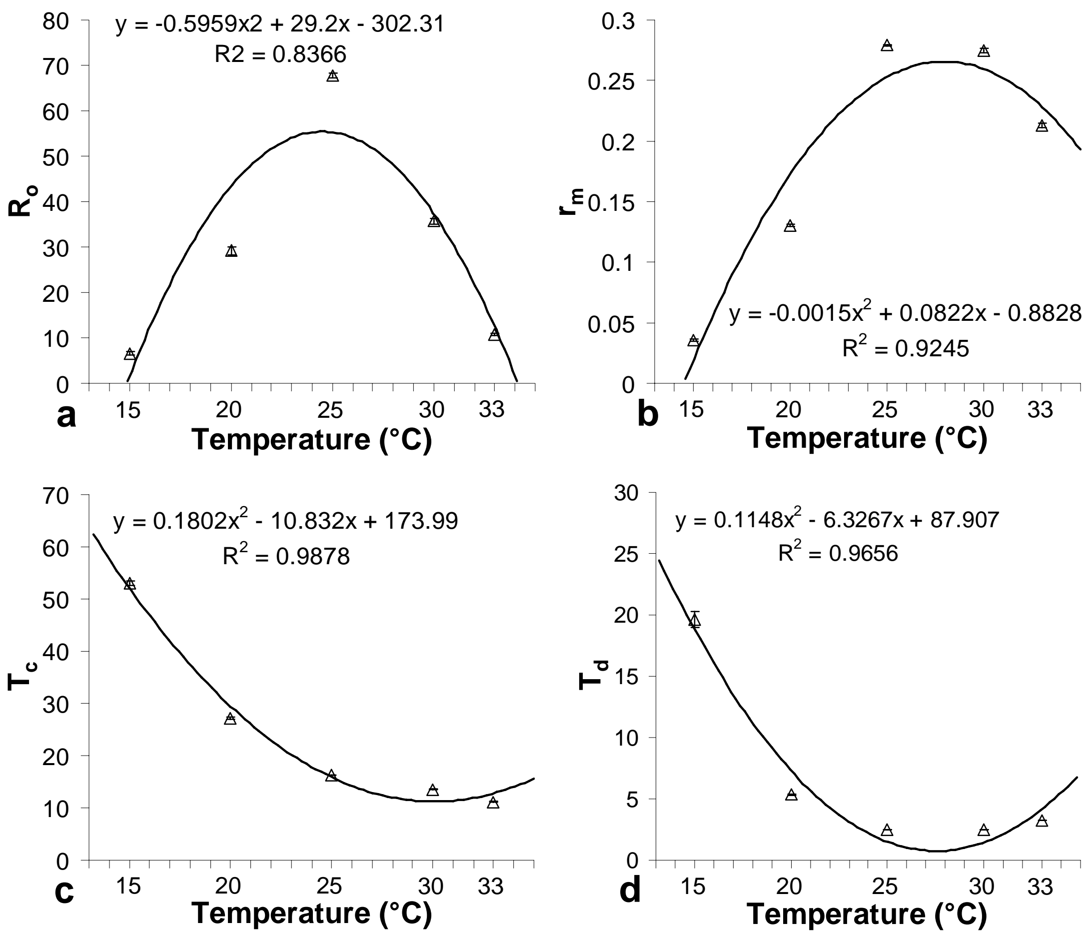

Figure 1. Fitted quadratic lines for life table statistics R_o (a), r_m (b), T_d (c) and T_c (d) for *G. triguttatus* at each of five experimental temperatures.

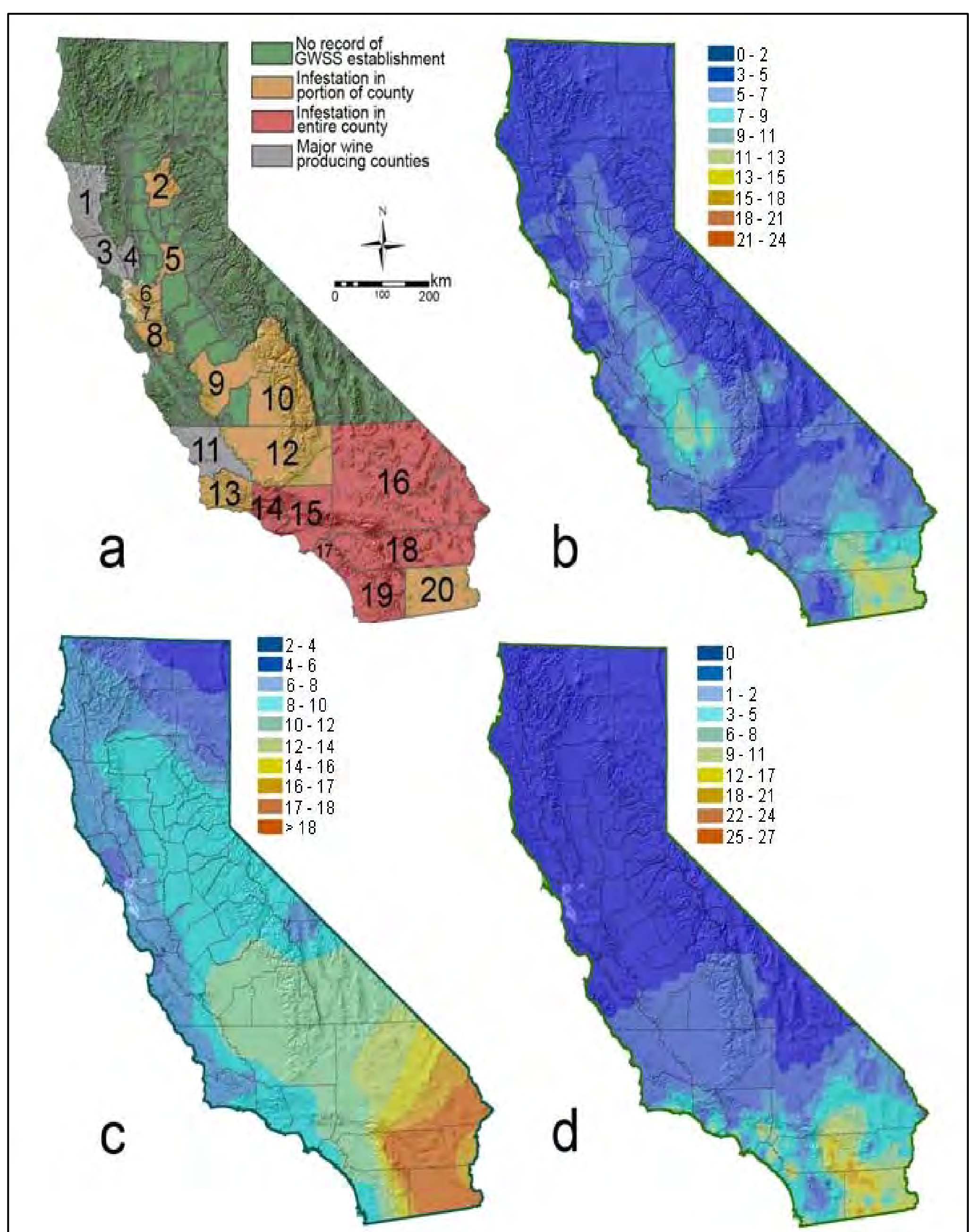

Figure. 2. Geographical information systems mapping of estimated life table statistics for the parasitoid *G. triguttatus* in California, U.S.A.; A) Counties in California and the status of GWSS populations in each area 1. Mendocino 2. Butte 3. Sonoma 4. Napa 5. Sacramento 6. Contra Costa 7. Alameda 8. Santa Clara 9. Fresno 10. Tulare 11. San Luis Obispo 12. Kern 13. Santa Barbara 14. Ventura 15. Los Angeles 16. San Bernardino 17. Orange 18. Riverside 19. San Diego 20. Imperial; B) estimated number of generations populations of *G. triguttatus* may experience in each area calculated by dividing the year's accumulated degree-days by the total degree-days required by *G. triguttatus* for development; C) estimated number of generations that hypothetical populations of *G. triguttatus* may experience in each area calculated by applying historical weather data to the formula for yearly generations, T_{num}, derived from the life table statistic for generation time, T_c. and; D) estimation of the yearly value for net reproductive rate, R_o, derived by applying historical weather data to the ormula for R_o. For Figs. 1b-d the colored legend indicates the value of the particular statistic of interest for an entire year.

NON-TARGET IMPACT OF THE GLASSY-WINGED SHARPSHOOTER PARASITOIDS *GONATOCERUS ASHMEADI* AND *G. FASCIATUS* ON SHARPSHOOTERS NATIVE TO CALIFORNIA, WITH NOTES ON INDIGENOUS PARASITOIDS OF THE BLUE-GREEN SHARPSHOOTER AND THE GREEN SHARPSHOOTER

Project Leaders:

Mark S. Hoddle	Elizabeth A. Boyd
Department of Entomology	Department of Entomology
University of California	University of California
Riverside, CA 92521	Riverside, CA 92521
mhoddle@ucr.edu	elizabeth.boyd@email.ucr.edu

Reporting period: The results reported here are from work conducted May 2003 to September 2006.

ABSTRACT

A rigorous testing strategy involving choice and no-choice test arenas was developed to explore the potential non-target impacts of classical biological control agents. The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis* (Germar) (Hemiptera: Cicadellidae), biological control program was studied as a model system for analyzing choice and no-choice host preferences for natural enemies and also for performing retrospective non-target impact assessments. *Gonatocerus ashmeadi* Girault and *G. fasciatus* Girault (both Hymenoptera: Mymaridae), egg parasitoids of the exotic GWSS, were tested against three potential non-target sharpshooters indigenous to southern California. Work conducted here showed that the solitary *G. ashmeadi* was able to successfully parasitize eggs (i.e., viable progeny resulted) of smoketree sharpshooter (STSS), *H. liturata* Ball, but not eggs of the blue-green sharpshooter (BGSS), *Graphocephala atropunctata* (Signoret), or the green sharpshooter (GSS), *Draeculacephala minerva* Ball (all Hemiptera: Cicadellidae). *G.ashmeadi* exercises no parasitization preference when presented with a choice of STSS and GWSS eggs simultaneously. The gregarious *G. fasciatus* parasitized eggs of STSS and GSS, but not eggs of the BGSS. BGSS and GSS eggs were collected from the field and reared to catalog their indigenous egg parasitoid fauna. Any parasitoids reared from these eggs were reciprocally exposed to 'clean' lab colony eggs. Two parasitoids, *G. latipennis* Girault and a *Polynema* sp. (Hymenoptera: Mymaridae) were confirmed as parasitoids of BGSS eggs. Three parasitoids, *G. mexicanus* Perkins and two unidentified trichogrammatids were confirmed as parasitoids of GSS eggs.

INTRODUCTION

Examining possible non-target effects of biological control agents is becoming a more common requirement for many biological control programs targeting arthropod pests. Currently, for classical biological control of weeds, the centrifugal method provides a robust theoretical framework for identifying potential natural enemies that could cause harm to non-target plants. However, a rigorous, reliable, and universally applicable testing standard for arthropod biological control with a strong theoretical basis is currently lacking. No-choice and choice testing strategies are a common way to test for possible non-target effects of new biological control organisms. However, these lab studies are often carried out in small testing arenas where the study organism is forced onto the host which may be adequate for determining physiological host range but may seriously overestimate its ecological host range in nature. Under these conditions, efficacious natural enemies may be unnecessarily eliminated from the candidate natural enemy pool as being insufficiently host-specific. To more accurately determine the host range of a natural enemy our research involved the use of rigorous testing strategies utilizing standard Petri dish test arenas, coupled with larger-scale entire plant test arenas in no-choice and choice comparisons. As retrospective studies in ongoing biological control programs can yield valuable information on non-target impacts, we chose the glassy-winged sharpshooter (GWSS) classical biological control program in California as a model for our non-target impact studies. We are examining the possible non-target impacts of the self-introduced and omnipresent *Gonatocerus ashmeadi* and the recently introduced *G. fasciatus*, both egg parasitoids of GWSS, on three sharpshooters native to California, U.S.A.: the smoketree sharpshooter (STSS) *Homalodisca liturata* Ball (Hemiptera: Clypeorrhyncha: Cicadellidae: Cicadellinae: Proconiini) (native congener to GWSS), blue-green sharpshooter (BGSS) *Graphocephala atropunctata* (Signoret), and green sharpshooter (GSS) *Draeculocephala minerva* Ball (the latter two, all Hemiptera: Clypeorrhyncha: Cicadellidae: Cicadellinae: Cicadellini). Our experiments with small-scale Petri dish studies and larger-scale full plant studies were supplemented with deployment of sentinel plants bearing eggs from laboratory colonies of BGSS or GSS and habitat surveys to determine the invasiveness of GWSS parasitoids into habitats occupied by BGSS or GSS.

OBJECTIVES

1. Classify the native egg-parasitoid fauna in California associated with sharpshooters native to California, primarily the STSS, BGSS, and GSS.
2. Assess the possible non-target impacts of *G. ashmeadi* Girault and *G. fasciatus* (both Hymenoptera: Mymaridae), parasitoids being used for the classical biological control of GWSS, on the above mentioned native sharpshooters.

RESULTS

Indigenous parasitoids of BGSS and GSS

Eggs of BGSS and GSS were collected from natural habitats in California and held at laboratory temperatures to rear egg-parasitoids. Sentinel eggs from lab colonies of either BGSS or GSS were placed in the field to expose eggs to resident parasitoids. Parasitoids reared from field collected or sentinel eggs were exposed to 'clean' eggs from lab colonies to confirm their host association with the proposed native sharpshooter. Two egg-parasitoids, *G. latipennis* and a *Polynema* sp., were reared from field collected BGSS eggs. *Polynema* sp. was confirmed as a parasitoid of BGSS. Three parasitoids, *G. mexicanus* and two unidentified trichogrammatids were reared from field collected GSS eggs. All three were confirmed as parasitoids of GSS eggs via reciprocal attacks on "clean" eggs from the GSS lab colony.

Host specificity testing

Choice and no-choice tests were conducted with *G. ashmeadi* and *G. fasciatus* on BGSS, GSS, and STSS eggs using GWSS eggs as a control. Tests were conducted on two scales, micro (= petri dish, 100 x 15 mm) and macro (= full plant, approximately 30 cm height), using single, one day old, mated, honey-water-fed *G. ashmeadi* or *G. fasciatus*. Micro scale tests were used to estimate the physiological host range of the parasitoid by reducing the area of search and increasing host contact thereby forcing the parasitoid onto a host. By contrast, the macro scale tests were utilized to estimate the ecological host range of the parasitoid by incorporating host finding on an entire plant thereby enabling the parasitoid to use a greater part of its searching repertoire for assessing host suitability. BGSS eggs have not yet been tested at the micro scale. Each test was conducted utilizing two different host plants for each of the sharpshooters examined. An effort was made to include at least one native or naturalized California host plant. Eureka Lemon (*Citrus limon* (L.) Burm.f. cv. 'Eureka'; Sapindales: Rutaceae) and jojoba (*Simmondsia chinensis* (Link) Schneid.; Euphorbiales: Simmondsiaceae) were used for STSS, sweet basil (*Ocimum basilicum* L.; Lamiaceae: Lamiales) and wild grape (*Vitis girdiana* Munson; Rhamnales: Vitaceae) for BGSS, and milo (*Sorghum bicolor* L. Moench; Cyperales: Poaceae) and rescuegrass (*Bromus catharticus* Vahl.; Cyperales: Poaceae) for GSS. In choice tests, the parasitoid was exposed to approximately 20 eggs each of the test species and control on the same host plant type simultaneously. For no-choice testing, each parasitoid was supplied approximately 40 target eggs. All target eggs were < 48 h of age. In all tests, the parasitoid was provided honey water as a food source and allowed 24 h to parasitize eggs before removal from the testing arena.

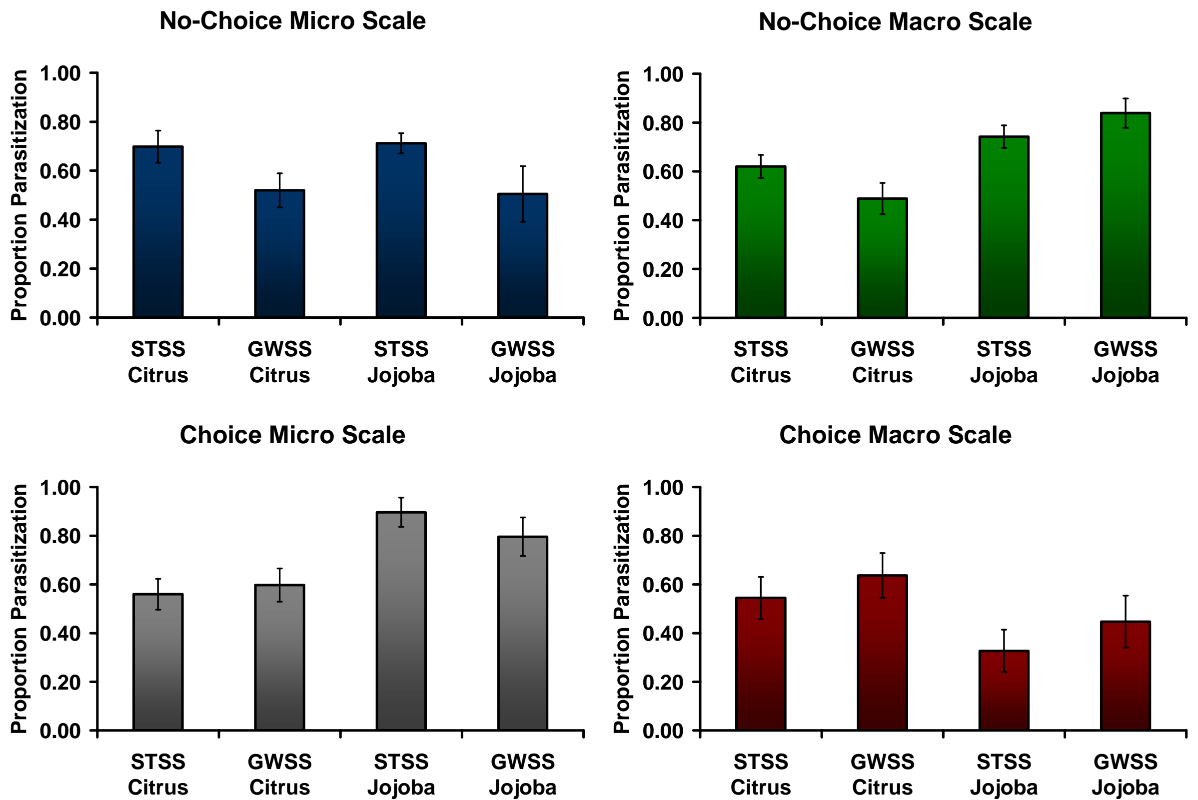

Figure 1. Proportion parasitism of STSS and GWSS eggs by *G. ashmeadi* in choice and no-choice studies.

For no-choice micro scale tests, proportion parasitization of STSS eggs by *G. ashmeadi* appears to be higher than that of GWSS eggs for both host plants tested (Figure 1). This trend appears to hold true for no-choice macro scale tests on lemon, but not for jojoba. For both micro and macro choice arenas, there does not appear to be any difference in proportion parasitism when *G. ashmeadi* is offered a choice between GWSS and STSS on either host plant. *G. ashmeadi* did not

parasitize BGSS eggs on basil or grape in either macro choice or no-choice test arenas, and this parasitoid did not parasitize GSS eggs on milo or rescuegrass in any of the four test arenas.

Preliminary data shows that *G. fasciatus* will parasitize STSS eggs in all four test arenas (Figure 2) and can produce 2-5 offspring per egg. It did not parasitize BGSS eggs on basil or grape in either macro choice or no-choice tests. *G. fasciatus* parasitized GSS eggs (producing two offspring per egg) on rescuegrass in both choice and no-choice micro scale test arenas, but did not parasitize eggs in either macro choice or no-choice tests. Many replicates for *G. fasciatus* are in progress for both STSS and GSS, thus results are preliminary.

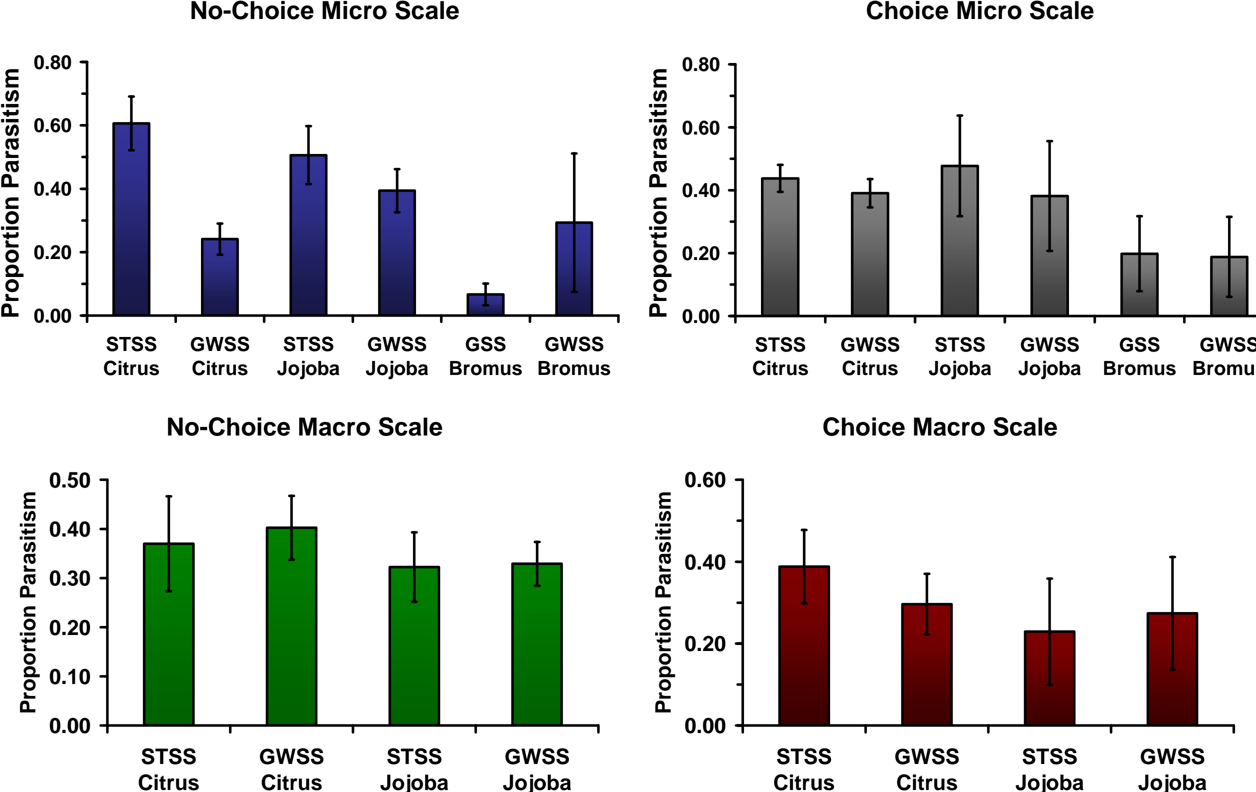

Figure 2. Proportion parasitism of STSS, GSS, and GWSS eggs by *G. fasciatus* in choice and no-choice studies.

CONCLUSIONS

BGSS, GSS, and STSS are all vectors of Pierce's disease and documentation of the indigenous natural enemy complex for these sharpshooters is an essential step when developing sustainable control options that rely on resident natural enemies for pest control. Now that progress has been made on identifying the indigenous parasitoid fauna associated with *Xylella*-vectoring native sharpshooters it may be possible to conserve or augment populations of these natural enemies in future management programs. Additionally, documentation of these parasitoids will allow for subsequent research on the depth of any indirect non-target impacts associated with the GWSS biological control program.

It is interesting that the solitary egg-parasitoid, *G. ashmeadi*, will parasitize more STSS eggs than it will GWSS eggs in a no-choice test arena. STSS eggs are smaller then GWSS eggs (Al-Wahaibi 2004) and *G. ashmeadi* offspring are smaller and less fecund from STSS eggs than those reared from GWSS eggs (N. A. Irvin unpublished data). It is possible the parasitoid is aware that the offspring will be smaller and less fit, so the mother may compensate by parasitizing more of the smaller host eggs. However, when offered a choice between the target and non-target eggs, there is no difference in parasitism regardless of host plant. This might suggest that the parasitoid is distributing her chances of offspring survival evenly between the two hosts. Given the substantial availability of GWSS eggs, these parasitoids may impact the native *Ufens* spp. (Hymenoptera: Trichogrammatidae) parasitoid complex if large numbers of *G. ashmeadi* spill out of GWSS infested areas and attack STSS eggs, the preferred host for *Ufens* spp. We would speculate these 'runts' may have an overall reduced fitness, and that STSS eggs may ultimately be a dead-end host for *G. ashmeadi*, especially if no selection of evolutionary significance occurs for use of STSS eggs. However, if this parasitoid were to establish in large numbers in the xeric habitats where STSS is most abundant and where GWSS is relatively rare or absent, and if *G. ashmeadi* larvae are able to out-compete *Ufens* spp. larvae, then we might expect an impact on the native natural enemy fauna of STSS in desert regions. Presently, we suspect that *G. ashmeadi* and most likely *G. fasciatus* are unlikely to physiologically withstand the harsh environments of desert areas of eastern California, but the possibility and the consequences of such an incursion, should it occur, are worth consideration.

While results for the gregarious *G. fasciatus* are still in progress, it will be interesting to see if similar trends for STSS parasitism are observed for this parasitoid as were observed for *G. ashmeadi*. Since *G. fasciatus* has parasitized GSS eggs successfully producing two female offspring per GSS egg, despite the egg length being approximately one-half the length of a GWSS egg, it suggests GSS is a physiologically acceptable host for *G. fasciatus*. However, no *G. fasciatus* were reared from field collected egg masses, and the parasitoid has not parasitized GSS eggs at the macro scale in either choice or no-choice arenas. This suggests that although *G. fasciatus* is physiologically capable of parasitizing GSS eggs and will attack eggs from this species in a Petri dish, it is unlikely that GSS eggs are an ecologically suitable host for this parasitoid as this parasitoid has failed to parasitize GSS on entire plants. Consequently, it is unlikely this parasitoid will be a significant threat to indigenous egg-parasitoids of GSS.

Our research approach with GWSS parasitoids has attempted to include physiological, ecological, temporal and spatial elements in determining possible native sharpshooter (and associated native parasitoids) non-target effects. Via choice and no-choice testing at two scales, parasitoid field surveys, non-target habitat monitoring and natural enemy classification, and by determining oviposition, egg, and habitat characteristics of the possible non-target species, we are obtaining important information for retroactively assessing the possible risk posed by two exotic natural enemies of GWSS (*G. ashmeadi* and *G. fasciatus*) to native members of the receiving ecosystem.

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

Project Leader:
Joseph G. Morse
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:
David J.W. Morgan
Calif. Dept. Food & Agric.
Mount Rubidoux Field Station
Riverside, CA 92501

Jonathan M. Lytle
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT
The California Department of Food & Agriculture (CDFA) has a number of sites in southern California where they are releasing egg parasitoids of the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*; formerly *H. coagulata*). To date, species released include *Gonatocerus ashmeadi, G. fasciatus, G. walkerjonesi,* and *G. triguttatus.* Very recently, two new species, *G. morrilli* (first released at our sites 5/2/06) and a strain of *Anagrus epos* from Minnesota (first released at our sites 5/17/06) have been released. We recently recovered *G. morrilli* (on 8/22/06) but not yet *Anagrus epos* (*A. epos*). CDFA monitors for parasitoid establishment and population dynamics at release sites. This project is intended to complement and expand the scope of this monitoring with an eye towards improving our understanding of the benefit of releasing alternative parasitoid species and how well they are surviving, dispersing, and impacting GWSS populations in southern California.

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

In the two-year (July 2001 – June 2003) study by Al-Wahaibi (2004) around CDFA's release site on the UC Riverside campus, parasitism was due to a total of eight parasitoid species with *Gonatocerus ashmeadi*, *Ufens principalis* (previously *Ufens* A, Al-Wahaibi et al. 2005), *Ufens ceratus (*previously *Ufens* B), and *G. walkerjonesi* being the most abundant. *Ufens* spp. were dominant on jojoba while on other plants, *Gonatocerus* species tended to dominate. Across all ten host plants sampled, ranked percent parasitism was *G. ashmeadi* (27.4%), *U. principalis* (19.8*%), U. ceratus* (2.9%), *G. walkerjonesi* (2.1%,), *G. incomptus* (0.4%), *G. novifasciatus* (0.3%), *G. triguttatus* (0.1%), and *G. fasciatus* (0.01%). Note, however, that these data may have been biased by the proximity of nearby hosts harboring smoke-tree sharpshooter and high levels of *Ufens* spp. on jojoba.

The taxonomy of the "*G. morrilli*" complex was revised when it was discovered that what had been called *G. morrilli* was actually 2 species that were difficult to tell apart. These are the newly names *G. walkerjonesi* which is either native to California or was unintentionally introduced from some other area (similar to species from Central America) and *G. morrilli* which is native to Florida, Texas, and northeastern Mexico. In the past, CDFA released what they were calling "*G. morrilli*" but when genetic methods became available to distinguish the two species, it was realized that the culture was actually *G. walkerjonesi*. At that time, *G. morrilli* was recollected from Texas, a colony was started in California, and releases have been made starting 6/6/06 at four of our release sites.

Another new listing in our recovery data is the newly named *G. morgani*. This species is likely native to California or was unintentionally introduced into California. It was overlooked in the past, perhaps because it was not common on smoke-tree sharpshooter and was thus missed prior to GWSS's establishment and proliferation in California.

Anagrus epos was collected in Minnesota by Dr. Roman Rakitov (Center for Biodiversity, Illinois Natural History Survey, Champaign, Illinois) near Glyndon, Clay Co., Minnesota, from egg masses of *Cuerna fenestella* Hamilton (a native, univoltine proconiine sharpshooter) on *Solidago* sp. (goldenrod, *Compositae*) and *Zigadenus* sp. (death camus, *Liliaceae*) and sent to Dr. Serguei Triapitsyn at the UC Riverside quarantine facility under an appropriate permit (Hoddle & Triapitsyn 2004, Triapitsyn & Rakitov 2005). A permit for release from quarantine was obtained in 2005 by Dr. David Morgan and this strain is presently being reared by CDFA and has already been released at a few field sites in California.

OBJECTIVES (As Modified)
Monitor GWSS egg parasitoids in six areas in southern California (three coastal, three interior sites) on citrus within CDFA's parasitoid release sites. Focus on evaluation of two new species, i.e. *A. epos* and *G. morrilli*.

The type of monitoring data we collect at each site is listed below. We are using CDFA's basic monitoring protocol with modifications. Note that we have three replicated sampling plots at our Mission Viejo and San Juan Capistrano sites, two at UC Riverside Field 7H, only one at Irvine (because the site is too small for two), and six at Temecula. For 2006, we are releasing and sampling in one sampling plot per site in order to maximize chances of detecting establishment of newly released parasitoids.

1. Sticky traps to monitor for adult GWSS levels: Use 10 yellow sticky traps in each plot to assess adult GWSS activity levels every two weeks.
2. Leaf sampling: Count and collect the number of fresh GWSS egg masses on 10 leaves collected from the end of branches on each of 10 trees in each plot every two weeks. In contrast to method three, this is intended to return a less-biased estimate of GWSS egg mass levels. Old egg masses are counted, but not collected. The egg mass sampling is mainly intended to estimate recent GWSS egg mass levels and to serve as a means of collecting egg masses for parasitoid rearing.
3. Time search for GWSS egg masses: Do six two-minute time searches near the center of each plot every two weeks, looking for, counting, and collecting viable (new) GWSS egg masses. Continue sampling an additional 30 minutes until a minimum of five egg masses are found from methods two and three combined.
4. Parasitoid emergence data: Using egg masses collected in methods two and three (aim for 5-10 egg masses per date if possible), return egg masses to the lab and rear out and identify parasitoid species that are present.

RESULTS
Based on discussions with our CDFA cooperators, we have made several changes in project objectives, experimental design, and methodologies because of low levels of GWSS at several initial monitoring sites, changes in the species / strains of parasitoids CDFA has reared and released, the number of parasitoids they have been able to produce over this past year (this has been a very difficult year as far as rearing GWSS egg masses which are the cornerstone of the rearing program), and what makes practical sense within an applied management program (Shea et al. 2002) given advances in our knowledge regarding *Gonatocerus* species and the new strain of *A. epos* from Minnesota (see below).

To briefly summarize our research activity to date, we have monitored parasitoid activity at a total of 13 sites in southern California. Three sites were dropped because GWSS and parasitoid activity were too low (Mecca 1 and 2, BC = UC Riverside Biological Control grove), two were dropped when the grove was sold and the grower turned off the water for over a month resulting in about one-half of the trees dying (Temecula 2 and 3), two were dropped because the organic grower did not control weeds and let the Argentine ant population get completely out of control resulting in a crash in GWSS egg mass levels (Temecula 1 and 4), and one was dropped when we decided to switch to lemon blocks at all sites (Crafton Hills was navel orange). At present, we have six sampling / parasitoid release sites, three in the coastal area (Irvine, Mission Viejo, San Juan Capistrano) and three in the interior area (Corona, UC Riverside Field 7H, and Temecula).

2005 Parasitoid data
In 2005, we made a total of 98 collections from 13 different field sites. Out of a total of 2,647 parasitoids recovered, 61.9% (1639) were *G. walkerjonesi*, 29.5% (782) *G. ashmeadi*, 4.5% (120) *Ufens* spp. (either *U. principalis* or *U. ceratus*), 2.5% (66) *G. novifasciatus*, 1.2% G. sp. (32) (identity could not be determined due to specimen condition), and 0.3% (8) were *G. triguttatus*. This latter species was collected only once at a single site.

2006 Parasitoid data
A total of 3,610 *G. morrilli* have been released in 2006 at four of our release sites (UC Riverside Agricultural Operations, Irvine, Mission Viejo, and San Juan Capistrano). Due to limitations in how many of these parasitoids can be reared for release, we have not yet released this species at the Corona or Temecula sites.

A. epos has proven difficult for CDFA to rear and we have also experienced problems rearing this species in one of the two colonies at UC Riverside (it is doing very well on the second floor insectary room but not at all well on the third floor quarantine room). Additional work is needed to determine why this species is difficult to rear in some cases but not others. To date, we have made only a single release of *A. epos* at each of two release sites (180 wasps on 5/17/06 at Agricultural Operations, 300 wasps on 5/25/06 at Irvine).

We are only part way through our 2006 survey at the six parasitoid release sites (parasitoids are still being reared out from egg masses collected in early September) but so far we have recovered 595 parasitoids in total. Within the three interior sites, our best site by far is Agricultural Operations and both the Corona and Temecula sites are yielding few GWSS egg masses with minimal parasitoid diversity (we have recovered only *G. ashmeadi* at these two sites). A key finding is a single

G. morrilli recovered at Agricultural Operations on 8/22/06, only 35 days after it was first released at this site on July 18, 2006.

Among the three coastal sites, all three sites started out quite strong (good GWSS egg mass and parasitoid recovery) but Argentine ants have taken over at the Irvine site despite our instituting a very aggressive ant baiting program. This site is an organic lemon grove and thus we were restricted to using boric acid bait stations as the only organically approved treatment. A bait station was placed under each of 31 sample trees and despite baiting continuously for 16 weeks so far, we have not caused a significant reduction in Argentine ant levels (compared to the level of ants in 16 untreated control trees at the end of the block). Argentine ants feed on GWSS egg masses and disturb nymphs and adults. GWSS egg mass levels have dropped at this site and because non-organic treatments cannot be used (e.g., a very effective chlorpyrifos ground spray), we may have to abandon this site.

The coastal parasitoid data is an interesting contrast to data from the interior region. *G. ashmeadi* (only 5.7% of 350 parasitoids recovered so far) is present on the coast but the most common species are *G. walkerjonesi* (67.4%) and *G. morgani* (25.1%). We have also seen low numbers of *G. novifasciatus* (6.0%) and *Ufens* spp. (1.7%) on the coast.

CONCLUSIONS

Despite 2005 releases of *G. fasciatus* and *G. triguttatus*, limited numbers of these species have been recovered. The latter species is still produced and released as some success in recoveries has occurred in past years. The CDFA facility in Riverside has ceased producing and releasing *G. fasciatus* as no recoveries have been recorded in the past two years. We find it interesting that we are recovering so many *G. walkerjonesi* in the coastal region whereas a common impression held by many is that *G. ashmeadi* predominates in California. We believe this is because many biological control researchers have worked in the Riverside and other interior areas but relatively few have studied GWSS egg parasitoids in coastal regions. It is not surprising that species levels vary with geographic region and climate. We are very encouraged by recovering *G. morrilli* from field samples only 35 days after it was first released and believe it is much too early to be discouraged by our not yet recovering *Anagrus epos*. It will be important to continue these studies an additional year once greater numbers of *A. epos* have been released in the field. In particular, we are hoping *A. epos* may show up early in the year on first generation eggs of GWSS when *Gonatocerus* egg mass parasitism is generally quite low.

As a consequence of these and similar studies undertaken by the CDFA, a greater investment is being made toward the production, release, and monitoring of *G. morrilli*. This species is currently the second most produced biological control agent by the CDFA in both of its production facilities and it is being released over a range of environments including urban, organic, coastal, and inland locations.

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FUNDING AGENCIES

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THE *ANAGRUS EPOS* COMPLEX: A LIKELY SOURCE OF EFFECTIVE CLASSICAL BIOLOGICAL AGENTS FOR GLASSY-WINGED SHARPSHOOTER CONTROL

Project Leaders:
Joseph G. Morse
Department of Entomology
Universiey of California
Riverside, CA 92521

Richard Stouthamer
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:
Serguei V. Triapitsyn
Department of Entomology
University of California
Riverside, CA 92521

David J. W. Morgan
CDFA
Mount Rubidoux Field Station
Riverside, CA 92501

Jonathan M. Lytle
Department of Entomology
University of California
Riverside, CA 92501

Rodrigo Krugner
Department of Entomology
University of California
Riverside, CA 92501

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT

The purpose of the work planned in this project is to determine whether the "Minnesota strain" of the mymarid, *Anagrus epos* Girault, we have in culture on the glassy-winged sharpshooter (GWSS) is the same species as *A. epos* strains previously released in California, how it compares with other "*A. epos"* strains, and whether there are other strains of "*A. epos"* that should be imported for biological control of GWSS. Without understanding what species we have and how the Minnesota strain is related to similar strains, it is difficult to know how to proceed in selecting strains of this species to culture for mass-rearing and release in California for GWSS control. Concurrently, we will evaluate field releases and establishment of the Minnesota *A. epos* strain at release sites in southern California.

INTRODUCTION

Anagrus epos is a common and seemingly widespread egg parasitoid of leafhoppers (Cicadellidae) in North America. It was first described from a collection in Illinois in 1911 (Girault 1911). Location records for this species also include Colorado, Kentucky, New Mexico, and New York in the U. S. as well as Baja California and Sonora in Mexico (Triapitsyn 1998). While commonly collected as a parasitoid of grape leafhopper species (*Erythroneura* spp.), a recent collection of *A. epos* from the egg mass of the sharpshooter genus *Cuerna* in Minnesota was the first time this species had been collected from a sharpshooter species (Hoddle & Triapitsyn 2004). Wasps from this collection have been reared continuously since June 2004 in the University of California Riverside (UCR) Quarantine facility on eggs of the glassy-winged sharpshooter (GWSS). This species is particularly promising for application in the biological control of GWSS because it is a gregarious species and fourteen or more wasps emerge from each egg. Another apparent advantage of this species is that it will also parasitize the eggs of several other leafhopper species (R. Krugner, unpublished data), thus allowing it to expand its numbers even at times of the year when GWSS eggs are not present. We also expect this strain may do quite well in the colder regions of central and northern California based on where it was collected.

Like many minute parasitoids, identification to species in this group is exceedingly difficult because of the lack of adult morphological features. Species identifications have been made using light microscopy to determine the presence of key morphological features for *A. epos.* A recent taxonomic revision of the genus *Anagrus* associated with vineyards in North America (Triapitsyn 1998) has shown that: 1) more species are present than previously thought; 2) some species have a very wide geographic distribution; and 3) relatively few morphological characters are available for distinguishing these species, leaving several authors to think that *A. epos* is not a single species but a complex of different species (e.g., Pickett et al. 1987). The morphological characters that are used for differentiating closely related *Anagrus* spp. can be variable and thus, species limits are often difficult to assess without supporting data from their biology and from DNA sequences. Due to limitations on what is practical (economically) to rear and mass-release and also because of restrictions on importing and releasing exotic parasitoids in California without understanding their taxonomy, we feel we must better understand this species complex. We intend to use three approaches to determine the species identity of different *A. epos* populations: (1) reassess key morphological features using scanning electron microscopy (SEM) to determine if subtle morphological differences exist between *A. epos* populations which could indicate species differences (Dr. Triapitsyn will conduct this work); (2) conduct mating compatibility studies to determine if different populations of *A. epos* are reproductively isolated, or if mating occurs, whether offspring from different strains are viable, thereby defining species groups on the basis of successful interbreeding (Ph.D. student John Lytle working with Dr. Morse); (3) determine if molecular differences exist between *A. epos* populations collected from different regions by comparing mitochondrial and ribosomal DNA sequences

(Dr. Stouthamer). Molecular dissimilarities generally indicate the existence of different species. Results from these three methods of investigation (morphology, behavior, and genetics) will be evaluated together to establish the identity of the species in the *A. epos* complex. Once the different species have been determined, we will test them for their suitability in the biological control of GWSS using laboratory studies and field release evaluations (Dr. Morgan and Dr. Morse).

OBJECTIVES

1. Examination of male and female *A. epos* complex populations for unique morphological characters
2. Molecular characterization of mitochondrial and ribosomal DNA *of A. epos* populations
3. Mating compatibility studies between *A. epos* strains
4. Field release and evaluation of the "Minnesota strain" of *A. epos*

RESULTS

Progress on Objectives 1-3

Our revised experimental design is to collect dead specimens of various *A. epos* strains and related species for taxonomic examination (Objective 1) and genetic work (Objective 2) in years 1-2. Objective 3 is scheduled for year 3 once we have the results of Objective 1 and 2 research, which will tell us which strains of *A. epos* to concentrate on other than the Minnesota strain we currently have in culture on GWSS egg masses.

Triapitsyn (1998) re-described *A. epos* from the type material and other specimens collected in Centralia, IL, and also indicated its additional distribution in North America (Mexico: Baja California, Sonora; USA: Colorado, Illinois Kentucky, New Mexico). In CO and NM, it is a parasitoid of *Erythroneura* leafhoppers on grapes; also indicating that morphologically, it is a variable species (and thus possibly a complex of several cryptic species). The specimens from Minnesota are within this variation range and are possibly also members of such a complex. The species closely related to *A. epos* are *Anagrus daanei* S. Triapitsyn (Canada: British Columbia; USA: California, Michigan, New York, Washington) and *Anagrus tretiakovae* S. Triapitsyn (Mexico: Baja California, Coahuila; USA: Arizona, Delaware, Illinois, Michigan, Maryland, New Mexico, New York, Washington); in AZ and NM (and Mexico), *A. tretiakovae* is a known parasitoid of *Erythroneura* leafhoppers on grapes.

Collection Material

See our 2005 progress report (Morse & Stouthamer 2005) for a listing of *Anagrus* spp. collections made in summer 2005. Additionally, during fall 2005, we obtained large numbers of *A. daanei* from Fresno Co. (courtesy of K. Daane and G. Yokota, UC KAC) and *A. tretiakovae* (reared at UCR quarantine from eggs of *Erythroneura* spp. on grape leaves, collected in Albuquerque, New Mexico by S. Triapitsyn). In 2006, the following collections were made: *A. daanei* from Washington (courtesy of L. Wright, Washington State University, Prosser), *A. erythroneurae* Trjapitzin & Chiappini from Oasis and Temecula, California (reared from eggs of *Erythroneura variabilis* Beamer on grapes), and *A. epos* from Grand Junction and Palisade, Colorado (reared by S. Triapitsyn from eggs of *Erythroneura vulnerata* Fitch on grapes and also from eggs of *E. ziczac* Walsh on Virginia creeper). Thus, all necessary collections for this study have been made, with the exception of *A. epos* from Sonora, Mexico, where all the grapes were treated with insecticides against the vine mealybug, resulting in elimination of *Erythroneura variabilis* leafhoppers there. Luckily, there are enough preserved *A. epos* vouchers stored in a freezer at the UCR Entomology Research Museum, reared by S. Triapitsyn in 1994 in Sonora from eggs of *E. variabilis* on grapes. These were successfully sequenced in Dr. Stouthamer's lab, and also were used for morphological studies.

Morphological Studies

Scanning electron micrographs (SEMs) of the antennae and bodies were taken for the following specimens: *A. epos* (Grand Junction, Colorado), *A. epos* (Sonora, Mexico), and *A. epos* (Minnesota origin). Digital photographs (using the Automontage system) of the antennae, forewings, and bodies were taken for the following specimens: *A. epos* (Grand Junction, Colorado), *A. epos* (Sonora, Mexico), *A. epos* (Minnesota origin), and *A. epos* (Illinois). Certain body part measurements were taken from the following specimens: *A. epos* (Grand Junction, Colorado), *A. epos* (Sonora, Mexico), *A. epos* (Minnesota, both original and CA progeny), and *A. epos* (Illinois). Morphometric studies of these specimens are now underway and should be completed shortly.

Specimens and DNA Preparation

Nine *Anagrus* species were obtained from 15 collection sites for molecular identification (Table 1). Two individuals from each population were chosen to prepare template DNA by using 5% Chelex-TE solution. Each individual was ground in 45 ul 5% Chelex solution and then five ul of proteinase K were added in a 0.6 ml centrifuge tube. The mix was incubated at 55°C for one hr and 99°C for 10 min.

PCR Methods and Results

PCR was performed to yield the 28sD2 (ribosomal cistron) and CO1 (mitochondrial gene) regions with template DNA. Reaction conditions for the 28sD2 region were three min at 94°C, followed by 30 cycles of 45 sec at 94°C, 30 sec at 55°C, 90 sec at 72°C, and a final extension for three min at 72°C. Reaction conditions for the CO1 region were three min at 94°C, followed by 34 cycles of 45 sec at 94°C, 30 sec at 43.5°C, 90 sec at 72°C, and a final extension for five min at 72°C. We

were unable to obtain the CO1 region from the species. Therefore only the 28sD2 region was further treated. Specimens showing weak or no bands were excluded from sequencing. Thus, one PCR amplicon per group was sequenced.

The species of the genus *Anagrus* are very small and lack easy morphological characters that can be used for identification. The D2 sequence has been shown to be a sequence that is quite conserved within a species but is different between species. The work here illustrates the preponderance of species that would morphologically be classified as *A. epos*, but are different species (new species one, two, and three, each different from the MN "*A. epos*" and each other) whereas species four morphologically resembles *A. daanei*.

Table 1. *Anagrus* species used for molecular identification.

Collection	Genus	Species	Collection site
1	*Anagrus*	*epos*	UCR culture, originally collected near Glyndon, Clay Co., MN, 2004
2	*Anagrus*	*nr epos new species 1*	Campo Experimental INIFAP, Sonora, Mexico, 1994
3	*Anagrus*	*nr epos new species 1*	Near Caborca, Sonora, Mexico, 1994
4	*Anagrus*	*nigriventris*	UCR, Riverside, Riverside Co., CA, 2004
5	*Anagrus*	*daanei*	Kingsburg, Fresno Co., CA, 2005
6	*Anagrus*	*erythroneurae*	WSU-Prosser Research Center, Prosser, Benton Co., WA, 2005
7	*Anagrus*	*erythroneurae*	Oasis, Coachella Valley, Riverside Co., CA, 1994
Rearing in progress	*Anagrus*	*erythroneurae*	Temecula, Riverside Co., CA, 2006
8-10, 12	*Anagrus*	*tretiakovae*	Albuquerque, Bernalillo Co., NM, 2005
11	*Anagrus*	*tretiakovae*	Pavich vineyard, Harquahala Valley, Maricopa Co., AZ, 1994
13	*Anagrus*	*nr epos new species 2*	Grand Junction, Mesa Co., CO, 2006 (ex. *Erythroneura vulnerata* eggs)
14	*Anagrus*	*nr epos new species 3*	Palisade, Mesa Co., CO, 2006 (ex. *Erythroneura ziczac* eggs)
15	*Anagrus*	*nr daanei new species 4*	WSU-Prosser Research Center, Prosser, Benton Co., WA, 2006

Progress on Objective 4

We have initiated monitoring of endemic and released parasitoids of GWSS at each of six field sites in southern California (for details see the progress report in this Proceedings by Morse, Morgan, and Lytle). CDFA has had trouble rearing *A. epos*, so far we have made only two releases, of 300 and 180 *A. epos,* respectively at a single coastal and interior site using wasps from the UCR's colony (5/17/06 interior; 5/25/06 coastal), but we hope that CDFA will be able to produce wasps that can be released at additional sites in October 2006. So far we have not recovered *A. epos* from the two release sites but we feel that more than a single release may be needed to allow establishment, so it is difficult to know at this point what a failure to recover specimens means.

CONCLUSIONS

Genetic analyses have confirmed our hypothesis that there are cryptic species hidden within specimens which morphologically appeared to be identical *A. epos*. In addition, what was thought to be *A. daanei* in Washington appears to be a different species from the California *A. daanei*. Given these genetic results in hand, it will be interesting to see if morphometric and SEM examination can differentiate between these cryptic species. Project cooperators plan to meet at the Pierce's disease symposium in San Diego to discuss our next steps. Obviously, field release and sampling should continue with the Minnesota strain *A. epos* to see what impact it may have on GWSS. If permits can be obtained allowing us to do so, we would also like to take GWSS egg outplants to Colorado to determine if *A.* n. sp nr *epos* two and three will parasitize GWSS eggs (these would be shipped back to the UCR's quarantine facility to allow parasitoids to emerge for confirmatory genetic analysis).

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LABORATORY AND FIELD EVALUATIONS OF NEONICOTINOID INSECTICIDES AGAINST THE GLASSY-WINGED SHARPSHOOTER

Project Leaders:
Nick Toscano
Dept of Entomology
University of California
Riverside, CA
nick.toscano@ucr.edu

Frank Byrne
Dept of Entomology
University of California
Riverside, CA

Cooperators:
Carmen Gispert
UC Cooperative Extension
Riverside, CA
cgispert@ucdavis.edu

Ben Drake
Drake Enterprises
Temecula, CA

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ABSTRACT
Imidacloprid is still the most widely used neonicotinoid for the protection of grapevines against glassy-winged sharpshooter (GWSS) feeding and Pierce's disease (PD) transmission. This insecticide has now entered the generic age and within the past year, several new formulations of imidacloprid have been launched onto the market. To assist with grower acceptance of these new formulations, we are currently evaluating the uptake of different products in table and wine grapes. Bayer CropScience introduced Admire Pro to replace their original Admire 2F formulation. In Coachella Valley, the imidacloprid uptake profiles for vines treated with either Admire Pro or Admire 2F were similar, with peak uptake occurring within four days. In a further study, the uptake profile for Admire 2F was also consistent with a second soluble concentrate formulation (Nuprid 2F, marketed by Nufarm Americas Inc.).

We evaluated the performance of the neonicotinoid thiamethoxam (applied as Platinum) at three rates of application in a Temecula Valley wine grape vineyard. The concentrations of thiamethoxam in xylem fluid extracts were highest at the top application rates, and would provide good protection to vines against a sharpshooter infestation.

INTRODUCTION
Effective vector management through the use of the neonicotinoid insecticide, imidacloprid, has played a pivotal role in suppressing glassy-winged sharpshooter (GWSS) populations in California vineyards and citrus orchards (Castle et al., 2005; Byrne and Toscano, 2006). This in turn has greatly decreased the incidence of new Pierce's disease (PD) outbreaks in vineyards. With the expiry of the imidacloprid patent, there are now more formulations of this active ingredient becoming available to growers. To assist with grower confidence in the new products, we are evaluating their performances by measuring the uptake into vines (table and wine grapes) by extracting xylem fluid and quantifying the insecticide concentrations therein.

There are several insecticides within the neonicotinoid class with good systemic activity and each has its own distinct chemical properties that influence the efficacy with which the insecticide will work in the field. Systemic insecticides are commonly applied to vines through drip irrigation systems. This type of application is designed to deliver the insecticide close to the roots of the vines where more effective uptake into the plant xylem system can occur. In this way, systemic insecticides can directly exploit the xylophagous feeding behavior of the sharpshooter. Distribution of the insecticides within the plant xylem system can also provide more effective coverage of sharpshooter feeding sites and better persistence compared with foliar applications of the same product. As the number of available neonicotinoids increases, it is important to continue research efforts in order to better understand their behavior in California vineyards and to optimize their use by growers. Our studies in Coachella and Napa, for example, have shown that imidacloprid does not work consistently under all conditions experienced in California vineyards (Toscano and Byrne, 2005; Weber et al., 2005). We have, therefore, established a research program to examine the behavior of the different neonicotinoid insecticides within California vineyards.

In this report, we provide data on (1) the uptake and persistence of imidacloprid applied as different formulations, and (2) the impact of different rates of Platinum application on the uptake of thiamethoxam into grapevines.

OBJECTIVES
1. Determine the impact of soil type and irrigation on the uptake and residual persistence of neonicotinoid insecticides.
2. Develop an ELISA for the detection and quantification of dinotefuran residues within plant tissues.
3. Determine the uptake and persistence of imidacloprid, thiamethoxam and dinotefuran in grapevines in order to maximize protection of vineyards.

RESULTS

Evaluation of Imidacloprid Formulations

We evaluated the uptake of imidacloprid applied as Admire Pro, Admire 2F and Nuprid 2F (Figure 1). Admire Pro was introduced by Bayer CropScience to replace Admire 2F, while Nuprid 2F was introduced by Nufarm Americas Inc to compete for the Admire 2F market. Nuprid 2F and Admire 2F are both formulated as soluble concentrates.

In a study conducted at a Coachella Valley vineyard (two year old Superior), the profiles for the uptake and persistence of imidacloprid applied as either Admire Pro or Admire 2F were similar. Peak levels within the xylem fluid were reached within four days and persisted within the vines at sharpshooter threshold levels of 10 ppb for approximately 30 days.

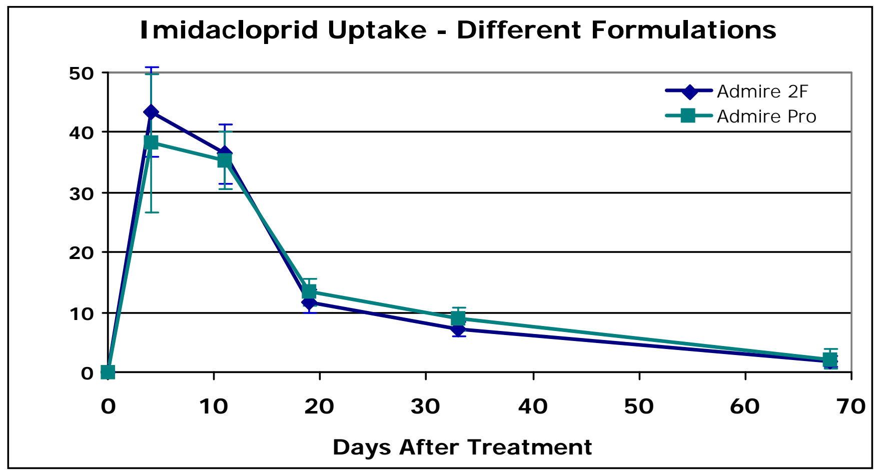

Figure 1. Uptake of imidacloprid applied as Admire Pro (7 fl oz/acre) and Admire 2F (16 fl oz/acre) to two-year old Superior table grapevines in a Coachella Valley vineyard. Although the product application rates were different, they deliver the same amount of active ingredient to the vines. Each point represents the mean (± SEM) for six vines.

In a second Coachella Valley vineyard (20 year old Perlettes), we compared the uptake of imidacloprid applied as either Admire 2F or Nuprid 2F. The uptake and persistence profiles for both products tracked each other well (Figure 2). Although the target thresholds for sharpshooter mortality were reached within five days, the peak uptake was not observed until about 12 days after the applications were made. The differences in uptake dynamics between the two sites (Figures 1 and 2) are likely to reflect differences in vine age.

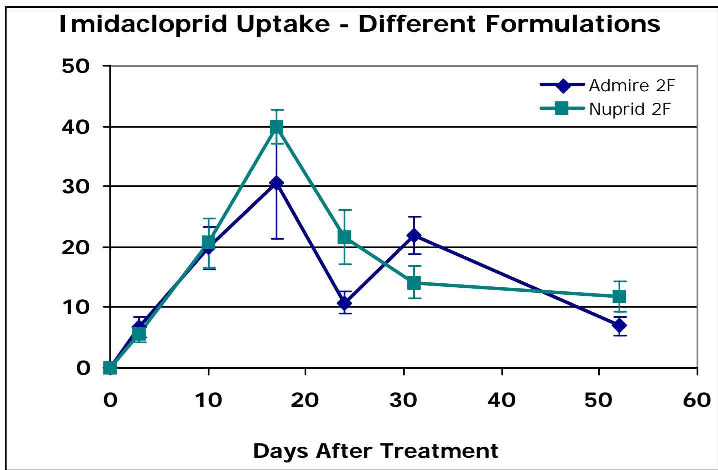

Figure 2. Uptake of imidacloprid applied as Admire 2F and Nuprid 2F to 20-year old Perlette table grapevines in a Coachella Valley vineyard. Each product was applied at 16 fl oz/acre. Each point represents the mean (± SEM) for 6 vines.

In a wine grape vineyard in Temecula Valley, we compared the uptake of Admire Pro and Admire 2F applied by chemigation. The profiles of imidacloprid uptake for both products tracked each other very well (Figure 3). The initial rate

of uptake was disappointing; however, the study was conducted in a commercial vineyard under normal operating practices. Water was minimal for several weeks following the initial application. The major peak in uptake coincided with increased water usage during a very hot period in the local weather. The data reinforce our earlier affirmation that irrigation is absolutely necessary to drive the imidacloprid into the vines.

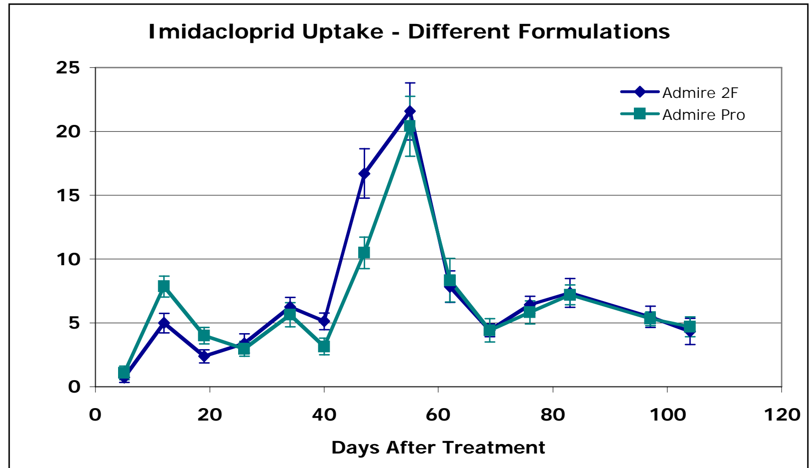

Figure 3. Uptake of imidacloprid applied as Admire Pro (7 fl oz/acre) and Admire 2F (16 fl oz/acre) to seven-year old Cabernet wine grapevines in a Temecula Valley vineyard. Although the product application rates were different, they deliver the same amount of active ingredient to the vines. Each point represents the mean (± SEM) for 16 vines.

We assessed the uptake of thiamethoxam (Platinum) applied by chemigation at three rates (Figure 4). The detection of thiamethoxam within extracts of xylem fluid was again determined by the frequency of irrigation, with peaks in concentrations matching the water usage. The 8 fl oz application rate provided an average of least five ppb thiamethoxam throughout the assessment period. This concentration of insecticide should provide good protection to vines against the glassy-winged sharpshooter given the two-fold greater level of toxicity of thiamethoxam compared to imidacloprid (Byrne and Toscano, 2004).

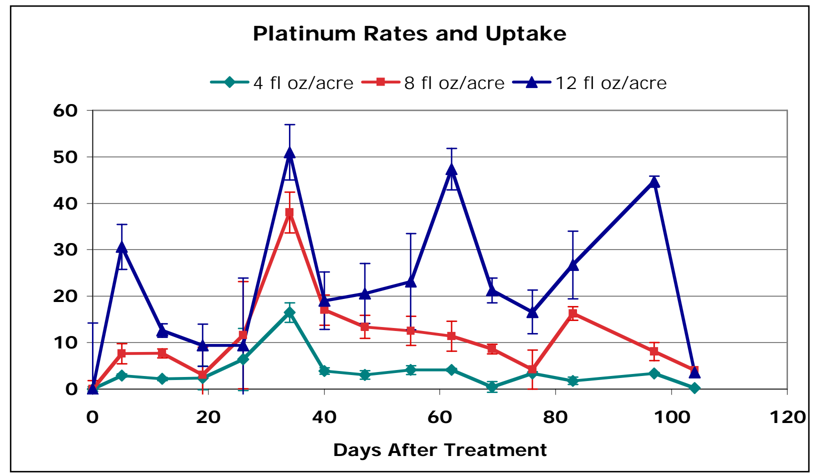

Figure 4. Uptake of thiamethoxam applied as Platinum to seven-year old Cabernet wine grapevines in a Temecula Valley vineyard. Each point represents the mean (± SEM) for 8 vines.

CONCLUSIONS

Management of sharpshooter populations is key to minimizing the spread of PD. The neonicotinoids have been effective at achieving area-wide management of this important disease vector, resulting in a dramatic decrease in the incidence of PD. In our studies, the newer imidacloprid formulations performed equally well in both table and wine grape vineyards. Thus, growers can be confident that, if correct application guidelines are adhered to, the use of generic formulations of imidacloprid will provide them with continued success in their efforts at managing the glassy-winged sharpshooter.

The use of Platinum in vineyards looks extremely promising. At application rates lower than those for the imidacloprid formulations, there was excellent uptake and persistence of thiamethoxam in the xylem system at levels toxic to GWSS. Applications of Platinum at rates much lower than the maximum allowable label rate will also minimize potential residue problems that were a concern to us in our previous studies.

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

Project Leaders:
Nick C. Toscano
Department of Entomology
University of California
Riverside, CA 92521
nick.toscano@ucr.edu

Carmen Gispert
University of California
CooperativeExtension
Indio, CA 92201
cgispert@ucdavis.edu

Operations Coordinator:
Gregory Ballmer
Department of Entomology
University of California
Riverside, CA 92521
Gregory.ballmer@ucr.edu

Cooperators:
John Snyder
Riverside County
Riverside, CA 92502
jsnyder@co.riverside.ca.us

Robert Mulherin
Riverside County
Riverside, CA 92502
rmulherin@co.riverside.ca.us

Reporting Period: The results reported here are from work conducted October 2005 through September 2006.

ABSTRACT

Riverside County has two general areas where citrus groves interface with vineyards, the Coachella and Temecula Valleys. The Coachella Valley with 10,438 acres of table grapes in proximity to 12,000 acres of citrus and the Temecula Valley with 2,000 acres of wine grapes in proximity to 1,600 acres of citrus are vulnerable to Pierce's disease (PD), *Xylella fastidiosa* (*Xf*). The grapes in the Coachella and Temecula areas of Riverside County are in jeopardy because of the glassy-winged sharpshooter (GWSS), the vector of the PD bacterium, build up in adjacent citrus groves. Citrus is an important year around reproductive host of GWSS in Riverside County, but also one that concentrates GWSS populations over the winter months during the time that grapes and many ornamental hosts are dormant. GWSS weekly monitoring in citrus and in grapes began in March 2000 in Temecula Valley and 2003 in Coachella Valley by trapping and visual inspections. Systemic insecticides such as Admire (imidacloprid), gave excellent control. Coachella Valley GWSS populations have increased since the treatment program was initiated in 2003 but have declined substantially relative to the pre-action levels due to insecticide applications.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) vectors a bacterium that causes Pierce's disease (PD). This insect and bacterium are a severe threat to California's 890,000 acres of vineyards and $30 billion dollar industry. An area-wide GWSS management program was initiated in Temecula in 2000 to prevent this vector's spread into other California grape growing regions. In Temecula valley itself, the wine grape industry and its connecting tourist industry generate $100 million of revenue for the economy of the area. GWSS/PD caused a 30% vineyard loss and almost destroyed the connected tourist industry. The area wide GWSS management program initiated in the spring of 2000 saved the industry from a 100% loss. Only a continuation of an area-wide GWSS management program will keep the vineyards viable in Temecula. The table grape industry in the Coachella Valley is represented by 10,465 acres of producing vines, which generate fresh market grapes valued at an average of over $110 million annually. The GWSS was identified in the Coachella Valley in the early 1990's. Population increases of this insect in Coachella Valley in the last three years have increased the danger of PD occurrence in this area, as has occurred in similar situations in the Temecula and San Joaquin Valleys. In July 2002, the occurrence of *Xylella fastidiosa (Xf),* the PD bacterium, was found in 13 vines from two adjacent vineyards in the southeastern part of the Coachella Valley. With this discovery, and the increasing GWSS populations, there was and is a real need to continue an area-wide GWSS/PD management program, to prevent an economic disaster to the work forces and connect small businesses of Mecca, Thermal, Coachella, Indio, etc. that depend upon the vineyards for a big portion of their incomes. Only a continuation of an area wide GWSS/PD management program will keep the vineyards viable in Coachella. At present there are no apparent biological or climatological factors that will limit the spread of GWSS or PD. GWSS has the potential to develop high population densities in citrus. Insecticide treatments in citrus groves preceded and followed by trapping and visual inspections to determine the effectiveness of these treatments are needed to manage this devastating insect vector and bacterium. Approximately 1,750 acres of citrus in Riverside County were treated for GWSS in February through July of 2006 between a cooperative agreement with USDA-APHIS and the Riverside Agricultural Commissioner's Office under the "Area-Wide Management of the Glassy-Winged Sharpshooter in the Coachella and Temecula Valleys." The cost of the 2006 Riverside County GWSS treatments was close to $700,000. This is down from the 5,200 acres treated in 2005 at a cost of $1,000,000.

OBJECTIVES

1. Delineate the areas to be targeted for follow-up treatments to suppress GWSS populations in the Temecula and Coachella Valleys for 2006.
2. Determine the impact of the 2005 GWSS area-wide treatments to suppress GWSS populations in citrus groves and adjacent vineyards.

RESULTS AND CONCLUSIONS

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

The GWSS/PD citrus groves and vineyards within the GWSS/PD management areas were monitored weekly to determine the need and effect of insecticide treatments on GWSS populations. Yellow sticky panel traps (7 x 9 inches) were used to help determine GWSS population densities and dispersal/movement within groves and into vineyards (Figures 1 and 2). A total of 986 GWSS yellow sticky panel traps are monitored weekly. Based on trap counts and visual inspection, approximately 1,500 and 250 acres of citrus were treated in Coachella and Temecula, respectively, for GWSS control in 2006. In Temecula and Coachella Valley treatments for GWSS in citrus were initiated when at least one to two GWSS adults were found at the same trap location for two consecutive weeks. In Temecula Valley, only the citrus where the GWSS were found was treated. In Coachella Valley, all citrus located within a 0.5 mile radius from the trap finds were treated as a preventive measure to protect surrounding groves. The decision to treat more area from GWSS finds in Coachella than what was treated in Temecula differed because of terrain, urban development and the history of GWSS blow-ups in Kern County and Temecula Valley the fourth year after GWSS area-wide programs were initiated. Approximately 91% of the citrus was treated with a single application of Alias (imidacloprid) at 36 ounces per acre. Organically grown citrus (9%) was treated with PyGanic (1.4% Pyerthrins) at 7 pints per acre. In most areas where PyGanic was used to manage GWSS, follow up treatments of PyGanic were applied one month after the first application for two consecutive months.

For a successful area-wide GWSS management program with large acreages of citrus, a management program has to be initiated. Organic insecticides are not as effective as the neonicotinoid insecticides, such as imidacloprid, for controlling GWSS. Therefore, organic insecticides will have to be applied more frequently than their synthetic counterparts. In our Riverside County GWSS area-wide program, organic citrus groves pose challenges to area-wide GWSS management programs (Figure 3).

FUNDING AGENCIES

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

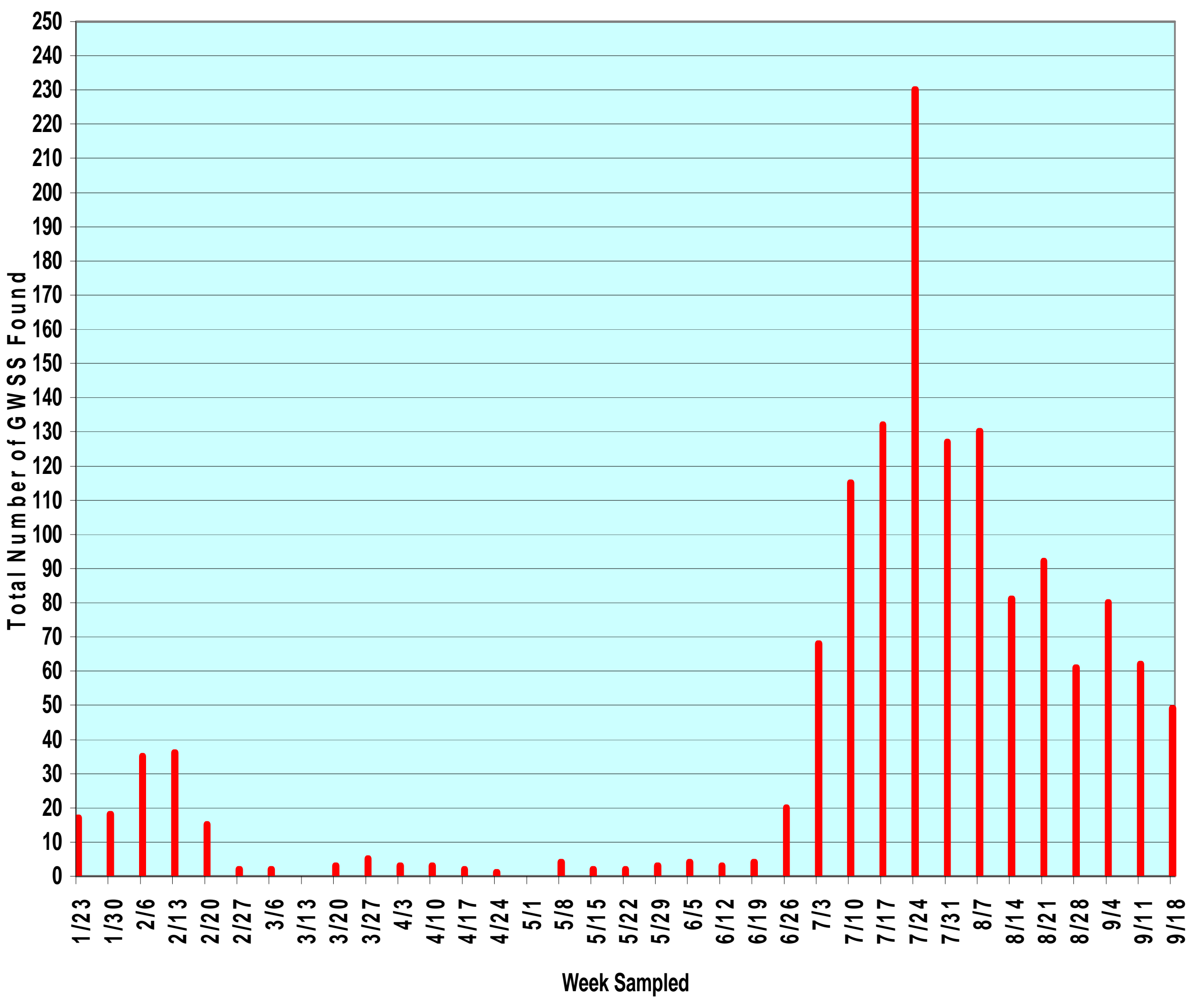

Figure 1. In 2006, high numbers of adult GWSS were caught on the yellow sticky panel traps in Temecula, with populations peaking in July, reaching a total of 230 trapped.

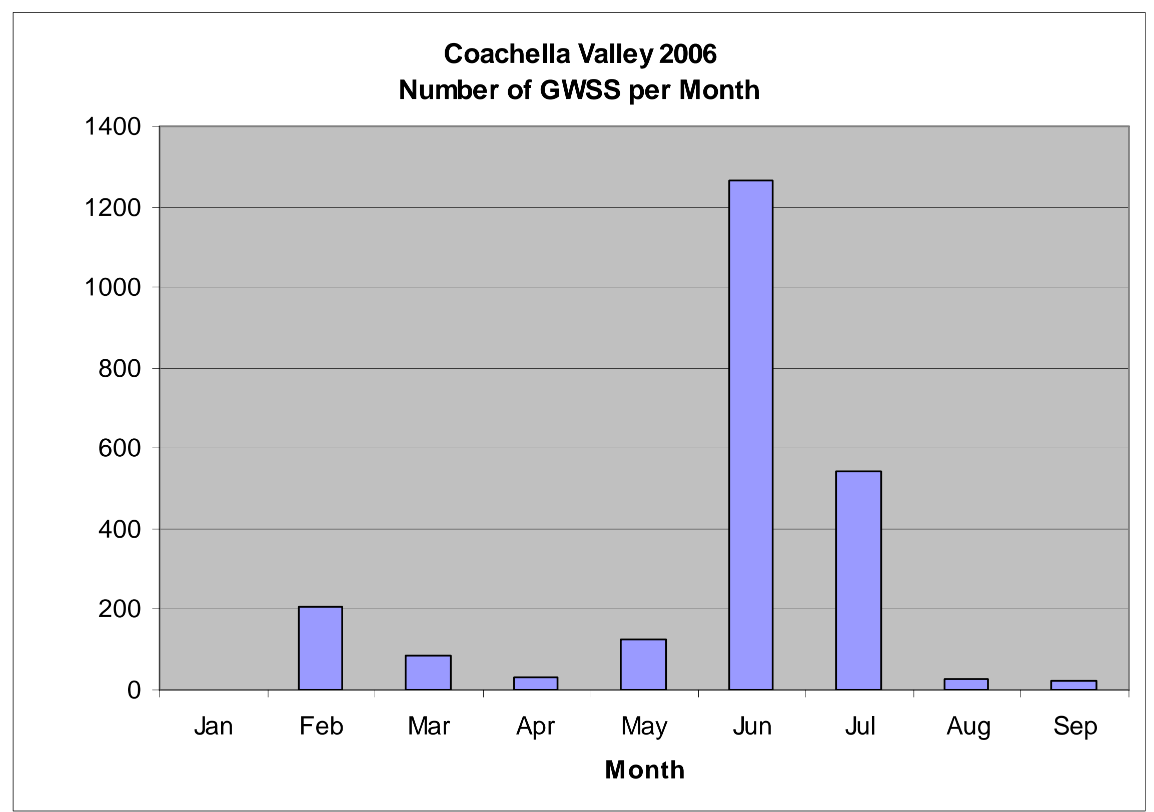

Figure 2. GWSS populations in Coachella Valley peaked in June 2006 with a high of 1,266 trapped.

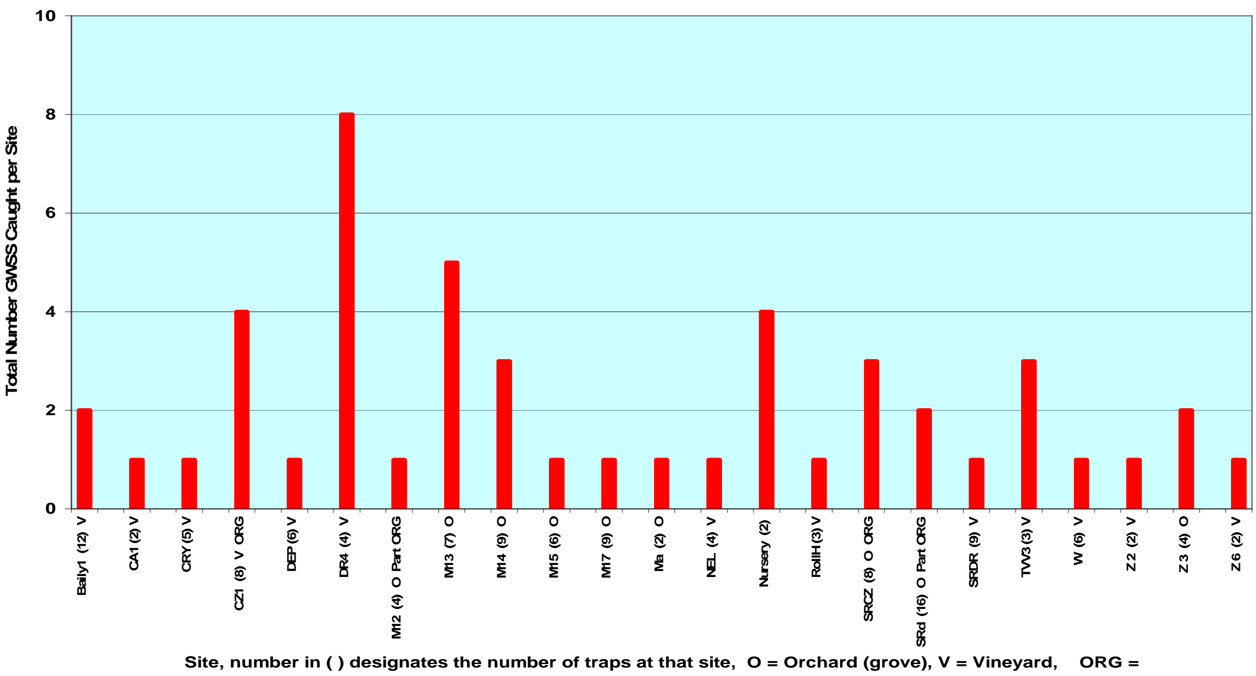

Figure 3. Temecula GWSS adults caught for the week of September 18, 2006.

COMPATIBILITY OF SELECT INSECTICIDES WITH NATURAL ENEMIES OF THE GLASSY-WINGED SHARPSHOOTER AND OTHER PESTS

Project Leaders:

Nick. C. Toscano
Department of Entomology
University of California
Riverside, CA 92521
nick.toscano@ucr.edu

Joseph. G. Morse
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:

N. Prabhaker
Department of Entomology
University of California
Riverside, CA 92521

Steven Castle
ALARC
USDA-ARS
Maricopa, AZ 85239

S. Naranjo
ALARC
USDA-ARS
Maricopa, AZ 85239

T. J. Henneberry
ALARC
USDA-ARS
Maricopa, AZ 85239

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ABSTRACT

To evaluate the compatibility of insecticides that have both a broad and limited spectrum of activity against biological control agents, laboratory studies were carried out to compare the relative susceptibilities of seven foliar and two systemic insecticides against four common species of beneficial insects: *Aphytis melinus* Debach, *Gonatocerus ashmeadi* Girault, *Eretmocerus eremicus* Rose & Zolnerowich, and *Encarsia formosa* Gahan. Evaluations with systemic insecticides also included two species of predators, *Geocoris punctipes* Say and *Orius insidiosus* Say. Foliar insecticides were evaluated by a petri dish technique across a range of concentrations to measure their effect on direct mortality of the parasitoids. A systemic uptake bioassay technique was used to determine the toxicity of systemics against the six species of beneficials. Insecticides tested are used against citrus and agricultural pests, and included acetamiprid, imidacloprid, thiamethoxam (all 3 are neonicotinoids); chlorpyrifos, (organophosphate); bifenthrin, cyfluthrin, fenpropathrin (all 3 are pyrethroids); and buprofezin and pyriproxyfen (two insect growth regulators = IGRs). Chlorpyrifos, a conventional organophosphate insecticide with broad-spectrum activity, was consistently the most toxic pesticide to all four species of beneficial insects tested. Among the pyrethroids, fenpropathrin demonstrated lower toxicity to parasitoids compared with bifenthrin or cyfluthrin. Acetamiprid, although efficacious against GWSS, exhibited fairly selective toxic characteristics to *G. ashmeadi* and *E. eremicus* until four days post-treatment while being toxic to *A. melinus* within 24 h after treatment. *Aphytis melinus* was the most susceptible hymenopterous parasitoid to all test insecticides. Buprofezin and pyriproxyfen, with a relatively narrow spectrum of activity, were less toxic to the parasitoids. Imidacloprid has been considered to be relatively selective, with limited impact on parasitoids because of its systemic activity. However, results from this laboratory study suggest that both systemics, imidacloprid and thiamethoxam, may not be as selective against parasitoids as was expected. To understand the bioassay results with the two systemics, quantification of imidacloprid and thiamethoxam in both the parasitoids and the test citrus leaves was evaluated using ELISA kits. Additional studies are underway in the laboratory to determine how the parasitoids are exposed to these two systemic materials through various routes of exposure. Selectivity of these two compounds to natural enemies is also being examined under field conditions. The results presented here will provide pest managers with specific information on the degree to which the tested insecticides are likely to be compatible with various natural enemies.

INTRODUCTION

The current management plan for glassy-winged sharpshooter, (GWSS), *Homalodisca vitripennis*, includes the use of a number of insecticides that are quite effective (Akey et al. 2001, Bethke et al. 2001, Prabhaker et al. 2006). However, if selected insecticides are effective against GWSS while showing minimal impacts on beneficial insects, biological control can be maximized. There has been little information available on the long-term impact that different control measures are having on GWSS populations and its natural enemies on citrus and grapes. Although biological control has been the foundation of citrus IPM in California for many years, it is now threatened by the arrival of several new pests and greater use of non-selective insecticides to control these new species. In particular, the recent registration of new insecticides for use on citrus is creating uncertainty over the long-term impact they may have on established IPM programs (Grafton-Cardwell and Gu 2003). Therefore, there is a need for accurate assessment of the impact of agrochemicals on both GWSS and nontarget insects, including parasitoids and predators. Such information is essential to attain greater understanding of the various control options for GWSS in citrus and how they can be best integrated with existing, successful management programs. The overall objective of this research project is to help determine IPM compatible management tactics by focusing on chemical controls being used against GWSS and evaluating their impact upon several important biological control agents. To address this goal, the impact of selected insecticides including those that are used against GWSS and other pests on citrus was assessed against a number of common beneficial parasitoids including *G. ashmeadi* (an egg parasitoid of GWSS), *A. melinus* (an endoparasitoid of armored scale insects on citrus), *E. eremicus* and *E. formosa* (two whitefly parasitoids), and two predators, *G. punctipes* and *O. insidiosus*. The relative selectivity of insecticides was determined in the laboratory using two bioassay techniques, a petri dish bioassay for foliar insecticides and a systemic uptake bioassay for systemic insecticides

(Prabhaker et al. 2006). The insecticides evaluated in this study were selected on their basis of utility and potential use, which included four conventional compounds, chlorpyrifos, bifenthrin, cyfluthrin and fenpropathrin; two IGRs, pyriproxyfen and buprofezin; and three neonicotinoids, acetamiprid, imidacloprid, and thiamethoxam.

OBJECTIVES

1. Monitor citrus orchards in Riverside, Ventura Co., and Coachella Valley to determine the relative abundance of select parasitoids and predators before and after treatment.
2. Evaluate select foliar and systemic GWSS pesticides used on citrus and grapes for their impact on GWSS egg parasitoids such as *G. ashmeadi* and *G. triguttatus* as well as other parasitoids in the system such as A. melinus.
3. Determine if honeydew produced by homopteran insects on citrus can be contaminated with systemic insecticides such as imidacloprid and thiamethoxam.
4. Determine the impact of imidacloprid and thiamethoxam residues within plant or within plant-feeding intoxicated insects, on the survivorship of *G. ashmeadi*, *G. triguttatus*, and *A. melinus.*

RESULTS

Objective 1. Assessment of relative numbers of egg parasitoids of *H.vitripennis*.

Relative numbers of different species of egg parasitoids of *Homalodisca* spp. were assessed through collection of leaf samples infested with egg masses. The estimation process was repeated on a weekly basis at the same sites and by collecting the same number of egg-infested leaves from two hosts, citrus and willow, for a period of five months. Results showed emergence of four species of egg parasitoids, *G. ashmeadi*, *G. novifasciatus, Ufens principalis*, and *U. ceratus*. The majority of the parasitoids of *Gonatocerus* spp. were *G. ashmeadi* whereas less than 1% of total collections were *G. novifasciatus*. Large number of parasitoids emerged during the summer months compared to the fall. These results provided a picture of relative GWSS activity within each orchard in addition to providing limited information on the activity and abundance of natural enemies.

In addition to the above-mentioned method, a survey for estimation of the relative abundance of natural enemies, including parasitoids and predators that are active against GWSS and other pests in citrus orchards in Riverside Co., was done using yellow sticky traps. Yellow sticky traps were posted at multiple locations within each orchard for continuous monitoring of GWSS and natural enemies and were changed once a week. Large differences were found in the numbers of parasitoids collected on sticky traps compared to the numbers collected from GWSS egg masses in petri dishes. Data collected from the sticky traps showed a significantly lower number of parasitoids relative to GWSS (<10%). These results are in contrast to those obtained through direct observations of the numbers of parasitoids that emerged from egg masses in petri dishes, which were much higher than were GWSS immatures.

Objective 2. Toxicological responses of four species of parasitoids.

Our study has focused on non-target effects of commonly used insecticides for control of agricultural pests against beneficial insects as measured by direct toxicity. We compared the relative toxicities of seven contact insecticides and two systemics to a number of beneficial insects that are important in biological control of both citrus pests and whiteflies. Variation in susceptibility to different insecticides was observed among the different species of natural enemies. *A. melinus* appears to have a generally lower susceptibility to many insecticides tested compared to *G. ashmeadi* or *E. eremicus* (Table 1). Compared to *A. melinus*, responses of *G. ashmeadi* and *E. eremicus* were similar to the test chemicals as exhibited by lower sensitivities in general. However, some similarities in trends were also observed among the four species of parasitoids. For example, sensitivity to chlorpyrifos was highest among the seven contact insecticides for all beneficial species. Bioassay responses of *G. ashmeadi* under laboratory conditions to the two IGRs by the petri dish method generated LC_{50}'s that were higher than with the neonicotinoids (Table 1). Although, differences in responses to certain pyrethroids were observed with respect to toxicity among the various species of natural enemies, fenpropathrin appeared to be less harmful to most of the beneficials compared to bifenthrin or cyfluthrin. A larger difference in toxicity was observed between the two systemics, imidacloprid and thiamethoxam against the parasitoids. Both compounds were toxic to *G. ashmeadi* but thiamethoxam was more toxic after 24 h exposure compared to imidacloprid which was not toxic to these insects at tested doses during the first 24 h of exposure. A follow-up study involving detection of imidacloprid using ELISA revealed variable amounts of this compound in insects even though they do not feed on plant tissue.

Evaluation of the susceptibility of two predators, *G. punctipes* and *O. insidiosus,* to imidacloprid and thiamethoxam revealed that both systemic compounds were toxic to these predators (Table 2). The LC_{50} values were low but only after 96 h exposure. These results are not surprising because *Geocoris* spp and *Orius* spp. will feed on plants.

Objectives 3 & 4. Impact of systemic compounds on parasitoid survival.

Work is on-going for objectives 3 and 4, evaluating the impact of imidacloprid and thiamethoxam within plants on the survivorship of *G. ashmeadi* and *A. melinus*. Our preliminary results have shown that systemics have an impact on GWSS egg parasitoids and *A. melinus*. The potential for mortality caused by systemic insecticides in non-plant feeding insects such as parasitoids of GWSS is being evaluated under field conditions. The lethal effects on *G. ashmeadi* and *A. melinus* that occur when exposed to systemically treated plant surfaces will be measured by determining the titers of both compounds

within the leaf tissue as well as in GWSS eggs or scale nymphs in which parasitoids develop. In future tests, we will attempt to relate survivorship of parasitoids to the titers of either material within the treated leaf tissue. The effect of imidacloprid and thiamethoxam treatments on *G. ashmeadi* within GWSS eggs will be studied.

Tests are in progress to expose *A. melinus* to cottony cushion scale honeydew after exposure of the scale to systemic treatments of imidacloprid. Preliminary tests assessing dose-mortality responses of cottony cushion scale to three rates of imidacloprid applied to potted citrus have been initiated. The mortality of cottony cushion scale was determined at seven, 14 and 21 d post-treatment. Preliminary tests also included quantifying titres of imidacloprid in cottony cushion scale, leaf and stem extracts and honeydew using commercially available ELISA kits. Imidacloprid was detected variably in the insect, leaf and stem extracts and honeydew based on the rate applied. Higher levels were found in the leaf extracts within one week of treatment with lower titres detected in the insect and honeydew. Future tests will determine the impact if any, of concentrations of imidacloprid present in the honeydew on *A. melinus* and *H. covergens*. These tests will assess toxicity in general to parasitoids because these parasitoids will feed on available honeydew on citrus produced by cottony cushion scale. If there are residues of imidacloprid in honeydew, ELISA tests can detect the presence of systemic chemicals.

CONCLUSIONS

This study showed differences in the relative number of natural enemies of GWSS using two monitoring methods. Numbers of parasitoids and rates of parasitism were higher using the egg mass collection method versus the yellow sticky trap technique. Both techniques showed seasonal differences in numbers of natural enemies, with higher levels in summer than in fall. This study helped fill the gap in knowledge regarding the effect of selected insecticides against natural enemies of GWSS. The work reported here investigated the toxicological effects of three neonicotinoids, imidacloprid (Admire), acetamiprid (Assail), and thiamethoxam (Platinum); two IGRs, buprofezin (Applaud) and pyriproxyfen (Esteem); three pyrethroids, bifenthrin (Capture), cyfluthrin (Baythroid), and fenpropathrin (Danitol); and an organophosphate, chlorpyrifos (Lorsban) against four parasitoids. Contrary to widespread assumption that systemic insecticides may not be toxic to natural enemies, our data showed that systemically applied imidacloprid and thiamethoxam were toxic to parasitoids that do not feed on plant tissue. Additionally, naturally occurring honeydew on citrus leaves may be toxic to *A. melinus*. These data will help determine the relative compatibility of particular insecticides to foraging natural enemies. However, results presented here are presently limited to laboratory observations. Field confirmation is needed and is underway.

Table 1.: Toxicity of various insecticides to *G. ashmeadi* and *A. melinus.*

Compound	Bioassay Technique	Exposure Time	*A. melinus* No. Tested	*A. melinus* LC_{50} μg(AI)/ml	*G. ashmeadi* # Tested	*G. ashmeadi* LC_{50} (μg(AI)/ml)
Chlorpyrifos	Petri dish	24	4148	0.0008	2106	0.006
Bifenthrin	Petri dish	48	4117	0.001	1006	0.010
Cyfluthrin	Petri dish	48	3683	0.007	1215	0.067
Fenpropathrin	Petri dish	48	4140	0.010	1554	166.88
Acetamiprid	Petri dish	48	3257	0.005	1744	0.134
Buprofezin	Petri dish	96	4531	0.764	1804	315.52
Pyriproxyfen	Petri dish	96	3767	0.421	1794	132.53
Imidacloprid	Uptake	48	2248	2.14	1278	11.06
Thiamethoxam	Uptake	48	2156	0.044	1209	0.312

Table 2.: Toxicity of two neonicotinoids to two predators using a systemic uptake bioassay.

Compound	Exposure time	# Tested	LC_{50} (μg(AI)/ml)
Orius insidiosus			
Imidacloprid	24	352	1.63
	96		0.013
Thiamethoxam	24	341	0.297
	96		0.005
Geocoris punctipes			
Imidacloprid	96	334	2.01
Thiamethoxam	96	311	4.83

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IDENTIFY THE SPECIES OF MYMARIDAE REARED IN ARGENTINA AND MEXICO FOR POTENTIAL INTRODUCTION TO CALIFORNIA AGAINST THE GLASSY-WINGED SHARPSHOOTER AND PREPARE AND SUBMIT FOR PUBLICATION A PICTORIAL, ANNOTATED KEY TO THE *ATER*-GROUP SPECIES OF *GONATOCERUS* – EGG PARASITOIDS OF THE PROCONIINE SHARPSHOOTERS (HEMIPTERA: CICADELLIDAE: PROCONIINI) IN THE NEOTROPICAL REGION

Project Leader:
Serguei V. Triapitsyn
Department of Entomology
University of California
Riverside, CA 92521

Cooperators:
Guillermo A. Logarzo
USDA, ARS South American
Biological Control Laboratory
Hurlingham, Buenos Aires, Argentina

John T. Huber
Canadian Forest Service
c/o Canad. Natl. Collection of Insects
Ottawa, Ontario, K1A OC6, Canada

Jesse H. de León
USDA, ARS
Beneficial Insects Research Unit
Weslaco, TX 78596

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ABSTRACT
At least sixteen species of *Gonatocerus* were reared in Argentina and Neotropical Mexico from eggs of the proconiine sharpshooters in the course of classical and neoclassical biological control projects against the glassy-winged sharpshooter (GWSS) *Homalodisca vitripennis* (Germar) in California. The objectives of this project are to identify them taxonomically and to prepare a pictorial, easy-to-use, annotated key to more than 60 Neotropical species of the *ater* group of *Gonatocerus* – mymarid egg parasitoids of the proconiine sharpshooters including *Homalodisca*. Results obtained during the first three months of this new project are being reported.

INTRODUCTION
In North America, eggs of proconiine sharpshooters, which are known vectors of *Xylella fastidiosa* (*Xf*), are parasitized by various Mymaridae and Trichogrammatidae. An illustrated, annotated key to the genera and species of proconiine-parasitizing Trichogrammatidae is available (Triapitsyn 2003), and a key to such North American Mymaridae was published recently (Triapitsyn 2006).

Recommendation 3.12 (NRC 2004) calls for support for the classical biological control (over augmentative approach) against the glassy-winged sharpshooter (GWSS). Recently (during 2000-2006), major efforts have been undertaken to survey for egg parasitoids of GWSS and the related proconiine sharpshooters in Mexico (Hoddle & Triapitsyn, 2004, 2005; Morgan et al., 2000; Pilkington et al., 2005; Triapitsyn et al. 2002; Triapitsyn & Hoddle 2001, 2002; Triapitsyn et al. 2006) as well as in Argentina (Jones 2001; Jones et al. 2005; Logarzo et al. 2004; Pilkington et al. 2005; Virla et al. 2005). As the result, 12 species of *Gonatocerus* (Mymaridae) were reared in Argentina from eggs of the proconiine sharpshooter genera related to *Homalodisca* (G. Logarzo, unpublished USDA-ARS South American Biocontrol Lab. reports for 2004 and 2005). During 2002-2004, some of these species were imported under permits into the Universiey of California, Riverside (UCR) and USDA-APHIS, Mission, Texas quarantine facilities and their colonies were established on GWSS eggs. Several species are still being maintained and evaluated (Jones, Logarzo, Triapitsyn et al. 2005; Jones, Logarzo, Virla et al. 2005; Hoddle & Triapitsyn 2005). However, importation and quarantine evaluation of other available species from Argentina have not been initiated because their identification is not possible without a careful comparison with more than 60 already described Neotropical species of *Gonatocerus*. Several other species of *Gonatocerus* were also reared from eggs of proconiine sharpshooters in Mexico (Triapitsyn et al. 2002; Hoddle & Triapitsyn 2005), Chile (Logarzo et al. 2006), and Peru (Logarzo et al. 2004). The major problem, however, is taxonomic identification of these species, which has been impossible, except for a few of them. Thus, further introductions of these unnamed species (including applications for their release) are hampered because no positive identifications could be made before this project was initiated.

The *ater* species group of the genus *Gonatocerus* is mostly associated with Proconiini in the New World (Triapitsyn 2002; Triapitsyn et al. 2002). It is extremely speciose in the Neotropical region, with at least 60 described species, mainly from Argentina by A. A. Ogloblin, and probably with at least 100-150 undescribed species. Some of them have wide distributions from Mexico to Argentina (Triapitsyn et al. 2006). Unfortunately, almost 50 species described from Argentina and Ecuador by A. A. Ogloblin cannot be positively identified at present because there are no taxonomic keys for their separation, no adequate illustrations that accompany their descriptions in Spanish, and because the type specimens of almost half of these species were not available. Some of these were not marked by A. A. Ogloblin as types; they were located among the miscellaneous slides of *Gonatocerus* in his collection deposited in La Plata Museum in La Plata, Argentina, and needed to be identified. Therefore, to make identification of any specimen of *Gonatocerus* reared from eggs of proconiine sharpshooters anywhere from Mexico to Argentina, it needs to be compared with about 60 already described Neotropical species from the

same group. Thus, a key is needed, which would include all the previously described species of *Gonatocerus* (*ater* group) and also all the new species reared in Argentina and Mexico in the course of the recent surveys. Such a key will also be a useful tool to distinguish the species already present in California from other species of the same genus with similar host associations following possible release and establishment of the exotic egg parasitoids from Argentina and Mexico. Moreover, because of the easy availability of proconiine sharpshooter eggs in California due to the establishment and outbreak of the GWSS, non-intentional introductions of exotic egg parasitoids from countries in Central and South America are also quite possible.

OBJECTIVES

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

RESULTS

Progress on Objective 1.

Types

To locate the unmarked or previously unavailable types of the *Gonatocerus* species, described by A. A. Ogloblin from Argentina and Ecuador, S. Triapitsyn and G. Logarzo visited the entomological collection of La Plata Museum in La Plata, Argentina, in August 2006 and studied the entire Ogloblin collection of *Gonatocerus*. Sorted and examined were numerous specimens stored in more than 20 boxes, each containing 100 microscopic slides. We found all but one primary types of Ogloblin's *Gonatocerus* species, which were borrowed for further study; digital photographs have already been taken from some of those (unfortunately, all the holotypes of the species described from Ecuador will need to be remounted as the mounting medium is so dark that the specimens are not visible). The holotype of *G. dorsiniger* (Ogloblin), described from Ecuador, is still missing but that would not affect our work as it belongs to a different (*membraciphagus*) species group of *Gonatocerus*. We were also able to curate a significant portion of the Ogloblin collection of *Gonatocerus*, labeling unmarked syntypes, paratypes, etc.

Identification

Morphologically, we recognized three more unidentified species among altogether at least 15 species of *Gonatocerus* reared in Argentina by G. Logarzo from eggs of the proconiine sharpshooters. So far we were able to positively identify the following species (including the three species identified during the reporting period, i.e., spp. #2, 8, and 12): *G. nigrithorax* (Ogloblin) [sp. #2], *G. annulicornis* (Ogloblin) [sp. #4], *G. metanotalis* (Ogloblin) [sp. #5], *G. tuberculifemur* (Ogloblin) [sp. #7]; *G. abbreviatus* (Ogloblin) [sp. #8], *G. uat* S. Triapitsyn [sp. #9], *G. atriclavus* Girault [sp. 10], and possibly *G. nigriflagellum* (Girault) [sp. #12]. Sp. # 1 from Argentina and a similar, yet clearly different species, reared from eggs of *Homalodisca* or *Oncometopia* in Veracruz, Mexico, and also sp. #6 from Argentina (a colony of which is being maintained in UCR quarantine) seem to be new, undescribed species, which will be described taxonomically later in the course of this project. The identities of other species remain to be figured out.

Specimen preparation

Due to the late availability of funding (in mid-September), work on slide-mounting of the hundreds of specimens will be conducted mostly during October 2006 – October 2007.

Preparation of the illustrations (mostly Objective 2)

High quality digital photographs (later arranged in plates) were taken, using the Automontage system, of the first 12 species reared in Argentina by G. Logarzo from eggs of the proconiine sharpshooters. Additionally, scanning electron micrographs were taken from some of them to facilitate their recognition and to illustrate some key morphological features.

CONCLUSIONS

Although work on this project has just begun, we are pleased to report some major accomplishments. We were able to locate all but one missing type of the South American species of *Gonatocerus*, described by A. A. Ogloblin from Argentina and Ecuador. High quality digital photographs were taken from both females and males of the first 12 species of *Gonatocerus*, reared by G. Logarzo in Argentina from eggs of the proconiine sharpshooters. Positive identifications of the additional three species from Argentina will make possible submission of several publications of the available biological and molecular data on these species (unpublished data by G. Logarzo, J. de León, and E. Virla). Results of this project will be of significant benefit to biological control (especially to the CDFA/PD Biological Control Program) specialists, ecologists, and other researchers that manage the Pierce's disease threat posed by GWSS. When completed, this key will make possible identifications of the mymarid egg parasitoids of proconiine sharpshooters in America south of the USA, differentiation of native vs. introduced species of *Gonatocerus*, and also will provide information on the candidate species of Mymaridae for

introduction as part of biological control programs, facilitate surveys for assessing levels of egg parasitism of the proconiine sharpshooters, and indicate all known host associations of the mymarid species important for classical and neoclassical biological control of GWSS and other Proconiini.

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Section 3: Pathogen Biology and Ecology

BIOLOGY OF THE *XYLELLA FASTIDIOSA*-VECTOR INTERFACE

Project Leader:
Rodrigo Almeida
Dept. of Env. Science, Policy & Mgmt.
University of California
Berkeley, CA 94720
rodrigo@nature.berkeley.edu

Collaborators:
Steven Lindow
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Harvey Hoch
Department of Plant Pathology
Cornell University
Geneva, NY 14456

Tom Burr
Department of Plant Pathology
Cornell University
Geneva, NY 14456

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

ABSTRACT
This project will build (and test) a framework to study the interactions between *Xylella fastidiosa* and insect vectors at the molecular, cellular and transmission biology levels. Understanding these interactions will lead to a better understanding of the transmission process and its biology, and potentially highlight promising strategies to disrupt pathogen retention by vectors and inoculation into plants. In addition, it will further elucidate how these genes function in infected grape and other hosts.

INTRODUCTION
Current data suggest that *Xylella fastidiosa* (*Xf*) is transmitted to plants from the precibarium of vectors where it attaches, multiplies, forms a 'carpet' of cells and eventually detaches to be injected into plants (Almeida and Purcell 2006). The details about the mechanics of an inoculation event (from the insect's probing behavior perspective) are still to be determined. In addition, there is no information on the interactions between *Xf* and vectors. To our knowledge, there is no datum on any molecular aspect of the vector-*Xf* interface, with the exception of Newman et al. (2004) who demonstrated that a cell-cell signaling mutant was not transmissible to plants by insects (which showed that signaling controls transmission, but did not identify genes associated with attachment or retention per se). This project will start to fill an essential gap in *Xf* transmission and biology research with a study on the molecular determinants of the vector-pathogen interface.

OBJECTIVES
1. Determine the effects of rpfC mutant on vector transmission.
2. Determine the transmission biology of *Xf* mutants hypothesized to be important in early and late stages of insect colonization.

RESULTS
This project is being initiated. We will first focus our studies on *Xf* attachment mutants characterized for some biological attributes by Meng et al. (2005), in addition to cell-cell signaling (rpf) mutants currently being studied by Steve Lindow's group at UC Berkeley.

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XYLEM FLUID CHEMISTRY MEDIATION OF PIERCE'S DISEASE: STIMULATION OF AGGREGATION AND BIOFILM FORMATION

Project Leader:
Peter C. Anderson
University of Florida, NFREC
Quincy, FL 32351
pcand@ufl.edu

Reporting Period: The results reported here are from work conducted July 2005 through September 2006.

ABSTRACT
Xylella fastidiosa (Xf) is the causal agent of Pierce's disease in grapevines. The mechanisms of pathogenicity are largely due to occlusion of xylem vessels by aggregation of *Xf* and biofilm formation. Our previous work has documented the effects of xylem constituents on both *Xf* proliferation and biofilm formation. This current research utilizes 1) addition of xylem constituents to defined media *in-vitro*, and 2) exposure of *Xf* to xylem fluids of different *Vitis* germplasms to investigate effects of xylem components on *Xf* growth and biofilm formation. Xylem fluid is typically low in O_2 and our *in-vitro* studies have established the capability of *Xf* to grow under hypoxic conditions. The growth in the defined (minimal) media is often superior or equal under the oxygen-limited conditions as compared to the air-saturated media. These effects were found to be variable with *Xf* strain and media formulation indicating interactive effects between O_2 and specific xylem components. Short term (1 hour) to long term (12 days) exposure of *Xf* to *Vitis* xylem fluids showed highly significant differences in both *Xf* growth rates and biofilm formation dependent on *Vitis* genotype. *Xf* growth in *Vitis* xylem fluid was correlated to the concentration of the organic acid citric acid, many of the amino acids including glutamic acid, glutamine, histidine, valine, methionine, isoleucine and phenylalanine and inorganic ions including copper, magnesium, phosphorous and zinc. Biofilm formation was also correlated to specific xylem constituents. Our next experiments will test the growth and biofilm formation of *Xf* in response to changing the concentration of these constituents noted above in xylem fluid so that the role of each constituent can be assessed.

INTRODUCTION
We have previously established that a functional relationship exists between Pierce's disease (PD) expression and *Xylella fastidiosa (Xf)* colony growth within *Vitis* germplasms. Colony growth results from proliferation of individual *Xf* (cfu), aggregation, and biofilm formation. Both *in-vitro* and *in-vivo* experiments have shown that both *Xf* proliferation and biofilm formation may be impacted by a variety of constituents including specific inorganic ions, O_2, antioxidants, amino and organic acids and sulfhydryl groups. Newly developed defined media result in variable patterns of *Xf* colony growth (i.e. PW+ results in rapid bacterial proliferation with comparatively little biofilm formation whereas our newly defined media CHARDS provides slower bacterial growth but high biofilm formation). CHARDS media is equivalent to CHARD2 (Leite et al. 2004) except that starch at 0.2g/liter is added. These media provide an array of tools to test effects of individual compounds on *Xf* colony growth. Lastly, we can also examine *Xf* colony formation within xylem fluids of varying *Vitis* germplasms, and correlate patterns of growth to composition of the xylem fluid.

OBJECTIVES
1. Utilize defined media to examine the effects of O_2 and other xylem components on *Xf* growth.
2. Quantify the relationship between naturally occurring xylem constituents (inorganic ions, amino acids and organic acids) and *Xf* colony growth utilizing xylem fluid from a variety of *Vitis* germplasms.

Objective 1. The effects of O_2 on *Xf* growth and biofilm
Our initial work focused on the role of O_2 in *Xf* colony growth. The levels of oxygen found in xylem fluid are highly variable between almost atmospheric to anoxic levels (Gansert et al. 2001; Eklund, 2000). There may also be great variation within a plant (Dongen et al. 2003). Levels documented in xylem are generally well below atmospheric levels, and *Xf* has been defined as an obligate aerobe incapable of growth without O_2 (Wells et al. 1987). We subjected *Xf* growing in liquid PW+ media to 5 levels of O_2 ranging from atmospheric O_2 (21%) to anaerobic conditions (0%). Gas treatments were applied for 5 minutes every 24 hours. *Xf* was cultured under these conditions for 15 days at which time *Xf* growth, quantified by optical density (OD), and biofilm formation were measured. OD was measured using a Genosys 8 spectrophotometer at a wavelength of 600nm. The formation of biofilm on the surface of polypropylene tubes was assayed by the crystal violet method (Espinosa-Urgel et al. 2000). The oxygen levels were determined using a LaMotte's Dissolved Oxygen Test Kit (model EDO•code 7414) to insure the accuracy and persistence of treatment conditions.

Our results established a relationship between O_2 concentrations and *Xf* growth. For many strains tested, *Xf* growth rates were highest under atmospheric conditions and declined as O_2 levels declined. Growth comparison of *Xylella fastidiosa* pv. Pierce's disease strain 'Temecula' and *Xanthomonas campestris* under 21% oxygen and 0% oxygen headspaces, indicate that there was a discrepancy among the ability to grow under a hypoxic condition. There was no change in the optical density for *Xanthomonas* but there was continued growth for *Xylella* under the nitrogen gas treatments (0% O_2; Figure 1). This was also

found for other strains of *Xylella*, such as the Pierce's disease strain 'UCLA' and the almond leaf scorch strain 'Tulare' (data not shown). Effects of oxygen on biofilm appear more variable than effects on OD, yet in about half of the experiments conducted biofilm increased significantly under hypoxic conditions. Media formulation had more significant effects on biofilm than did O_2 concentrations.

The absolute rates of *Xf* growth and the subsequent reactions to declining O_2 were strain dependent. Furthermore, the effects of oxygen varied greatly depending on media used during assays (Table 1). When grown in the defined media XDM2 effects of varying oxygen were highly significant, whereas effects were not evident for *Xf* grown in CHARDS. The latter is a defined media based on xylem fluid composition (Leite et al. 2004), whereas the former is based on genomic analysis (Lemos et al. 2003). Variations in effects suggest interactions between oxygen and other xylem constituents that may be important to *Xf* growth.

Preliminary analysis of media composition, CHARDS and XDM2, after sustained *Xf* growth under differing oxygen levels (21% versus 0%) showed both qualitative and quantitative variations in organic acid profiles. These results, along with analysis of terminal oxidase (the high through-put, low affinity cytochrome bo) in the electron transport pathway and the lack of production of hydrogen sulfide, suggest the possibility that the bacterium may be employing an anaerobic energy production pathway. Current research is addressing if such pathways may be fermentative. We are also analyzing these metabolic products to determine if these products are related to pathogenicity.

Objective 2: *Xf* colony growth in xylem fluid from varying *Vitis* germplasms.
Xylem fluid was collected from *Vitis* cultivars ranging in PD susceptibility. These included *Vitis rotundifolia* cvs. Carlos and Noble, *Vitis rupestris* cv. St. George, *Vitis simpsoni* cv. Pixialla, *Vitis champinii* cvs. Dogridge and Ramsey and *Vitis vinifera* cvs. Chardonnay, Chenin blanc and Exotic all collected from cut bleeding spurs (Andersen and Brodbeck 1991) at the NFREC-Quincy Research Center in March 2005 and also from *Vitis vinifera* cv. Chardonnay and *Vitis rotundifolia* cv. Noble in California during March and April 2006. Profiles of inorganic nutrients (phosphorous, potassium, magnesium, zinc, manganese, copper, boron and sodium) amino acids (glutamine, asparagine, aspartic acid, glutamic acid, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, cysteine, isoleucine, leucine, phenylalanine and lysine), organic acids (oxalic, citric, tartaric, malic, malonic, lactic and succinic acids), electrical conductivity and pH were established for the xylem fluid used in each assay. Cell suspensions of the Temecula strain were pelleted from PW+ medium and re-suspended in xylem fluid from the varying cultivars for periods of one hour, 5 days and 12 days. Bacterial concentration and biofilm formation were quantified for each treatment and correlated to individual chemical constituents in the xylem fluid. Tests were run in March 2006 and again in June 2006 to insure repeatability. We note that the only way to collect sufficient quantities of xylem fluid for this experiment is to use bleeding xylem fluid from cut *Vitis* spurs which is only available in late winter. These fluids from dormant vines may or may not be representative of xylem fluid from specific germplasms in summer when *Xf* is actively proliferating. Thus, this methodology was developed to provide a range of xylem profiles to assess effects of individual xylem components on *Xf* growth rather than to quantify characteristics of *Xf* growth as a function of specific *Vitis* germplasms.

Our results emphasize how dramatically even short term exposure of *Xf* to xylem fluid of varying composition quickly alters *Xf* growth and biofilm formation (Table 2). Significant effects in biofilm ($p<0.0001$) were present after 1 hour and persisted throughout 12 days. Effects on OD were delayed (not apparent after 1 hour) but became highly significant with time ($p<0.0001$ for the 5 and 12 day intervals). Optical density varied greater than 3-fold and biofilm formation greater than 5-fold in the various fluids during the experiments. In all cases both OD and biofilm formation increased with time, but rates of increase varied dramatically between xylem fluid treatments. For example, biofilm formation in Carlos fluid was significantly higher than in the other fluids when measured after one hour, was intermediate in value after five days and was lower than in all other fluids after 12 days. Exposure to specific xylem fluids often had the opposite effect on overall growth rates (OD) and biofilm formation. Optical density for *Xf* incubated in Ramsey fluid was consistently higher than *Xf* in other fluids at each time period, but also consistently lower in biofilm formation.

Analyses of xylem fluids showed that many xylem constituents were highly correlated to optical density and biofilm formation. Optical density (growth) became more highly correlated with these constituents over time with only two significant correlations after 1 hour (Table 3). Glutamine, the predominant amino acid in *Vitis* xylem fluid, was weakly but consistently correlated with OD after 5 and 12 days. Some of the minor amino acids (histidine, valine, methionine, isoleucine and phenylalanine) were much more strongly correlated to *Xf* growth. The organic acid citric acid was also very highly correlated to OD after 5 and 12 days. For the inorganic ions, phosphorous, copper and zinc were well correlated with OD. For all of the constituents mentioned above, results appeared consistent over time as significant relationships apparent after 5 days also persisted through 12 days. We have previously hypothesized the importance of calcium, magnesium, phosphorous and citric acid to *Xf* growth (Andersen 2005). Equations utilizing these as variables yielded higher correlations than regression analyses based on any single chemical constituent ($P<0.0001$; $R^2=0.90$). The strength of these relationships suggest that our original hypothesis merits further investigation.

Biofilm formation was also correlated to xylem constituents Table 4). Both the specific compounds correlated to biofilm formation and the timing of possible effects varied drastically from those found with OD. Six amino acids (glycine, alanine, threonine, arginine, leucine and lysine) and one organic acid (tartaric acid) were related to biofilm formation, but only for a short time (one hour). None of these relationships persisted. The only correlations between xylem constituents and biofilm formation were with xylem pH.

These consistent correlations between xylem constituents and *Xf* growth are important, but further work is needed to suggest a causative relationship between *Xf* growth patterns and individual xylem components. To further investigate potential mechanistic relationships, we are currently repeating these experiments but manipulating xylem chemistry via supplementation of xylem constituents that appear well correlated with *Xf* growth and development. These experiments will allow us to discriminate mechanistic relationships from those that were strictly correlative.

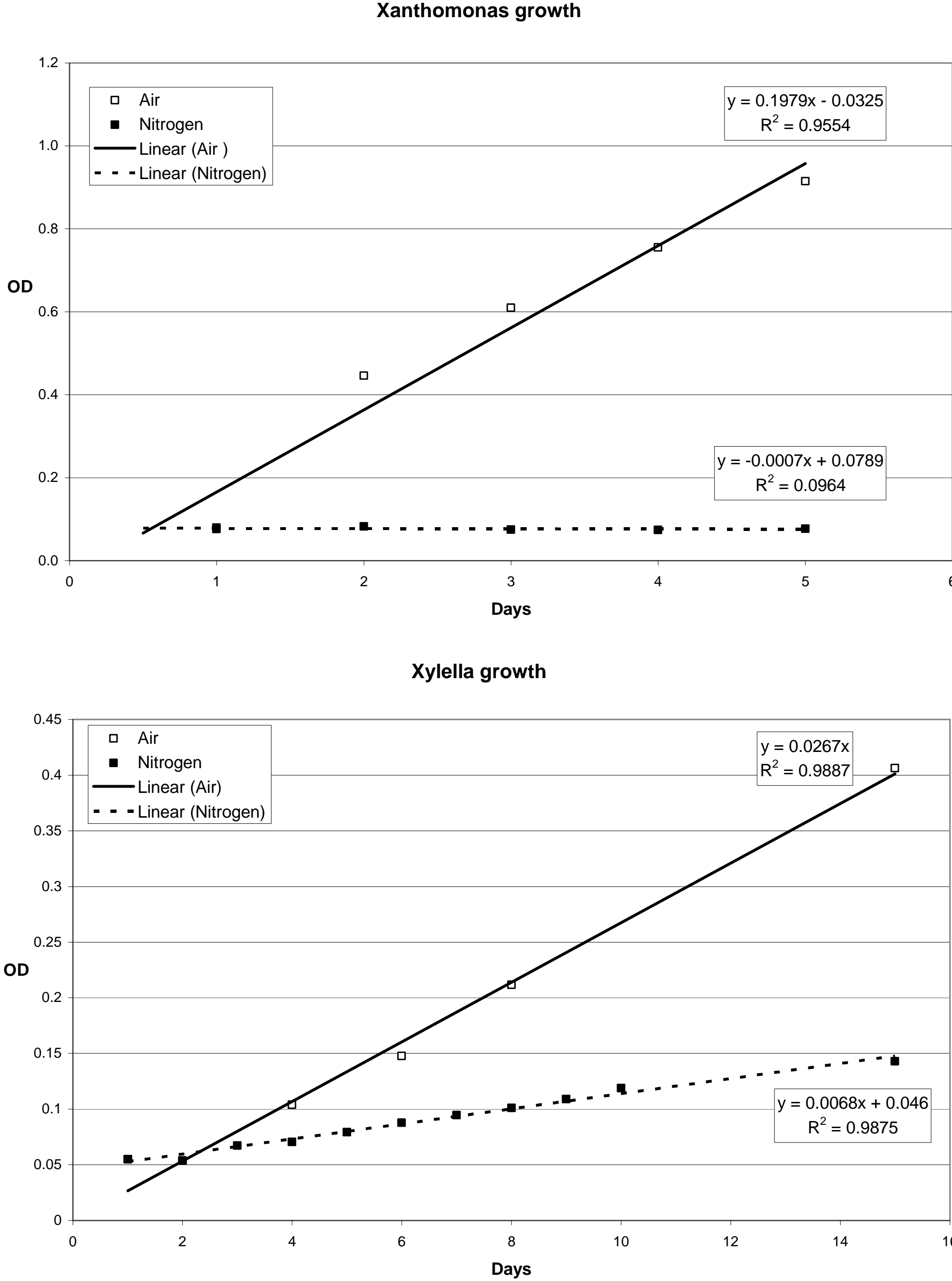

Figure 1. Mean bacterial growth of *Xanthomonas campestris* and *Xf* 'Temecula' in PW+ broth under various oxygen concentrations. Air is 21% oxygen and nitrogen is 0% oxygen. For each treatment level n=3.

Table 1. Optical densities for *Xf* growth under air (21% oxygen) and nitrogen (0% oxygen). On 11/2005 a pooled culture (rather than individual replications) was spun down and resuspended in the appropriate media and then dispensed into the falcon polypropylene test tubes. The experiments in 2006 both had initial optical densities of ~0.1 and the 11/2005 experiment started with OD=0.07.

Media-Gas	**OD** 03/2006	**OD** 04/2006	**OD** 11/2005*
CHARDS - Air	0.165 ±0.005	0.145 ±0.008	0.092 ±0.001
CHARDS - Nitrogen	0.154 ±0.007	0.155 ±.007	0.102 ±0.004
Statistics	P=0.2588	P=0.4123	P=0.0643
XDM2* - Air	0.119 ±0.004	0.107 ±0.005	0.079 ±0.001
XDM2* - Nitrogen	0.168 ±0	0.154 ±0.006	0.092 ±0.003
Statistics	**P=0.0002**	**P=0.0047**	**P=0.0017**

Table 2. Optical density and biofilm formation of *Xf* Temecula suspended in xylem fluid from nine different cultivars of *Vitis* for periods of 1 hour, 5 days and 12 days. Columns with different letters are significantly different for Duncan's MS ($p<0.05$).

	1 hour		5 days		12 days	
	OD	Biofilm	OD	Biofilm	OD	Biofilm
Carlos	0.111	0.139^{a}	0.104^{b}	0.469^{b}	0.106^{b}	0.688^{bcd}
Chardonnay	0.168	0.061^{bc}	0.155^{b}	0.223^{c}	0.137^{b}	0.550^{d}
Chenin Blanc	0.123	0.060^{bc}	0.128^{b}	0.474^{b}	0.140^{b}	0.636^{bcd}
Dogridge	0.129	0.075^{bc}	0.168^{b}	0.656^{ab}	0.160^{b}	0.724^{bcd}
Exotic	0.117	0.071^{bc}	0.124^{b}	0.520^{b}	0.120^{b}	0.847^{b}
Noble	0.131	0.094^{b}	0.112^{b}	0.801^{a}	0.153^{b}	1.200^{a}
Pixialla	0.133	0.065^{bc}	0.136^{b}	0.578^{ab}	0.130^{b}	0.586^{cd}
Ramsey	0.138	0.046^{c}	0.296^{a}	0.134^{c}	0.390^{a}	0.266^{e}
St. George	0.129	0.057^{bc}	0.140^{b}	0.549^{b}	0.166^{b}	0.794^{bc}
Statistics (p<)	NS	0.0001	0.0001	0.0001	0.0001	0.0001

Table 3. The relationship between xylem constituents and optical density of *Xf* Temecula strain suspended in xylem fluid from nine different cultivars of *Vitis* for periods of 1 hour, 5 days and 12 days.

	OD (600 nm)								
	1 hour			5 days			12 days		
	Equation	P<	R^2	Equation	P<	R^2	Equation	P<	R^2
Amino acids									
glu							y = 0.087 + 0.00153x	0.061	0.42
gln				y = 0.111 + 0.0000135x	0.051	0.44	y = 0.105 + 0.0000206x	0.043	0.47
his				y = 0.108 – 0.00144x	0.005	0.70	y = 0.994 + 0.00221x	0.0025	0.75
val				y = 0.100 + 0.000892x	0.024	0.54	y = 0.085 + 0.00143x	0.010	0.63
met				y = 0.111 + 0.0057x	0.047	0.45	y = 0.096 + 0.00276x	0.010	0.63
ile				y = 0.102 + 0.00157x	0.010	0.64	y = 0.0914 + 0.00241x	0.006	0.68
phe				y = 0.103 + 0.00115x	0.005	0.70	y = 0.095 + 0.00172x	0.004	0.71
Organic acids									
cit				y = 0.0745 + 0.000276x	0.0002	0.89	y = 0.0569 + 0.000393x	0.0008	0.82
tar	y = 0.0977 + 0.000327x	0.014	0.60						
Inorganic ions									
P				y = 0.079 + 0.0052x	0.018	0.57	y = 0.0606 + 0.0076x	0.021	0.56
Mg	y = 0.109 + 0.000095x	0.036	0.49						
Zn				y = 0.106 + 0.0793x	0.002	0.77	y = 1.00 + 0.115x	0.003	0.74
Cu				y = 0.084 + 0.846x	0.017	0.58	y = 0.076 + 1.13x	0.04	0.48
(Cit * P) / (Ca * Mg)				y = 0.0939 + 0.0338x	0.002	0.78	y = 0.0776 + 0.0523x	0.0005	0.84
(Cit * P) / (Ca + Mg)				y = 0.101 + 0.00142x	0.0001	0.90	y = 0.0927 + 0.0021x	0.0001	0.90

Table 4. The relationship between xylem constituents and biofilm formation of *Xf* Temecula strain suspended in xylem fluid from nine different cultivars of *Vitis* for periods of 1 hour, 5 days and 12 days.

	Biofilm								
	1 hour			5 days			12 days		
	Equation	P<	R^2	Equation	P<	R^2	Equation	P<	R^2
Amino acids									
gly	y = 0.0529 + 0.00067x	0.032	0.51						
arg	y = 0.0638 + 0.0000839x	0.0015	0.78						
thr	y = 0.0642 + 0.0000695x	0.002	0.77						
ala	y = 0.057 + 0.00115x	0.002	0.76						
leu	y = 0.0611 + 0.00028x	0.0002	0.87						
lys	y = 0.0579 + 0.0033x	0.0008	0.82						
Organic acids									
tar	y = 0.126 – 0.000510x	0.038	0.48						
Inorganic ions									
Cu				y = 0.734 – 3.07x	0.013	0.61			
pH				y = 4.10 – 0.624x	0.0011	0.80	y = 4.51 – 0.659x	0.016	0.59

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THE ROLES THAT DIFFERENT PILI CLASSES IN *XYLELLA FASTIDIOSA* PLAY IN COLONIZATION OF GRAPEVINES AND PIERCE'S DISEASE PATHOGENESIS

Project Leaders:
Thomas J. Burr
Department of Plant Pathology
Cornell University, NYSAES
Geneva, New York 14456
tjb1@cornell.edu

Harvey C. Hoch
Department of Plant Pathology
Cornell University, NYSAES
Geneva, New York 14456
hch1@cornell.edu

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

Other Researchers:
Yaxin Li

Reporting Period: The results reported here are from work conducted December 2005 to September 2006.

ABSTRACT
Type I and type IV pili of *Xylella fastidiosa* play different roles in twitching motility, biofilm formation, and cell-cell aggregation. Thirty twitching mutants were generated with an EZ::TN transposome system and type IV pilus-associated genes were identified, including *fimT*, *pilX*, *pilY1, pilO*, and *pilR*. Mutations in all resulted in a twitch-minus phenotype except that *pilY1* mutant was twitching-reduced. A *fimA* mutant lacked type I pili and altered biofilm development and twitching. A *fimA/pilO* double mutant lacked both classes of pili, was twitch-minus and produced almost no visible biofilm. The gene for the type IV pilin (*pilA*), was cloned and expressed (predicted 15 KDa protein). The pilin sequence is 38% and 55% identical to that of the type IV pilin from *Pseudomonas aeruginosa* and *P. syringae* pv. tomato, respectively. A monoclonal antibody against the *pilA* gene product (prepilin) is being prepared.

INTRODUCTION
Xylella fastidiosa (*Xf*) has both type I pili and type IV pili located at one pole of the cells, and exhibits twitching motility and biofilm formation (Meng et al, 2005). Twitching functions in host colonization of many gram-negative bacteria. Approximately 40 genes have been identified that are involved in the biogenesis and function of type IV pili in *P. aeruginosa* (Mattick, 2002), including those encoding structural and regulatory proteins. In several bacteria, type IV pili are known to function in attachment and biofilm formation (Hélaine *et al*., 2005, Schilling *et al.,* 2001); known virulence factors. The main structural protein of IV pili, pilin, is encoded by *pilA* and is essential for the twitching motility in *P. aeruginosa* and *P. stutzeri (*Mattick *et al.*, 2002; Graupner *et al.*, 2000).

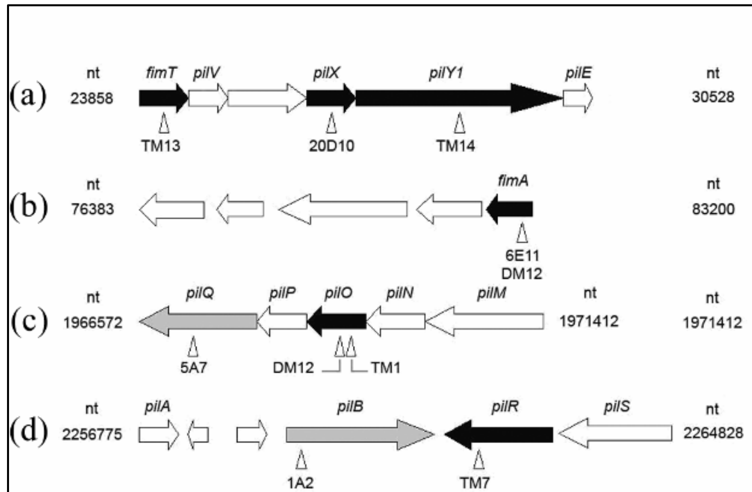
Our recent study revealed that type I pili play a central role in cell attachment and biofilm formation, and that type IV pili mediate twitching motility against a flowing current in microfluidic chambers. A *fimA* mutant (no type I pili) was capable of twitching motility. In contrast *pilB* and *pilQ* mutants (no type IV pili) did not twitch and were greatly impaired in their ability to migrate downward in grapevine shoots (Meng *et al.,* 2005). We have identified several previously undescribed genes in *Xf* that are associated with pili development and their associated phenotypes. We have cloned and expressed *pilA* and its product, pilin, the primary structural protein of type IV pili. Antibodies to PilA and other surface proteins will be used for development of diagnostic tests and eventually in the development of novel controls for Pierce's Disease.

OBJECTIVES
1. Characterize the putative type I pili gene cluster and phenotpes associated with genes.
2. Characterize two additional gene clusters that are likely to be involved in regulation of type IV pili and related functions.
3. Development of monoclonal antibodies to *Xf*.

RESULTS
Characterization of gene clusters associated with pil genes. Thirty twitching-defective mutants, representing 12 different open reading frames, were obtained from approximately 3000 Kan^R insertion mutants generated via the EZ::TN <Kan-2> system. Insertions occurred in homologs of pilus-related genes of *P. aeruginosa*, including PD0019 (*fimT*), PD0022 (*pilX*), PD0023 (*pilY1*), PD1693 (*pilO*), and PD1928 (*pilR*) that reside in four different gene clusters (Table 1; Figure 1). Open reading frame PD0062 corresponds to the *fimA* gene of *E. coli* (precursor for type I pili). A second round of mutagenesis in mutant 6E11 (lacks type I pili) was performed with EZ::TN <DHFR-1> system to select mutants that lacked both pilus types. Six non-twitching mutants were obtained having insertions in PD1923 (*pilC* in DM11, DM15), PD1693 *(pilO* in DM12), PD1671 (DM13), PD0609 (DM14) and PD0022 (*pilX* in DM16), respectively. DM12 (*fimA/pilO*) was selected for further study. *pilO* resides in operon *pilMNOPQ* (Van Sluys *et al*., 2003). Homologs in *P. aeruginosa* are required for type IV pilus assembly (Mattick, 2002).

Table 1.

Xylella fastidiosa		*Pseudomonas aeruginosa*		
Mutant	Gene (ORF)	Gene (ORF)	Predicted Gene Product	Percent Identity ‡
TM13 *	*fimT* (PD0019)	*fimT* (PA4549)	Type IV fimbrial biogenesis protein FimT	29
20D10 *	*pilX* (PD0022)	*pilX* (PA4553)	Type IV fimbrial biogenesis protein PilX	24
TM14 †	*pilY1* (PD0023)	*pilY1* (PA4554)	Type IV fimbrial biogenesis protein PilY1	31
5A7 *	*pilQ* (PD1691)	*pilQ* (PA5040)	Type IV fimbrial biogenesis outer membrane protein. PilQ precursor	39
TM1 *	*pilO* (PD1693)	*pilO* (PA5042)	Type IV fimbrial biogenesis protein PilO	41
1A2 *	*pilB* (PD1927)	*pilB* (PA4526)	Type IV fimbrial biogenesis protein PilB	56
TM7 *	*pilR* (PD1928)	*pilR* (PA4547)	Two-component response regulator PilR	58



TEM revealed that mutants TM1 (*pilO*), TM7 (*pilR*), TM13 (*fimT*), and 20D10 (*pilX*) lacked type IV pili, but still possessed type I pili. The double mutant DM12, lacked both classes of pili. Type I and type IV pili were both present in the *pilY1* mutant, TM14.

Twitching motility of wild-type and mutants. Mutants TM1 (*pilO*), TM7 (*pilR*), TM13 (*fimT*), and 20D10 (*pilX*) exhibited smooth colony margins on modified PW agar surfaces, indicating that they lacked twitching motility as shown in Figure 2. Mutant TM14 (*pilY1*) exhibited a crenulated colony margin, suggesting reduced twitching motility. The peripheral fringe of 6Ell colonies was nearly always wider than the fringe of the wild-type isolate, suggesting enhanced motility. This infers that the presence of type I pili may restrict motility, possibly by enhancing attachment and aggregation of cells. The double mutant DM12 (*fimA pilO)* exhibited no fringe or twitching.

Figure 2.

Biofilms, cell aggregates, and growth rates. Wild-type and twitching-defective mutants formed biofilms on polystyrene and polypropylene surfaces; 6E11 and DM12 exhibited significantly reduced biofilms (Figure. 3). Mutants lacking only type IV pili formed more robust biofilms on polystyrene and glass than the wild-type; however, no differences were observed on polypropylene suggesting that the surface material greatly impacts attachment of the bacteria Biofilm formation by wild-type, TM1, 6E11, and DM12 was tested in Erlenmeyer flasks with continuous agitation (Figure 4a). TM1 formed significantly more biofilm than did the wild-type isolate. The biofilm formed by 6E11 was visibly reduced as compared to the wild-type isolate or TM1 and DM12 developed no visible biofilm (Figure 4a). Biofilms formed by the wild-type and 6E11 were easily removed from the flask surfaces by swirling in distilled water, whereas the biofilm formed by TM1 remained intact (data not shown). Thus the presence of pili makeup also affects biofilm integrity.

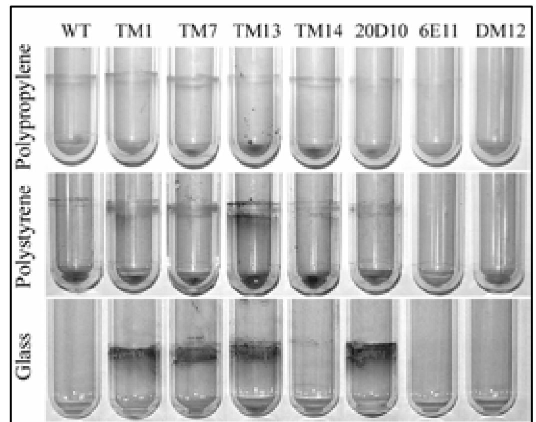


Figure 3.

The quantity of non-attached cell aggregates at the bottom of the culture vessels was proportional to the amount of biofilm on the flask side walls (Figure 4b) and distinct differences in the size and morphologies of aggregates was apparent. DM12 aggregates were

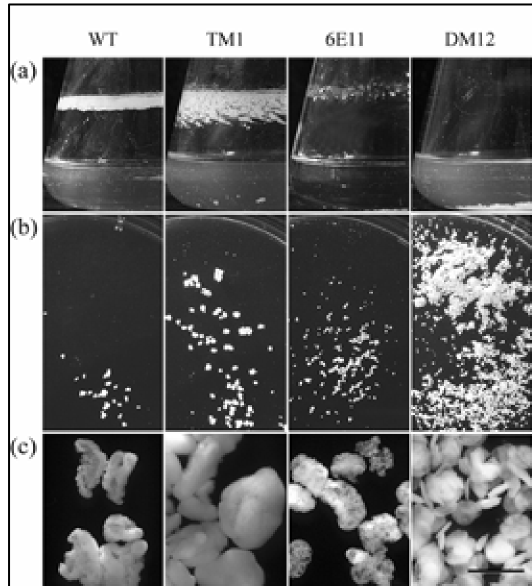


Figure 4.

numerous, small and lens-shaped compared to the other mutants and the wild-type (Figure 4c). TM1 consistently produced the largest aggregates, whereas wild-type and the 6E11 mutant produced loosely compact aggregates suggesting that afimbrial adhesions play a role in cell-cell aggregation. Aggregates of TM or 6E11 were larger than DM12, suggesting that pili affect the formation of larger aggregates. Growth rates of the various mutants did not differ significantly from wild-type (data not shown).

Cloning and expression of Type IV pilin. The gene for the type IV pilin (*pilA* gene product) from *X. fastidiosa* , was cloned and expressed in E. coli BL21 (DE3) cells. The pilin (deduced 15 KDa) sequence is 38% and 55% identical to those of the type IV pilin from *Pseudomonas aeruginosa* PAO1 and *P. syringae* pv. tomato, respectively (Figure 5). A monoclonal antibody against *pilA* encoded pilin, using phage display technology, is being prepared.

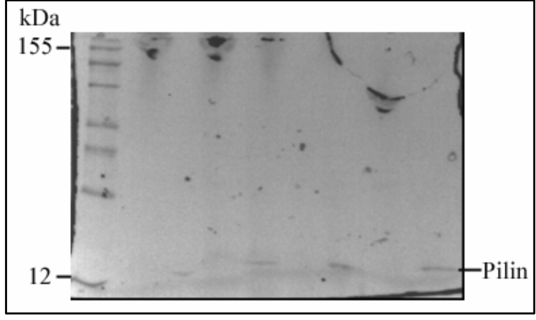


Figure 5.

CONCLUSIONS

Our results show that genes *pilO, pilR, fimT, pilX* are required for type IV pili formation and twitching motility in *Xylella fastidiosa*, and pilY1 affects twitching to a lesser degree. Twitching appears to be important in plant colonization by the pathogen. The type IV pili also are involved in biofilm formation and cell-cell aggregation and thus may play a role in virulence. Type I pili play a central role in biofilm formation and cell-cell aggregation. Cloning and expression of Type IV pilin have implications for understanding Type IV pili function and provide a useful background for the further characterization of the precise function of pilin protein in this process.

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TOWARDS THE DEVELOPMENT OF A SEQUENCE-BASED PCR SYSTEM FOR DETECTION AND GENOMIC STUDY OF *XYLELLA FASTIDOSA* STRAINS IMPORTANT TO CALIFORNIA

Project Leader:
Jianchi Chen
USDA – ARS
Parlier, CA 93648

Researcher:
Sam Livingston
Department of Plant Pathology
University of California
Davis, CA 95616

Cooperators:
Edwin Civerolo
USDA – ARS
Parlier, CA 93648

Bruce Kirkpatrick
Department of Plant Pathology
University of California
Davis, CA 96616

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT

We report two of our recent efforts for improvement of PCR detection and genomic analysis of *Xylella fastidiosa (Xf)*. We evaluated the use of PCR primers from a hyper-variable locus to monitor diversity within strains of the same pathotype. The *psp*B (PD1208) locus, encoding a serine protease, was selected and analyzed. It was observed that tandem repeat sequences in *psp*B locus were highly variable from strain to strain. The biological significance of this hyper-variation is unknown. We also evaluated a simple sample preparation method for template DNA. The pulverized freeze-dried tissue PCR (PFT-PCR) test was compared to the "gold standard" pathogen isolation method. Our results indicated that PFT-PCR had a high predictive value (90.8%) for true positive samples, but a low predictive value for true negative results (29.7%), indicating that a PFT-PCR result is best suited to confirm the presence of *Xf* in a sample.

INTRODUCTION

The complete sequencing of *Xylella fastidiosa (Xf)* genome and their availability allow easy access to every genomic locus of the pathogen. With the availability of many primer design softwares in the Internet, it becomes highly feasible for many plant pathology laboratories to design PCR primers and explore their applications. These form the foundation for developing a genome based detection system for *Xf* using an array of primers from different loci on the platform of polymerase chain reaction (PCR). PCR is theoretically a highly sensitive and versatile technique for pathogen detection. A PCR experiment can be illustrated in the following formula:

$$\text{PCR SUCCESS} = \text{PRIMER} * \text{SAMPLE PREPARATION} \qquad (1)$$

Where a PCR success is the function of utilization of primers designed based on genomic information and sample preparation that provides DNA template. Either factor is equally critical in affecting PCR outcome.

The evolution and nucleotide variation rates of each gene or genomic locus vary. Therefore, primers designed from different genomic loci have different value in evaluating the bacterial population diversity and usage to define *Xf* strains. Several specific PCR primer sets are currently available for *Xf* detection including the most thoroughly tested RST31/33 primer set (Minsavage, et al., 1994), derived from the RNA polymerase genomic locus, and those derived from 16S rRNA gene (Chen et al., 2005), an important taxonomical character for the description of *Xf* (Wells et al., 1987). These primers target the conserved genes. Variations in these gene sequences are closely associated with pathogen pathotypes. For example, in San Joaquin Valley of California, the 16S rDNA G-genotype (G-genotype) strains cause both Pierce's disease (PD) of grapevine and almond leaf scorch disease (ALSD). The 16S rDNA A-genotype (A-genotype) strains cause only ALSD. Few studied have been performed on the use of less conserved or highly variable loci to study *Xf*. Information from the more variable loci could facilitate our understanding of the bacterial pathogenicity and environmental adaptations.

In contrary to PCR primers, sample preparation methods have been subjected to much less vigorous evaluation. The most common PCR detection procedures for *Xf* detection involve DNA extraction to generate template DNA. However, this severely reduces the high throughput capacity of a PCR procedure. Efforts were made to simplify or omit the DNA extraction procedure by using expressed plant sap as PCR DNA template. However, the results were inconsistent. To address this problem, we previously reported the development of a procedure using pulverized freeze-dried almond tissues for PCR detection of *Xf* (Chen and Civerolo, 2005).

In this report, we present the results of our recent analyses on using primers from a hyper-variable locus to evaluate the population diversity of *Xf* strains within the same pathotype/genotype. We also evaluated the procedure using pulverized freeze-dried almond tissues for PCR (PFT-PCR) detection of *Xf*. These are part of our effort in developing a comprehensive genome sequence-based detection system for *Xf* strains important to California.

OBJECTIVES

1. To identify a hyper-variable locus in the genome of *Xf* and analyzed the variability of the hyper-variable locus among different *Xf* strains within the same pathotype/genotype
2. To evaluate the procedure of using PFT PCR for the detection of *Xf* ALSD strains with an array of PCR primers from the genomic loci important to pathogenicity.

RESULTS

Evaluation of intra-pathotype/genotype variations

Using bacteria like the *Haemophilus influenzae* (De Bolle et al., 2000) as a reference, that changes the number of tandem repeats through several genetic mechanisms to regulate gene expressions during the course of environmental adaptation; we, with the help of internet softwares, identified the locus of *psp*B (PD1208), encoding a putative serine protease, from the genome of *Xf* Temecula strain causing PD. The N terminus of *psp*B is characterized by the presence of 23 tandem repeats of TP (threonine and proline). This converts the tandem nucleotide repeat of $[ACG(A)CCA]_{23}$. Orthologs are also found in the genomes of *Xf* strain Dixon, causing ALSD, strain Ann-1, causing oleander leaf scorch disease (OLSD) and strain 9a5c, causing citrus variegated chlorosis disease (CVCD). The number of the repeat varied from strain to strain. PCR primers flanking the tandem repeat region were designed and used to amplify DNA from over 90 *Xf* strains isolated from different hosts and geographical regions. PCR amplicons were sequenced. The difference in tandem repeat numbers among different strains were estimated by amplicon sizes and/or counted from the sequences.

Evaluation of PFT-PCR

The evaluation process involved two experiments. 1). Symptomatic samples were collected from 102 almond trees in an orchard in Kern County, California, in November, 2004. Small branches showing leaf scorching symptoms were excised, placed in labeled plastic bags, and transported in an ice cooler to the SJV Agricultural Sciences Center, Parlier, California. Upon arrival, samples were stored at 4 ° C and processed within 24 hours. One symptomatic leaf was selected to represent one tree. Approximately, two cm petioles were removed from the leaves and used for both *Xf* isolation and for PFT-PCR. 2). During the growing season of 2006, two almond orchards in Fresno County, California, were selected based on the presence of ALSD in the previous year. One previously known ALSD infected tree and one asymptomatic tree were selected from each almond orchard. The branching patterns of each tree were mapped. Leaf samples were collected from labeled scaffolds starting in May when no leaf scorching symptoms were seen and throughout August when symptoms were obvious. Sample collection and processing were identical to experiment 1.

PCR procedure

PCR (25 µl) was carried out in 1x reaction buffer (10 mM Tris-HCl, pH8.3; 50 mM KCl; and 1.5 mM $MgCl_2$) with the addition of: 100 µM of dNTPs, 400 mM of each primer, 1 U of *Taq* DNA polymerase (TaKaRa taqTM Hot Start Version, Takara Bio Inc., Seta 3-4-1, Otsu, Shiga, 520-2193, Japan), and 2 µl of DNA templates. For PFT-PCR, The multiplex PCR procedure (Chen et al., 2005) was adapted for *Xf* detection. Amplification was performed in an MJ Research Tatrad II DNA engine with an initial denaturation at 96 ° C for 10 min, followed by 30 cycles consisting of: denaturation at 96 ° C for 30 s, annealing at 55 ° C for 30 s, and extension at 72 ° C for 30 s. The amplification products were then stored at 4 ° C. The amplified DNAs were resolved through 1.5 % agarose gel electrophoresis and visualized by ethidium bromide staining.

For PCR array, primers were designed and synthesized from 30 putative *pil* genes and 10 putative *gum* genes based on the strain Temecula genome sequence. *Pil* genes encode proteins needed for Type IV pili formation partially responsible for the bacterial motility. The *gum* genes encode enzymes related to the production of extracellular polysaccharides. Both *pil* and *gum* genes are believed to be related in *Xf* pathogenicity. All primers were used to construct a PCR array to amplify DNA from different strains of *Xf*. All PCR amplicons were planned to be sequenced and sequence variations will be determined.

Unlike the conserved 16S rDNA locus, *psp*B was a hyper-variable chromosomal region among the *Xf* strains. In general, A-genotype strains was found to have higher number of repeats (>20) than that of the G-genotype strains (<20) (Figure 1, Table 1). However, strain Temecula and 59-3 (causing PD) and a strain isolated from muscadine in Georgia showed higher repeat numbers, similar to some of the A-genotype strains (Figure 1 and Table 1). On the other hand, the A-genotype strain Ann-1 showed a low repeat number of 13 similar to those of G-genotype strains. All primers from *pil* and *gum* amplified DNA from pure bacterial culture and from pulverized freeze-dried almond tissues.

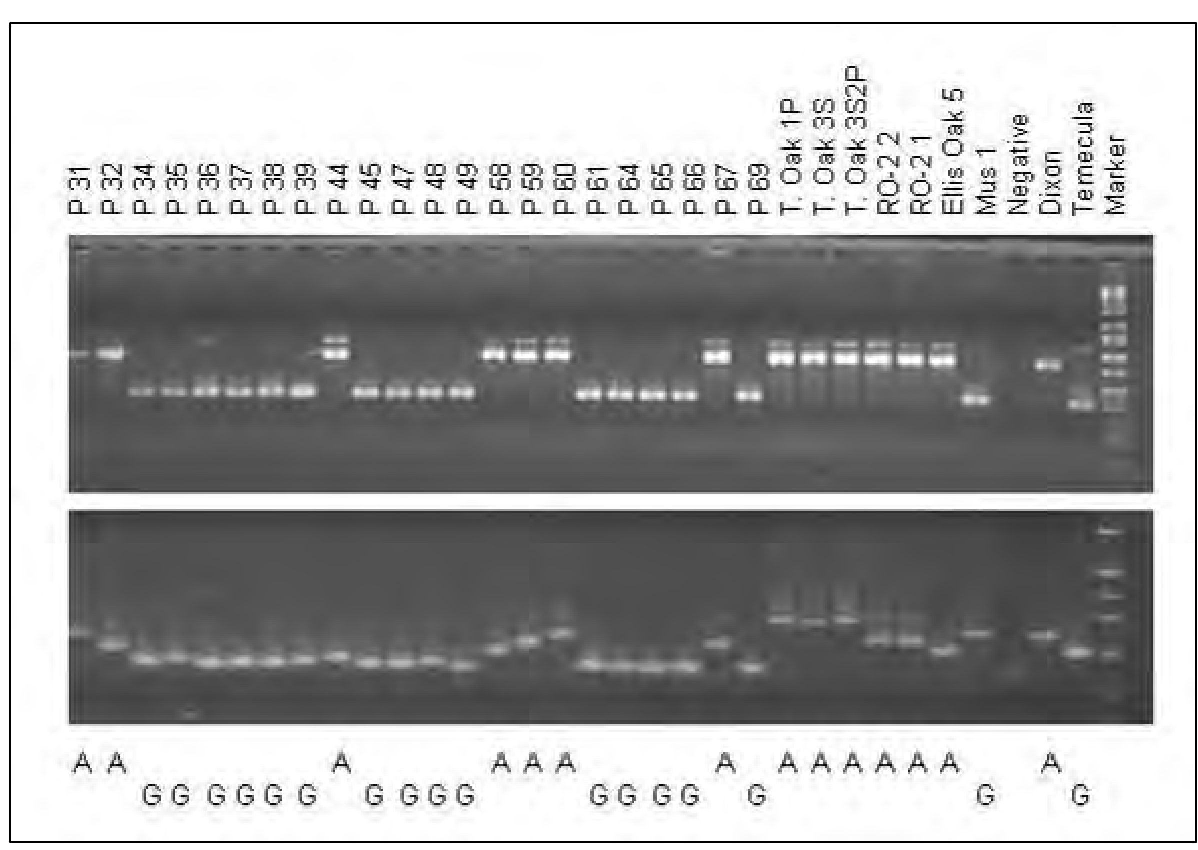

Figure 1. Comparison of DNA variations between the 16S rDNA locus (top) and the pspB locus (bottom) of *Xf* strains

Table 1. Comparison of tandem repeats of *Xf* G-genotype strains at the *psp*B locus.

	CCGCCA	ACGCCA	ACACCA	ACGCCA	ACACCA	Total
R77	1	3	9	0	0	13
R20	1	3	10	0	0	14
R29	1	3	10	0	0	14
R53	1	3	10	0	0	14
R10	1	3	11	0	0	15
R58	1	3	11	0	0	15
R75	1	3	11	0	0	15
R76	1	3	11	0	0	15
R14	1	3	12	0	0	16
R23	1	3	12	0	0	16
R63	1	3	12	0	0	16
R82	1	3	12	0	0	16
R27	1	3	13	0	0	17
Temecula	1	3	8	2	9	23
59-3	1	3	8	2	9	23

From the symptomatic trees, 85 out of 102 samples (83.3%) were positive based on pathogen isolation (Table 2). PFT-PCR detected 65 (63.7%) positive samples. Among the *X. fastidiosa* positive samples, 59 were shared by both methods. The true positive rate of PFT-PCR was 69.4%, 59/85) Table 2). Only 6 samples were PFT-PCR positive but isolation negative. These were considered to be false positive. The predictive value of PFT-PCR for a positive test was, therefore, calculated to be 90.8%. On the other hand, 11 *X. fastidosa*-negative samples by isolation and PFT-PCR were defined as true negative (64.7%, 11/17). Twenty-six PFT-PCR negative samples were in fact isolation positive and were considered to be false negative. The predictive value of the PFT-PCR method for a negative test was 29.7% (Table 2). The results of ALSD temporal development in four almond trees during 2006 are presented in Figure 2.

Table 2. Evaluation of PCR detection of *Xf* in pulverized freeze-dried almond petiole tissue.

		Pathogen isolation	
		Positive = 85	Negative = 17
PCR detection	Positive = 65	59 (a, True +)	6 (b, False +)
	Negative = 37	26 (c, False -)	11 (d, True -)

Predictive value of a Positive test = a/(a+b) = 59/(59+6) = 90.8%
Predictive value of a Negative test = d/(c+d) = 11/(26+11) = 29.7%

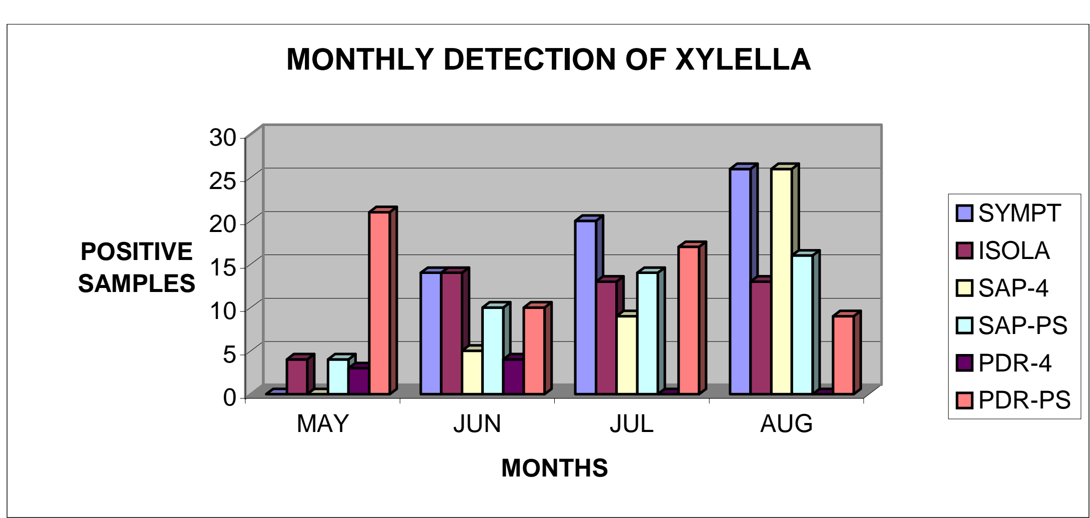

Figure 2. ALSD temporal development in during 2006. SYMPT: leaf scorch symptoms observed; ISOLA: isolation of *Xf* on PW-G media; SAP-4: sap PCR positive with 4 primers; SAP-PS: sap PCR positive with pspb primers; PDR-4: Freeze-dried powder positive with 4 primers; AND PDR-PS: Freeze-dried powder positive with pspb primers.

CONCLUSIONS

While SNPs in the 16S rDNA separate *Xf* strains into two genotypes/pathogtypes (Chen et al., 2005), sequences in *psp*B locus were found to have more variability (Figure 1). If repeat number (DNA fragment size) is considered, the distinction between the bacterial genotypes/pathotypes could be blurred. This means that the variation in *psp*B locus is independent from the bacterial pathotype. The *psp*B locus alone does not clearly define the two genotypes/pathotpes.

The continuous increment of hexomer number from strains in the same orchard (Table 1) implies that the *psp*B locus could be under a constant change. In other bacteria, DNA slippage during replication is one of the few mechanisms responsible for the change of tandem repeats. DNA slippage has not been reported in *Xf*. More interestingly, *psp*B encode a serine protease. Serine proteases participate in a wide range of cellular functions possibly including pathogenicity. Protease genes have been the research target for many bacterial pathogen including *Xf*. It is of high interest to investigate how the variation in tandem repeats will affect the expression of the serine protease.

Since PFT-PCR and pathogen isolation were performed simultaneously from the same petiole, we were able to evaluate PFT-PCR quantitatively by calculating the predictive value of a positive test and the predictive value of a negative test. As shown in Table 2, PFT-PCR had a high predictive value (90.8%) for the true positive samples, but a low predictive value for a true negative result (29.7%). These values suggested that the power of PFT-PCR is, in inferring a positive result rather than a negative result. That is, a PFT-PCR positive result suggests with high confidence, the presence of *Xf* in the sample. However, a PFT-PCR negative result does not appear to be a reliable indication of the absence of *Xf* in the sample.

As shown in Table 2, the pathogen isolation method detected 20 % more *Xf* positive samples than PFT-PCR. Pathogen isolation method is simple and is still a routine test in our laboratory when sample size is small and time is not a constraint. It should be noted that the bacteria were isolated from fully symptomatic leaves in September. Detection accuracy or reliability of both pathogen isolation and PFT-PCR are similar when asymptomatic samples were used (data not shown). For *Xf* detection in asymptomatic samples which is usually associated with a large sample size, PFT-PCR is advantageous. PFT-PCR is more rapid and much less labor intensive than *Xf* isolation and better suited for high throughput pathogen detection for other applications, such as epidemiological studies and evaluating germplasm for *Xf* resistance. The slight decrease in

detection sensitivity is justified by its throughput capacity. Our focus in future will be on the role of tandem repeat variation on the expression of *psp*B and its effects in the host-pathogen interactions using PFT-PCR techniques.

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CHARACTERIZATION OF REGULATORY PATHWAYS CONTROLLING VIRULENCE IN *XYLELLA FASTIDIOSA*

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

Cooperators:
Xiangyang Shi
Department of Plant Pathology
University of California
Riverside, CA 92521

Korsi Dumenyo
Department of Plant Pathology
University of California
Riverside, CA 92521

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ABSTRACT

We are pursuing a strategy to identify traits important in virulence of *Xylella fastidiosa* (*Xf*) through the mutagenesis of "global" regulatory genes, which are known to broadly regulate virulence functions in other microbes. In addition to phenotypic characterization of such mutants, we are using whole-genome microarrays to identify which genes are regulated by these global regulators and examine these genes as putative virulence factors. Here we report a specific example of this approach that has helped to define genes involved in aggregation, biofilm formation, and virulence of *Xf*. In previous work with *X. fastidiosa*, we mutated the global regulatory gene *rsmA*, and found a number of genes that were over-expressed in this mutant when grown in vitro, implying that these genes are normally repressed by the post-transcriptional regulator RsmA in the wild-type. In addition, the *rsmA*$^-$ mutant formed much more biofilm than wild type. Among the genes repressed by *rsmA* was another regulatory gene, *algU*, which regulates important virulence factors in *Pseudomonas*. In this study, an *algU::nptII* mutant had reduced cell-cell aggregation, attachment, biofilm formation, and lower virulence in grapevines. DNA microarray analysis showed that 42 genes had significantly lower expression in *algU::nptII* than wild type, including several genes which could contribute to cell aggregation and biofilm formation, as well as other physiological processes that could contribute to virulence and survival. Thus, *rsmA* appears to control biofilm formation and other traits partly through its repression of the positive regulator, *algU*.

INTRODUCTION

Many virulence genes in bacterial pathogens are coordinately regulated by "global" regulatory genes. The gene *rsmA*, for example, is known to regulate pathogenicity and secondary metabolism in a wide group of bacteria (Blumer and Haas, 2000; Mukherjee et al., 1996). Conducting DNA microarray analysis with mutants for such genes, compared with wild-type, can help to refine the list of genes that may contribute to virulence. We have reported on such an analysis with an *rsmA* mutant of *Xylella fastidiosa (Xf)*, and identified a number of genes that were overexpressed in the mutant (Cooksey, 2004). Among those were *pil* genes that have been subsequently confirmed to be important in twitching motility and long-distance spread of *Xf* in grapevines (Hoch and Burr, 2005; Meng et al., 2005), as well as enzymes or other structural proteins. In addition, a few genes controlled by *rsmA* were "secondary" regulatory genes, such as *algU*, which controls exopolysaccharide production in certain human and plant pathogens and contributes to virulence (Schnider-Keel, et al., 2001; Yu et al., 1995; Yu et al., 1999).

AlgU is a an alternative sigma factor whose role in regulation of biosynthesis of the exopolysaccharide (EPS) alginate has been extensively studied in *Pseudomonas aeruginosa* and *P. syringae*. Alginate functions as a virulence factor in *P. aeruginosa* during infection of cystic fibrosis patients (May and Chakrabarty, 1994), and also contributes to both virulence and epiphytic survival of the plant pathogen *P. syringae* (Yu et al., 1999). In *P. aeruginosa,* AlgU activates AlgU-dependent promoters of *algD* and *algR*. AlgR regulates *algC* and *algD* in cooperation with AlgU (Martin et al., 1994). *mucD* is a negative regulator of *algU* activity in *P. aeruginosa*. Homologs of *algU* (PD1284), *algZ* (PD1154), *algS* (PD0347), *algR* (PD1153), *algC* (PD0120), *algH* (PD1276) and *mucD* (PD1286) were detected in the *Xf* genome (Simpson et al., 2000; Van Sluys et al., 2003), but there are no homologs of the alginate biosynthesis genes *algA, algD, algG, algF, algI* and *algJ*. The alginate homolog genes in *Xf* are therefore probably not involved in alginate biosynthesis, but may be involved in synthesis of other EPS or of lipopolysaccharide (LPS), which could play a role in biofilm formation and cell attachment. In *P. aeruginosa*, the *algC* gene encodes a bifunctional enzyme that is involved in alginate production (phosphomannomutase activity) and lipopolysaccharide (LPS) production (phosphoglucomutase activity) (Coyne et al., 1994). We have constructed an insertional mutation in *algU* in *X. fastidiosa*, which reduced cell-cell aggregation, attachment, biofilm formation, and virulence. DNA microarray analysis of the *algU* mutant was then conducted to determine which genes it regulates.

OBJECTIVES

1. Conduct DNA microarray analysis of gene expression patterns in regulatory mutants of *Xylella fastidiosa*
2. Characterize mutants in regulatory genes and genes that they regulate for changes in virulence and other phenotypes

RESULTS

Phenotype of an *algU* mutant

The *algU* gene in *X. fastidiosa* strain A05 was amplified by PCR, cloned into pUC129 and randomly mutagenized *in vitro* with the EZ::TN^{TM} system. A mutant was selected with Tn5 inserted 79bp from the ATG code of the *algU* ORF and used to replace the wild-type *algU* gene in strain A05. Analysis by RT-PCR showed that there was no expression of *algU* within the *algU::nptII* mutant cells but strong expression was detected within wild-type cells. The *algU::nptII* strain exhibited a more random distribution of cells on agar surfaces, whereas the wild-type grew in clumps. The wild-type formed large aggregates in liquid culture, whereas the *algU::nptII* strain was impaired in its ability to aggregate in a quantitative assay (Fig.1, Left). The exponential and stationary phases of growth of the mutant were similar to those of the wild-type parent A05 in PD3 medium, but the ability of the mutant to adhere to glass surfaces was reduced (Figure1, Right). Biofilm analysis revealed that the *algU::nptII* strain had a greatly reduced ability to form biofilm (Figure 2, Left).

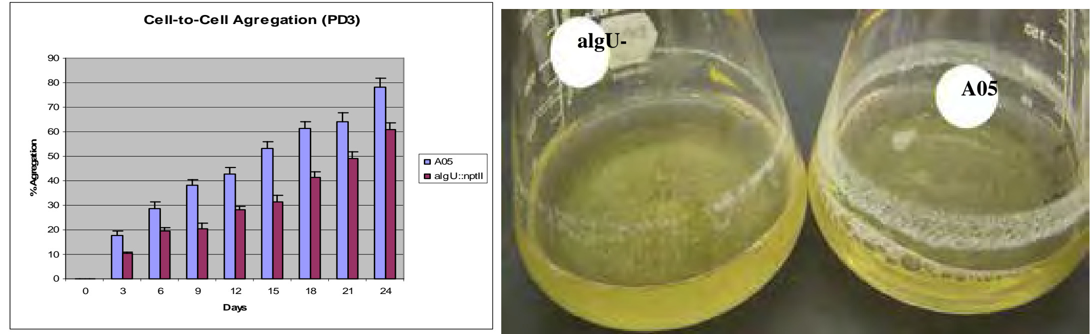

Figure 1. Cell-to-cell aggregation and attachment analysis of *Xf algU::nptII* mutant and wild-type. **Left.** *Xf algU::nptII* mutant was impaired in the ability to form cell-to-cell aggregates in liquid culture. **Right**. Reduced adherence of the *algU::nptII* mutant to a glass surface.

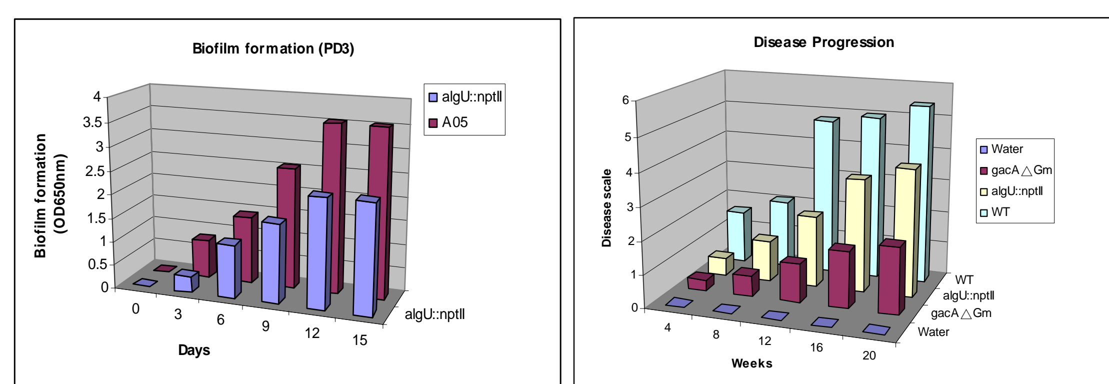

Figure 2. Left: Biofilm formation by *Xf* wild-type and *algU::nptII* mutant **Right**: Disease progression of grapevines inoculated with *Xf* wild-type and *algU::nptII* mutant. Disease severity was based on a visual disease scale of 0 to 5 and was assessed 4, 8, 12, 16, and 20weeks after inoculation. The data are an average of 10 independent replications. The water-inoculated control did not show any symptoms during 20 weeks.

Virulence assay

Grapevines inoculated with the *Xf algU::nptII* mutant developed less severe disease than did those inoculated with the wild type strain 20 weeks after inoculation (Figure 2, Right). Grapevines inoculated with the *algU::nptII* mutant showed i) later symptom development, ii) slower disease progression over a period of 20 weeks, and iii) late appearance of leaf scorching, in comparison with the wild type. Bacterial populations at 25cm and 50cm above inoculation points were estimated from ELISA assays by comparing the OD at 600 nm with that of positive control *Xf* concentrations. The cell populations of the *algU::nptII* mutant were less than that of the wild-type, indicating that *algU* affects the growth and possibly the movement of *Xf* inside the xylem resulting in reduced pathogenicity.

Table 1. Genes differentially expressed in the *Xf algU::nptII* mutant

ORF	Gene[c]	Description	Mutant /Wild type	Signfig.[b]	The expression in mutant
PD0347	*algS*	sugar ABC transporter ATP-binding protein	0.41	*	down
PD0120	*algC*	Phosphomannomutase	0.3	*	down
PD1153	*algR*	two-component system, regulatory protein	0.617	*	down
PD1276	*algH*	transcriptional regulator	0.652	*	down
PD1286	*mucD*	periplasmic protease	0.616	*	down
PD0664	*clpS*	ATP-dependent Clp protease adaptor protein	0.448	*	down
PD0665	*clpA*	ATP-dependent Clp protease subunit	0.485	*	down
PD1685	*clpB*	ATP-dependent Clp protease subunit	0.421	*	down
PD1371	*grpE*	heat shock protein GrpE	0.40	*	down
PD1370	*dnaK*	heat shock protein-Hsp70	0.378	*	down
PD1280	*hspA*	heat shock protein(Hsps)	0.469	*	down
PD2123	*rpmH*	50S ribosomal protein L34, unknown function	0.449	*	down
PD0439	*rplW*	50S ribosomal protein L23, unknown function	0.499	*	down
PD0444	*rplP*	50S ribosomal protein L16, unknown function	0.426	*	down
PD0445	*rpmC*	50S ribosomal protein L29, unknown function	0.479	*	down
PD0447	*rplN*	50S ribosomal protein L14, unknown function	0.477	*	down
PD0450	*rpsN*	30S ribosomal protein S14, unknown function	0.244	*	down
PD0451	*rpsH*	30S ribosomal protein S8, unknown function	0.256	*	down
PD0452	*rplF*	50S ribosomal protein L6, unknown function	0.323	*	down
PD0454	*rpsE*	30S ribosomal protein S5, unknown function	0.437	*	down
PD0455	*rpmD*	50S ribosomal protein L30, unknown function	0.348	*	down
PD0458	*rpsM*	30S ribosomal protein S13, unknown function	0.394	*	down
PD0488	*rpmB*	50S ribosomal protein L28, unknown function	0.436	*	down
PD0489	*rpmG*	50S ribosomal protein L33, unknown function	0.353	*	down
PD0749	*rpmE*	50S ribosomal protein L31, unknown function	0.350	*	down
PD0750	*gltA*	citrate synthase-Energy production and conversion	0.496	*	down
PD1926	none	Type II secretion system-pilus assembly fimbrial protein	2.478	*	up
PD1709	*mopB*	outer membrane protein	0.479	*	down
PD1807	*ompW*	outer membrane protein	0.391	*	down
PD1065	*SecB*	Type II secretion system-Preprotein translocase	0.409	*	down
PD1672	*bfr*	Bacterioferritin-ferritin-like diiron-carboxylate proteins	0.178	*	down
PD0095	*rsmA*	RsmA homologue-regulate virulence determinants	0.403	*	down
PD0066	*hfq*	host factor-I protein, ubiquitous RNA-bing protein hfq	0.32	*	down
PD0216	cvaC[d]	colicin V precursor-antibacterial polypeptides toxin	0.40	*	down
PD0159		unknown	0.479	*	down
PD0521		unknown	0.439	*	down
PD1354		unknown	0.392	*	down
PD0968		unknown (Helix-turn-Helix motif)	0.495	*	down
PD1028		unknown	0.425	*	down
PD1058		putative transcriptional regulatory protein	0.484	*	down
PD1295		putative integral membrane protein	0.469	*	down
PD1668		putative integral membrane protein	0.413	*	down
PD1667		HesB-like protein-unknown function	0.462	*	down

[a] Hybridization signal intensity obtained with the mutant was divided by that from wild-type to obtain the M/W ratio
[b] Based on standard deviation calculations, genes having $\geq$ 1.5 or $\leq$ 0.66 final M/W ratios were selected as statistically significant up-regulated or down-regulated genes, respectively. Significant T-test, $t < 0.001$.
[c] Genes were detected based on *Xf* Temecula genomic sequences at the NCBI site. [d] Currently annotated as colicin V precursor proteins.

DNA microarray analysis of gene expression.

RNA was prepared from the *algU::nptII* mutant and wild type AO5, and cDNA was synthesized to hybridize to a genomic DNA microarray from NimbleGen Systems. This oligo-based, high-density microarray contains multiple probes for every gene found in the Pierce's disease strain sequence as well as those unique to the CVC strain sequence. Expression levels of 2188 genes between wild type and were analyzed. Many of the differentially expressed genes were validated by RT-PCR. Forty three genes were differentially expressed in *algU::nptII* compared with the wild type (Table 1). One gene, predicated to encode a fimbrial protein (PD1926), had increased expression in the mutant, but the other 42 genes had decreased expression and are therefore considered to be positively regulated by *algU* in wild-type *Xf* (Table 1). These included homologs of the alginate genes *algS*, *algC*, *algR*, *algH*, and *mucD*, which may have a role in LPS or EPS biosynthesis. Several genes involved in cell structural components and secretion (*mopB, ompW,* and *secB*) are also positively regulated by *algU*,. MopB was shown to bind specifically to xylem tissue (Bruening et al., 2005), and its decreased expression in the *algU::nptII* may have contributed to its decreased attachment and aggregation. Genes involved in physiological metabolism under stress, such as heat shock protein genes *cplS, clpA, clpB*, *dnaK, grpE* and *hspA*,, and the iron storage and detoxification gene, *bfr*, are positively regulated by *algU,* as well as a colicin V precursor (PD0216) that may function in competition with other microbes (Pashalidis et al., 2005). Interestingly, *rsmA* and *hfq*, involved in posttranslational regulation, are also positively regulated by *algU* in *Xf*, while it was shown previously that RsmA negatively regulates *algU*.

CONCLUSIONS

Investigating expressed gene profiles of the *algU::nptII* mutant compared with wild-type via microarray analysis revealed that *algU* regulate various factors which contribute to attachment and biofilm formation, as well as physiological processes that may enhance tolerance to environmental stresses and competition within the xylem. Similar experiments will be conducted to examine differential expression of the *algU::nptII* mutant and wild type *in planta*. In addition, several select candidate pathogenicity genes that were regulated by AlgU will be mutated, and the effects of the mutations on phenotype and virulence will be assessed. The intent of this research is to identify essential virulence factors that may serve as targets for novel control approaches.

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

Project Leaders:

Peter Cousins
Grape Genetics Research Unit, USDA – ARS
New York State Agric. Expt. Station
Geneva, New York, 14456
Peter.cousins@ars.usda.gov

John Goolsby
Beneficial Insects Research Unit, USDA – ARS
Kika de la Garza Subtropical Agric. Res. Center
Weslaco, Texas, 78596
jgoolsby@weslaco.ars.usda.gov

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ABSTRACT

Chardonnay is a *Vitis vinifera* scion variety that is susceptible to Pierce's disease (PD). We are evaluating the effect of rootstock variety on PD symptom expression in Chardonnay grown in an experimental vineyard at Weslaco, Texas with high natural PD pressure and abundant vectors, including glassy-winged sharpshooter. The rootstocks under evaluation are Dog Ridge, Florilush, Tampa, Lenoir, and Freedom. Natural *Xylella fastidiosa* infection will be permitted to test the effect of rootstock variety on PD in the Chardonnay scions.

INTRODUCTION

Rootstocks are widely in use in viticulture to manage damage from soil-borne pests and provide adaptation to soils. In citrus (He et al. 2000) and peach (Gould et al. 1991), rootstock variety has been reported to impact expression of *Xylella fastidiosa* (*Xf*) diseases in scions. Pierce (1905) reported that rootstock variety affected expression of "California vine disease" (PD) 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 1952, 1965; 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 PD prone areas. Elite wine, juice, and table grape varieties could be grown in areas where viticulture is currently restricted to PD resistant and tolerant varieties whose consumer appeal is low.

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

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

OBJECTIVE

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

RESULTS AND CONCLUSIONS

Grafted vines of Chardonnay on five rootstocks (Freedom, Tampa, Dog Ridge, Florilush, and Lenoir) were planted at the Kika de la Garza Subtropical Agricultural Research Center in Weslaco, Texas in July, 2006. Evaluation of PD response of the vines will begin in 2007.

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EFFECTS OF GROUP, CULTIVAR, AND CLIMATE ON THE ESTABLISHMENT AND PERSISTENCE OF *XYLELLA FASTIDIOSA* INFECTIONS CAUSING ALMOND LEAF SCORCH

Project Leader:	**Cooperator:**	**Researcher:**
Kent Daane	Bruce Kirkpatrick	Christina Wistrom
Dept. Environ. Sci., Policy, & Mgmt.	Department of Plant Pathology	Dept. Environ. Sci., Policy, & Mgmt.
University of California	University of California	University of California
Berkeley, CA 94720-3114	Davis, CA 95616	Berkeley, CA 94720-3114

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ABSTRACT

Almonds are one of the most widely-grown crops infected by *Xylella fastidiosa (Xf)*. To get a better idea of the conditions that lead to almond leaf scorch outbreaks, and to determine the risks that *Xf* infections may pose to adjacent vineyards, three factors were assessed that may influence the establishment of *Xf* infections and almond leaf scorch development: almond cultivar, *Xf* genetic group, and winter severity. Experimental plots of 100 trees each were planted at two field sites, Armstrong Farm at UC Davis (UCD) and Intermountain Research and Extension Center at Tulelake (IRC). In field plots, equal numbers of highly susceptible 'Peerless' and less-susceptible 'Butte' almond trees were inoculated with grape and two almond *Xf* isolates. Because *Xf* infections must survive multiple winters in an almond tree cause almond leaf scorch, field sites were selected with moderate (UCD) and severe (IRC) winter temperatures. To better understand the role of cold temperatures in overwintering *Xf* infections, a controlled dormancy test was also done. Potted almond trees were inoculated with almond-type *Xf*, and infected trees held ind dormancy outside, or in cold rooms at 1.7ºC or 7ºC. Ten trees from each treatment were brought back into the greenhouse to break bud after 1, 2, or 4 months.

INTRODUCTION

Because almonds are one of the most widely-grown crops that can host *Xylella fastidiosa (Xf)* in the Central Valley, they might serve as a source of *Xf* infections in grapes, although for unknown reasons *Xf* dispersal between almond orchards and vineyards is uncommon (A. Purcell – *unpublished data*). Almond leaf scorch (ALS) is caused when *Xf* multiplies extensively within the xylem of infected trees, eventually severely limiting nut production (Davis et al. 1980). The disease was first formally described in 1974, and outbreaks occurred in Los Angeles and Contra Costa counties in the 1950's (Moller et al. 1974). Symptoms of ALS are similar to Pierce's disease in grapes and include leaves with marginal necrosis and chlorosis and poor terminal growth. Initial infections spread slowly and often occur only on one branch, but after a few years are visible on the entire tree (Almeida and Purcell 2003c), reducing almond productivity (Mircetich et al. 1976, Moller et al. 1974). In both grapes and almonds, *Xf* multiplies to high populations (1,000,000 bacteria per gram of plant tissue) and is acquired and transmitted by insect vectors (Almeida and Purcell 2003a, Almeida and Purcell 2003c, Purcell 1980a). In laboratory tests, *Xf* was transmitted to almonds by 5 species of xylem-feeding insects, including the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis;* formerly *H. coagulata*) (Almeida and Purcell 2003b, 2003c).

In previous studies, almond cultivars varied greatly in their susceptibility to ALS, with some developing extensive leaf scorch, and others showing little disease. *Xf* inoculations made from May through July had the best odds of surviving the following winter. (B. Kirkpatrick – *unpublished data*). We compared *Xf* infection establishment and survival in two cultivars, highly susceptible 'Peerless' and less-susceptible 'Butte'.

The genetic type of *Xf* may affect development of almond leaf scorch. Cross-inoculation studies in the greenhouse showed that the genetic type influenced the ability of the bacteria to over winter in grapes or almonds, as almond types died in grapes and grape types died in almonds (Almeida and Purcell 2003c). For this reason, we also used different genetic types of *Xf* in our field trials. Three genetic types of *Xf* have been identified from almond trees. One type was identical to *Xf* from Pierce's disease infected grapevines. The other two genetic types were unique to almonds (Hendson et al. 2001). The three types were distinguished by growth on selective media and DNA digestion with restriction enzymes (Almeida and Purcell 2003c).

After vector inoculation, *Xf* must survive multiple winters in an almond tree to reach sufficient populations for sharpshooter acquisition and economic impact disease levels. Growth chamber and field studies with grapevines showed that the degree of plant dormancy, as well as severe cold, affected the over winter survival of *Xf* (Feil and Purcell 2001, Purcell 1980b). To date, there is no information available on the effects of winter dormancy on *Xf* infections in almonds. Therefore, field sites were selected with moderate and severe winter temperatures (Armstrong Farm at UCD, and IRC at Tulelake, CA, respectively) in order to study treatment impact under different winter temperatures. A controlled dormancy severity test with potted plants and growth chambers was also started at Kearny Agricultural Center, near Parlier, California.

OBJECTIVES

1. Compare the establishment and multi-year persistence of *Xf* isolates belonging to three ALS genetic groups in almond cultivars with either low or high susceptibility to almond leaf scorch.
2. Compare effects of winter severity and the degree of plant dormancy on the infection rate, symptom severity, and titer of *Xf* in inoculated almonds.
3. Use collected data on almond leaf scorch development to determine if almond orchards may serve as a reservoir of *Xf.*

RESULTS

Field trials. One hundred bare-root almond trees, 50 of each cultivar, were planted in spring 2005 at two different field sites: Armstrong Farm at University of California, Davis, CA (UCD), and Intermountain Research and Extension Center, Tulelake, CA (IRC). Trees were planted in a complete randomized block design with a split plot (almond cultivars) in each block. There were ten replicates of each treatment combination (*Xf* isolate × almond cultivar). Trees are drip irrigated at UCD and sprinkler irrigated at IRC.

The almond trees were inoculated with different genetic types of *Xf*, either Fresno-ALS (isolated from almonds but genetically similar to *Xf* that causes Pierce's disease in grapes; PD-Xf), Dixon (ALS-*Xf* type 1) and ALS 6 (ALS-*Xf* type 2), Medeiros (from grapes), or buffer control. All isolates of *Xf* were isolated from infected plants in Solano, Fresno, or San Joaquin Counties, and were pathogenic in recent greenhouse tests. In 2005, inoculations were done in early May (UC Davis) and early July (IRC) when the young shoots were at least 6 mm in diameter. Inoculum was prepared in the field from two week old cultures of *Xf*. Each tree was inoculated with approximately 100,000 CFU of *Xf* following Hill and Purcell 1995. Inoculation sites were marked with permanent metal tags and paint. Twenty trees at UCD were re-inoculated on May 15, 2006 with ALS-6 or Dixon *Xf* strains. Leaves immediately adjacent to the inoculation sites were tested for *Xf* in fall 2005 to determine the number of successful inoculations, bacterial titer, and symptom development in field-grown almond trees.

Two trees at UCD died in winter 2006, and 64 trees (or inoculated branches) died at IRC. The infected trees remaining with living inoculated branches were evenly distributed among isolate treatments, with 7 buffer-inoculated, 5 ALS6-inoculated, 3 Dixon-inoculated, 2 Fresno-inoculated, and 6 Medeiros-inoculated trees surviving. While mortality was high, similar losses were seen in previous studies examining the over winter survival of *Xf* in grapevines (Purcell 1980b). Assessment of the number of over-wintering infections is ongoing in mid-September 2006. Trees will also be evaluated for the presence of *Xf* in 2007 and 2008. The severity of infection was rated by the number of scorched leaves on the inoculated stem. Almond petioles from each tree were cultured to determine *Xf* infection and population. Strain identification of *Xf* was done by re-streaking growing bacteria on two different artificial media, PD3 and PWG (Davis et al 1980, Hill and Purcell 1995). All types of *Xf* grow on PWG, while ALS-*Xf* type 2 and Pierce's disease types grow on PD3 as well. ALS-*Xf* type 1 does not (Almeida and Purcell 2003c). To separate ALS and PD isolates, polymerase chain reaction (PCR) was used to amplify DNA from the bacteria, and *Rsa 1,* a restriction enzyme, cut the DNA of ALS-*Xf* isolates into two pieces, but did not cut the DNA of PD-*Xf* (Almeida and Purcell 2003c).

In 2006, the onset of almond leaf scorch symptoms was delayed in trees at UCD. Despite sampling two weeks later than in 2005, *Xf* was recovered from only one tree inoculated in 2005 and one inoculated in 2006. The *Xf* populations in those two trees in 2006 were 10-fold lower than populations recovered from similarly infected trees one year previously. Almond leaf scorch symptoms were only visible on the trees that tested positive via culture. Re assessment of trees for disease symptoms and *Xf* presence is planned in late September.

In 2005, almond leaf scorch symptoms were much more severe at UCD, especially in 'Peerless' trees, with an average of 4.6 scorched leaves per tree, compared to 0.8 in 'Butte'. Both cultivars at IRC had no scorched leaves, an average of 0.2 and 0.1 leaf per tree for 'Butte' and 'Peerless', respectively. However, there was no difference in the proportion of infected trees at UCD (32 of 78 infected at UCD, 41 of 96 infected at IRC; Chi-square $P > 0.05$), nor in the median populations of *Xf* present in inoculated trees at UCD (6.2×10^{6} CFU/g) or IRC (1.3×10^{7}; $\log_{10}$-transformed; $P = 0.26$). The difference in symptoms may have two explanations: i) trees at UCD were tested for *Xf* 3.5 months after inoculation and had longer to develop symptoms, compared to trees at IRC, which were tested 2 months after inoculation; or ii) the infected trees were under more moisture stress at UCD, which led to the development of disease symptoms.

While it is too soon to tell how infections overwintered in 2006, in 2005 there were not large differences between infection percentage (41% of 'Butte, 38% of 'Peerless'; Chi-Square $P > 0.05$), or *Xf* population (2×10^{6}CFU/g for 'Peerless' and 9×10^{6}CFU/g for 'Butte; $\log_{10}$-transformed; $P = 0.11$) for the two cultivars. 'Peerless' had much fewer scorched leaves than 'Butte' at UCD, but not at IRC, as discussed in the previous paragraph. Also in 2005, grape strain *Xf* was more frequently recovered from inoculated trees than either almond strain. Fresno and Medeiros were recovered from 64 and 77% of trees, respectively, whereas ALS6 and Dixon were recovered from 27 and 28% of trees. Leaf scorch symptoms were more severe in trees inoculated with grape-type isolates Fresno and Medeiros (an average of 2.8 and 3.2 scorched leaves/tree), compared to almond isolates Dixon and ALS6 (0.3 and 0.9 scorched leaves/ tree), and background leaf scorch in buffer-inoculated trees (0.1/ tree).

In 2005, bacterial populations in trees infected with grape and almond isolates were similar, even though infection percentage and symptom severity was greater in grape isolates of *Xf*. Median populations of *Xf* in infected trees were: 6.2 x 10^6 CFU/g (ALS6), 2.8 x 10^6 CFU/g (Dixon), 5.5 x 10^6 CFU/g (Fresno), 2.4 x 10^7 CFU/g (Medeiros), and 0 CFU/g (buffer). Bacterial populations were high even in only a few trees in the treatment were infected with *Xf*, as in ALS6 inoculated plants at UCD. In future analyses, ANOVA will be used where applicable to detect differences in infection percentage and bacterial populations between cultivars and bacterial isolates. Temperature data collected from on-site weather stations will be compared for both sites as well, to determine the number of hours with temperatures outside the growth range of *Xf*.

Glasshouse and Growth Chamber trial. An additional experiment examined the effect of over wintering temperature in the survival of *Xf* infections in controlled environments. One hundred and fifty-five potted two-year-old 'Peerless' almond trees were inoculated in spring 2005, 125 with ALS 6 *Xf* and 30 with buffer alone. Trees were kept in the greenhouse and tested for infection in fall 2005. Ninety trees infected with *Xf,* and 27 buffer-inoculated trees were used for the rest of the experiment. Trees went dormant in screen cages outside, and were divided equally between treatments in January 2006. One-third remained outside in the screen cage, 1/3 were kept in a cold chamber at 7°C (45°F), and 1/3 at 1.7°C (35°F). *Xf* dies at these temperatures in grapevines (Almeida and Purcell 2003c, Feil and Purcell 2001). Trees were removed from each cold treatment at intervals of 1, 2 and 4 months, and allowed to break bud in the greenhouse. These intervals were reflective of dormancy periods used in previous studies with almonds and grapevines (1 month; Almeida and Purcell 2003c, Feil and Purcell 2001), typical dormancy in the central valley (2 months; going fully dormant in December and flowering in February) and an extreme treatment for abnormally long dormancy (4 months). Plants were kept the greenhouse until they developed almond leaf scorch, in mid-August, then assessed for disease severity, and *Xf* presence and population as previously described.

Preliminary analysis of data collected in September 2006 indicated no differences in the number of symptomatic leaves between cold treatments, with trees held at 1.7ºC averaging 23.3 symptomatic leaves per tree, trees at 7ºC 24.3 symptomatic leaves per tree, and trees kept outside an average of 18.5 symptomatic leaves per tree. Trees exposed to one or two months of cold treatment had fewer symptomatic leaves than trees left outside or in the cold box for 4 months (1 month = 17.8 leaves, 2 months = 15.4, 4 months = 32.2), regardless of temperature. Initial implications suggest that almond *Xf* strains have different overwintering survival characteristics that grape *Xf* strains, as has been indicated by previous studies (Almeida and Purcell 2003c).

CONCLUSIONS

Analysis of data collected in August and September 2006 is ongoing; however some interesting preliminary results emerged. In 2005, ALS symptoms at UC Davis were more severe than at IRC, probably the result of the longer interval between inoculation and sampling (3.5 months for UCD and 2 months for IRC). The current data do not suggest an explanation why ALS group isolates infected trees more frequently at IRC than at UCD. In spring 2006, an additional 20 trees at UCD were inoculated with almond-type isolates to determine if they do not infect the trees as frequently, or if the low infection rate observed in 2005 was an anomalous result. Potted almond trees in our controlled overwintering study showed no "curing," in fact trees exposed to a 4-month dormancy had greater numbers of symptomatic leaves than trees dormant for one or two months. Not all field-grown trees have been analyzed for infection severity and *Xf* presence; complete data will allow better documentation of the fate of overwintering *Xf* infections.

Although the effect of cold on *Xf* infection survival was investigated in grapes (Feil and Purcell 2001, Purcell 1980b), there is little data on the effect of dormancy on bacterial over wintering in almonds. Previous studies suggested (Almeida and Purcell 2003) that almond-type *Xf* had better over winter survival in almonds. Since both grape and almond strains reached approximately the same titers in plants, and the grape strains in this study initially infected almond trees at a greater rate, over winter survival may be explain why almond strain *Xf* is so prevalent in naturally-occurring infections.

Table 1. Number of leaves showing almond leaf scorch symptoms on trees held under varying overwintering conditions of different temperatures and cold storage periods.

Cold Storage (in months)	Temperature (°C)		
	1.7	7.0	Ambient (Fresno Co.)
1	17.33 ± 8.22 a, A	35.14 ± 9.26 a, B	23.71 ± 8.21 a, B
2	22.67 ± 5.51 a, AB	22.22 ± 6.82 a, B	16.50 ± 6.37 a, B
4	50.00 ± 9.75 a, B	43.25 ± 7.18 a, B	30.50 ± 5.08 a, B

Different letters (small case) indicate a significant different in each row among tested temperatures under similar cold storage periods, and different letters (upper case) in each column among different cold storage periods (upper case) under similar temperatures. Tukey's test, $P < 0.05$.

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EVALUATING THE ROLES OF PILI IN TWITCHING AND LONG DISTANCE MOVEMENT OF *XYLELLA FASTIDIOSA* IN GRAPE XYLEM AND IN THE COLONIZATION OF SHARPSHOOTER FOREGUT

Project Leaders:
Harvey C. Hoch
Dept. of Plant Pathology
Cornell University, NYSAES
Geneva, New York 14456

Thomas J. Burr
Dept. of Plant Pathology
Cornell University, NYSAES
Geneva, New York 14456

Post-doctoral Researcher:
Leonardo De La Fuente
Dept. of Plant Pathology
Cornell University, NYSAES
Geneva, New York 14456

Cooperators:
A.H. Purcell
ESPM-Division of Insect Biology
University of California
Berkeley, CA 94720-3112

Rodrigo Almeida
ESPM-Division of Insect Biology
University of California
Berkeley, CA 94720-3112

Steven E. Lindow
Dept. of Plant and Microbial Biology
Berkeley, CA 94720-3112

Other Researchers:
Cheryl Galvani and Yaxin Li

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ABSTRACT
Our investigations have focused on intra-plant movement and colonization by *Xylella fastidiosa* (*Xf*). This study is particularly directed toward elucidating how *Xf*, once introduced into xylem vessels, moves in these elements farther upstream (down shoots and canes). In addition, it reports on the influence of the physical and chemical environments of the xylem as they relate to *Xf* attachment, colony development, and biofilm formation in both plant tissues as well as microfludic chambers fabricated to mimic xylem elements. Toward these goals, we have made extensive use of mutants deficient for various traits of pili and fimbriae.

INTRODUCTION
How *Xylella fastidiosa* (*Xf*) moves in xylem elements upstream against the flow of the transpiration stream and into petioles from the leaf or down shoots and canes has long been a particularly puzzling and important question, the answer of which could provide clues about better disease control practices through knowledgeable timing of pruning or roguing. Certainly, it would provide significant advances in the biology of the pathogen. Our studies have been directed toward elucidating movement of *Xf in planta* and *in vitro*, as well as how these bacteria colonize and establish biofilms. Toward this, we have made use of a number of *Xf* mutants deficient in traits important for cell attachment, movement, and colonization. Previously, we reported that migration of individual *Xf* bacteria occurred both *in vitro* and *in planta*, and that such migration occurred against a strong current of flowing media (Meng et al., 2005; Hoch, 2005). Such movement is characteristic of twitching motility that occurs in some gram-negative bacterial species. It is mediated by type IV pili (Mattick, 2002). There are several important implications of this observation: this was not only the first observation of twitching movement by a non-flagellated plant pathogenic bacterium (albeit, *Ralstonia solanacearum*, that sometimes has flagella, has been shown to exhibit colony features characteristic of twitching (Liu et al., 2001)), it was also the first time that such movement by *Xf* was observed. Such motile behavior is important in explaining, in part, the query posed above about how the bacteria spread in the grapevine from an inoculation point to upstream locations.

Type IV pili are long (1-5.8 µm in length) filamentous appendages (a.k.a, fimbriae) located at either one or both poles, depending on the species (Bradley, 1980), are generally 5-7 nm in diameter, and may be up to several micrometers in length. They are assembled primarily from single structural protein subunits, pilin (PilA) (Mattick, 2002). Twitching movements are generated as the pili are retracted and dissembled. Because the pili tips are attached to the substratum, the cell moves toward that point of contact as the pili shorten (Mattick, 2002; Skerker and Berg 2001). Genomic analysis of *Xf* indicates that there are at least 26 genes related to pili synthesis and function (Simpson et al., 2000). In addition to the type IV pili, *Xf* has shorter (0.4-1.0 µm in length) type I pili, also positioned on the same cell pole. The unique dual-pili composition of the wild type *Xf* presents an opportunity to study the two types of pili comparatively in the same experimental setting. Biofilm deficient mutants (e.g., 6E11), the result of a disruption of the *fimA* gene (lacking type I pili), were previously shown to continue to migrate since they still possess the type IV pili; whereas, mutants deficient in genes that code for type IV pili (e.g., 1A2) are migration deficient and develop robust biofilms (Meng et al., 2005). Attachment of *Xylella* cells at their polar ends is well documented in the precibarium region of the sharpshooter foregut. At this point, however, little is known about how they attach in this orientation (other than the conjecture that the pili may be involved) to this preferred region, as opposed to other foregut regions. Additionally, little is known about how they detach from this region.

OBJECTIVES
Our goal is to understand how *Xf* colonizes plants and insects. One aim is to identify factors that contribute to attachment (and detachment) and migration of *Xf* cells on surfaces. Using wild-type and mutants of *X. fastidiosa*, we have proposed to

examine temporal and spatial interactions on both native and artificial surfaces using a microfabricated *in vitro* microfluidic system that mimics features of xylem vessels. It has thus far provided significant new insight into the dynamics of *Xf* cell-surface relationships.

RESULTS

Mutants. As previously reported (Hoch and Burr, 2005), the EZ::TN Transposome system was used to generate Kanamycin-resistant mutants from the Temecula isolate of *Xf* (Guilhabert et al., 2001). Mutants with deficiencies in pilus and/or fimbrial gene products were sought that would affect colony and biofilm development, and the ability to migrate via type IV pilus twitching motility. We previously reported that *Xf* mutants (1A2, 5A7, and 6E11) were deficient in the genes *pilB*, *pilQ*, and *fimA*, respectively (Meng et al., 2005). The first two mutants are deficient in twitching motility characteristics since they lack type IV pili, while the latter mutant retains its motility phenotype, having type IV pili, but lacking the shorter type I pili. We have now generated more than 30 single-site mutations representing deficiencies in more than 14 genes associated with pili and fimbria function. 'Double mutants' were generated through a second round of mutagenesis using trimethoprim (as the selection agent) of the 6E11 (*fimA*) *Xf* mutant and has resulted in mutants deficient for the genes *fimA/pilC*, *fimA/pilO*, *fimA/pilX* (Table 1).

Table 1. Partial listing of *Xf* Temecula mutants.

Gene	Single Mutation	ORF	Gene Product	Observed Characteristics *in vitro*
pilB	1A2	PD1927	Pilus biogenesis protein	No twitching motility.
pilQ	5A7	PD1691	Fimbrial assembly protein	No twitching motility.
fimA	6E11	PD0062	Fimbrial subunit precursor	Twitching motility. Colony 'fringe' wider than wild type.
pilX	20D10	PD0022	PilX protein	Twitching motility in chamber not examined. Colony 'fringe' deficient.
pilO	TM1	PD1693	Fimbrial assembly membrane protein	No twitching motility
pilO	TM5	PD1693	Fimbrial assembly membrane protein	Twitching motility in chamber not examined. Colony 'fringe' deficient.
pilC	TM6	PD1923	Fimbrial assembly protein	Twitching motility in chamber not examined. Colony 'fringe' reduced.
pilR	TM7	PD1928	Two component system regulator	Twitching motility in chamber not examined. Colony 'fringe' deficient.
pilY1	TM14	PD0023	PilY1 protein homolog, pilus tip protein	Twitching motility. Colony margin smooth to crenulate.
pilR	TM17	PD1928	Two component system regulator	Twitching motility in chamber not examined. Colony 'fringe' deficient.
	Double Mutation			
fimA, pilO	DM12	PD0062 PD1693	Fimbrial subunit precursor Fimbrial assembly membrane protein	No twitching motility. Colony 'fringe' deficient.
fimA, pilX	DM16	PD0062 PD0022	Fimbrial subunit precursor PilX protein	No twitching motility. Colony 'fringe' deficient.
fimA, pilC	DM15 DM11	PD0062 PD1923	Fimbrial subunit precursor Fimbrial assembly protein	No twitching motility. Colony 'fringe' deficient.

Transmission electron microscopy (TEM), atomic force microscopy (AFM), and/or confocal microscopy (LSCM) using Agdia's antibody to *Xylella* (Carbajal et al., 2004) were used to assess for the presence of pili. In addition, light microscopy in conjunction with time-lapse imaging and image analysis was used extensively to assess motility, motility rates, adhesiveness of *Xf* wild-type and mutants to substrata, colony development, and cell 'autoaggregation'. Microfluidic devices fabricated to mimic xylem vessel characteristics were prepared similar to the protocols previously described (Meng et al., 2005).

Disease development in grapevines infected by pili-defective mutants. To date, a number of pilus-defective mutants have been introduced into greenhouse grown Cabernet Sauvignon vines and evaluated for PD symptom development. Notable is that the wild-type Xf Temecula isolate and the mutant 6E11 (type IV pili only) expressed symptom development at about the same time (9 weeks following needle inoculation), where as the double mutant DM12 (Table 1) remained asymptomatic until the 18th week. The single mutant TM7 remains asymptomatic to date. Similar results with these mutants are being observed by our cooperator Rodrigo Almeida in an ongoing project..

Figure 1. PD symptom expression of WT *Xf* vine 10 weeks after inoculation. Asymptomatic vine (17 weeks) inoculated with mutant DM12

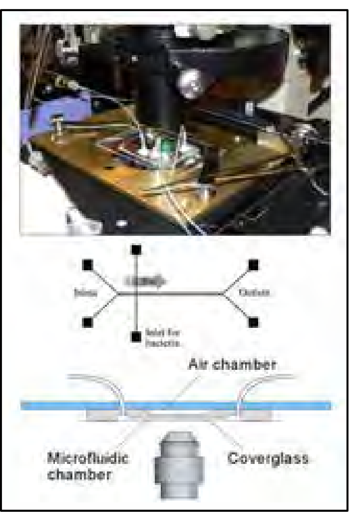

Figure 2. Microscope and dual channel (80 µm wide, 50 µm deep) microfluidic chamber configuration.

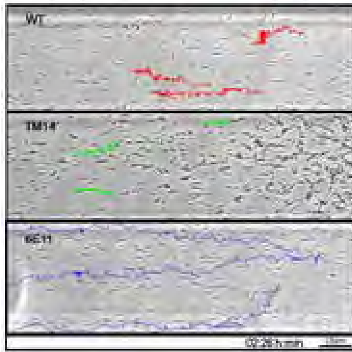

Figure 3. Relative rate of twitching motility of *Xf* wild type and pili-defective mutants. Color tracks correspond to twitching distance (right to left) over 2h 26min. Media flow, left to right.

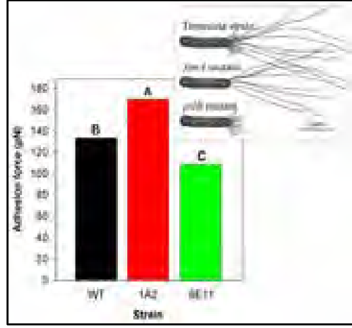

Figure 4. Adhesion forces required to remove *Xf*. Letters A, B, and C indicate differences in significance at 0.05.

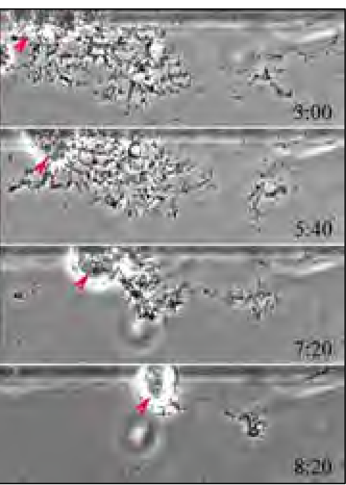

Figure 5. Autoaggregation in 9-day old culture, first noted as dispersed cells aggregated into developing cell mass (upper frame). Cells continued to be attracted to the developing aggregate (arrowheads). Times, h:min.

Motility of pili-defective mutants. We reported that *Xf* moves via type IV pili-dependent twitching-motility in 'artificial xylem' vessels against a flow (Meng et al., 2005). We now report that mutations in the pili genes influence not only the ability of the cell to exhibit twitching motility, but also the rate at which they move. Clearly, mutants without type IV pili, e.g., 1A2 (*pilB*), 5A7 (*pilQ*) are incapable of moving as has been previously demonstrated (Meng et al., 2005); however, there are a number of type IV pili mutations that do influence motility. To examine rates of movement, a microfluidic chambers was designed with side-by-side channels separated by a 50 µm-wide partition so that two mutants or isolates could be compared at the same time, under the same conditions (Figure 2). We compared the rate of twitching-motility of the wild type isolate with mutants TM14 (*pilY1*$^-$) and 6E11 (*fimA*$^-$) against a constant stream flow. The wild type isolate moved at 0.7 µm min^{-1}, whereas mutant TM14, which lacks a *tip protein* of the type IV pili, exhibited a significantly slower motility rate of 0.2 µm min^{-1} (Figure 3). Mutant 6E11 (lacking the short type I pili) moves 7 times faster than the wild type (5 µm min^{-1}). The short type I pili likely function as a "brake" reducing the speed of movement of wild type compared to the mutant 6E11.

These results agree with our observations (see **Adhesiveness of type I and type IV pili**) that type I pili support the strongest adhesion force between the bacterial cells and a surface, as well as developing greater amounts of biofilm (Li et al., 2006).

Adhesiveness of type I and type IV pili. Adherence to a surface is a crucial early event in the process of bacterial biofilm formation. Adherence is especially important for *Xf* since it inhabits xylem vessels where sap is flowing. Using microfluidic devices we measured the adhesion force of *Xf* wild-type and pili-defective mutants. *Xf* cells were introduced into the chambers where they attached to the substratum. Adhesiveness of the cells was assessed by gradually increasing the flow of media through the chamber with a syringe pump. The number of cells remaining attached to the glass surface after each flow rate increase was captured digitally and scored. By means of a computational model we determined the adhesion force exerted by each bacterial cell. Using selected pili mutants, the role of pili-type in adhesion was determined. Mutant 1A2 (short type I pili-only) had a significantly greater adhesion force (170 pN) than the wild type isolate (both type I and type IV pili) (134 pN). Mutant 6E11 which posses only long type IV pili had the weakest adhesion (108 pN) (Figure 4). These results further confirm that type I pili play a dominant role in *Xf* attachment to a substratum. The presence of type IV pili reduces the strong attachment exerted by type I pili, as was observed in the wild type isolate.

Cell aggregation and autoaggregation. When, where, and how individual *Xf* cells come together to form aggregates and biofilms to block xylem vessels remains poorly understood. It has been assumed that such cell masses develop as sessile cells divide, remain in place, and slowly accumulate mass. Such ‘slow’ aggregation does not explain how symptom development (reddening and drying of leaf margins) often occurs within a short time span—from overnight to a few days. We discovered using time-lapse imaging that ‘autoaggregation’ of many dispersed *Xf* cells into large cell masses occurs over relatively short time periods following 7-11 days of growth. Such large aggregates developed over periods ranging from 3-10 hours (Figure 5).

Influence of BSA on twitching motility. It was previously shown that the wild-type *Xf* Temecula isolate developed a ‘peripheral fringe’ around colonies (Meng *et al*., 2005) grown on PW agar, a trait associated with type IV pilus-mediated twitching motility. During the course of a survey of various wild-type pathogenic *Xf* isolates to ascertain that twitching motility is a characteristic of other wild-type isolates, we discovered that the concentration of bovine serum albumin (BSA) in the medium dramatically influenced whether or not a peripheral fringe was associated with the colonies. For some isolates, as well as the *fimA* mutant 6E11, a barely perceivable fringe was frequently observed at the highest BSA concentration (3.5-6.0 g L^{-1}), while medium with no or low BSA (0-1.8 g L^{-1}) produced the widest and most pronounced fringe. At the same time, the higher BSA concentrations nearly always produced larger colonies which, in part, substantiated the original report describing the need for BSA in PW medium for *Xf* (Davis *et al*., 1980).

CONCLUSIONS

Observations from this period demonstrate the pronounced role that pili and fimbriae have in *Xf* attachment, movement, colonization, and biofilm formation, as well as being involved in the timing of disease expression. We have again demonstrated that ‘artificial xylem vessels’ can be used to gain valuable information about the biology of *Xf*, and to infer roles for these phenomena *in planta*. Temporal and spatial data are not easily obtainable *in planta* without destructive sampling. In this report we describe autoaggregation in *Xf*, a phenomenon that could explain the rapid development of symptoms in grapevines affected by PD. The data on the speed of movement and the adhesiveness to a surface by different pili-defective mutants provide information on the relevance of type I and type IV pili regarding cell attachment and motility. Type I pili have a decisive role in “slowing” down cell motility and strongly attaching the cells to the surface. We demonstrated that the appearance of a peripheral fringe around *Xf* colonies is suppressed by higher BSA concentrations, thus explaining why such a colony morphology (and twitching motility) has not been readily observed.

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

Project Leader:
Michele M. Igo
Section of Microbiology
University of California
Davis, CA 95616
mmigo@ucdavis.edu

Cooperators:
Ayumi Matsumoto
Dept. of Food Science & Technology
University of California
Davis, CA 95616
amatsumoto@ucdavis.edu

Bruce Kirkpatrick
Department of Plant Pathology
University of California
Davis, CA 95616
bckirkpatrick@ucdavis.edu

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ABSTRACT

Autotransporters are multi-domain proteins that are responsible for secreting a single specific polypeptide (the passenger domain) across the outer membrane of Gram-negative bacteria. At last year's Symposium, we reported our studies characterizing a strain containing a kanamycin insertion in the putative autotransporter protein PD0528. Here, we report the results of our complementation analysis with the PD0528::Km mutant strain. Reintroduction of the wild-type PD0528 gene into the mutant strain restored the presence of PD0528 and a number of other proteins to the *Xf* outer membrane. However, reintroduction of PD0528 did not correct the phenotypic properties of the mutant strain or restore a wild-type phenotype. This would suggest that a second mutation(s) was acquired during the initial construction of the PD0528::Km mutant strain. Therefore, we decided to regenerate a null mutation in PD0528 using a variety of different strategies. None of these strategies were successful, which suggests that PD0528 might be an essential gene. During the course of these experiments, we also discovered a technical problem with the selection method we used for gene replacement in *Xf*. Although this may be a problem unique to our laboratory, we decided to include these data in case other researchers in the field are running into similar technical difficulties.

INTRODUCTION

The Gram-negative bacterium *Xylella fastidiosa* (*Xf*) is the causative agent of Pierce's disease (PD) of grapevine (Hopkins and Purcell 2002). The ability of *Xf* to colonize the plant and to incite disease is dependent upon the capacity of the bacterium to produce a diverse set of virulence factors. Many of these virulence determinants are proteins that are either secreted to the bacterial cell surface or released into the external environment (Meidanis *et al.* 2002, Smolka *et al.* 2003). In Gram-negative bacteria, secretion occurs through one of five major secretion pathways, numbered I to V (Preston *et al.* 2005). These pathways are highly conserved and exhibit functionally distinct mechanisms of protein secretion.

One of the simplest secretion mechanisms is exhibited by the AT-1 autotransporters, a subcategory of Type V secretion systems (for a review, see Henderson *et al.* 2004). AT-1 systems are dedicated to the secretion of a single specific polypeptide called the passenger domain across the outer membrane. Virulence functions associated with passenger domains include proteolytic activity, adherence, biofilm formation, intracellular motility, cytotoxic activity, or maturation of another virulence determinant. Based on genomic analysis, there are six members of the AT-1 autotransporter family in *Xf*-PD. Three of these proteins (PD0218, PD0313, PD0950) are predicted to encode subtilisin-like serine proteases (Bateman *et al.* 2004). The fourth protein (PD1879) is predicted to encode a member of the GDSL family of esterase/lipases (Bateman *et al.* 2004). The last two AT-1 proteins, PD1379 and PD0528, contain tandem repeats of a 50-60 amino acid motif within their passenger domains. PD1379 contains three copies of this repeat, whereas PD0528 contains six copies. Interestingly, this motif is only found in *Xf* species (Bateman *et al.* 2004). Given the importance of AT-1 autotransporters in pathogenicity, the secretion of this unique motif to the *Xf* cell surface could have important implications in the PD infectious cycle. To address this and other questions concerning the role of these species-specific tandem repeats in *Xf* virulence, we have been conducting a detailed characterization of the putative autotransporter protein, PD0528. Our studies of PD0528 were also designed to develop the protocols and genetic tools necessary for characterizing all six *Xf*-PD AT-1 autotransporters.

OBJECTIVES

1. Determine the role of the six *Xf*-PD autotransporter proteins and their passenger domains in *Xf* cellular physiology and virulence. Given the importance of AT-1 proteins in the virulence of other Gram-negative pathogens, it is highly likely that most of the *Xf*-PD AT-1 proteins will play an important role in *Xf* virulence.
2. Generate a mutation in each of the six AT-1 genes and determine their impact on *Xf* cell physiology and virulence.]
3. Examine the biochemical properties and location of the six AT-1 passenger domains. Priority will be given to any gene identified in Specific Aim 1.

RESULTS

One of the most abundant, integral *Xf-PD* outer membrane proteins is the gene product encoded by the PD0528 locus. Based on its predicted amino acid sequence, PD0528 is a putative AT-1 autotransporter protein that has a passenger domain containing six tandem repeats of a species-specific 50-60 amino acid motif. In order to investigate the role of PD0528 in *Xf-PD* cell physiology and virulence, we generated a null mutation in the PD0528 gene using the gene replacement method

described by Feil *et al.* (2003). This procedure involved generating the plasmid pAM12, which carried a kanamycin resistance marker flanked on each side by chromosomal sequences from immediately upstream and downstream of the PD0528 opening reading frame (ORF). This plasmid was then introduced into *Xf* by electroporation. The resulting kanamycin resistant transformants were selected on PD3 containing 5 µg/ml kanamycin and screened by PCR to identify a mutant strain in which the PD0528 ORF was completely removed and replaced by the kanamycin resistance marker.

Our initial characterization of the PD0528::Km deletion mutation was reported in the Symposium Proceedings for 2005. In these experiments, *Xf* membrane proteins were extracted using the BioRad ReadyPrep™ Protein Extraction Kit (Membrane 1). Although this method did not allow us to distinguish between outer and inner membrane proteins, it allowed us to quickly compare the total membrane profiles of different *Xf* strains. As expected, comparison of the membrane profiles of a wild-type *Xf-PD* strain and an *Xf-PD* strain carrying the PD0528::Km mutation revealed that the band corresponding to the PD0528 protein is missing in the PD0528::Km mutant. However, it was not possible to use membranes prepared with the protein extraction kit to obtain more detailed information concerning the impact of the PD0528::Km mutation on the *Xf* outer membrane protein profile. Therefore, we repeated this analysis using membranes prepared by a different method. In these experiments, the *Xf* strains were grown in 1 liter of PD3 medium and the harvested cells were then rupture with a French pressure cell as described previously (Igo 2003). The outer membrane fractions were isolated by sucrose density gradient centrifugation. The proteins were then analyzed using SDS-polyacrylamide (PAGE) gel electrophoresis.

Comparison of the membrane profiles of a wild-type *Xf* Temecula strain and an isogenic PD0528::Km mutant revealed that there are many differences in the outer membrane protein profiles of the two strains (Figure 1). Similar results were obtained with an *Xf* Travers strain containing the PD0528::Km mutation (data not shown). In addition to the absence of the PD0528 protein, the outer membrane of the PD0528::Km mutant is missing a number of other outer membrane proteins. One simple explanation for this result is that the absence of the PD0528 protein is causing a major perturbation in the protein composition of the outer membrane and of the *Xf* cell surface. However, it is also possible that a second mutation(s) was acquired during the construction of the original PD0528::Km mutation and that this second mutation(s) is responsible for the phenotype.

To distinguish between these possibilities, we performed complementation analysis using the plasmid pAM61. pAM61 carries the wild-type PD0528 gene and is a derivative of the plasmid pBBR1MCS-5 (Kovach *et al.* 1995). We chose this plasmid vector because a pBBR1MCS-5 derived plasmid was successfully used by Gabriel and his colleagues for complementation of the *Xf tolC* gene *en planta* (Gabriel 2005). We began our complementation analysis by introducing the plasmid pAM61 into the PD0528::Km mutant and then compared the membrane protein profile of the resulting strain to the wild-type strain and the PD0528::Km mutant. As shown in Figure 1, the PD0528 protein is present in both the wild-type strain (Lane 1) and the PD0528::Km mutant strain containing plasmid pAM61 (Lane 3). The identification of the band indicated by the arrow as the PD0528 protein was confirmed by MALDI-TOF mass spectrometry. Moreover, the reintroduction of the PD0528 gene into the mutant strain also restores many proteins to the outer membrane that were missing in the outer membrane of the PD0528::Km mutant. This would suggest that the absence of the PD0528 protein is having a profound impact on the protein composition of the outer membrane. However, it is worth noting that the reintroduction of PD0528 does not completely restore the wild-type outer membrane profile, suggesting that there might be a second mutation(s) in the PD0528::Km mutant strain.

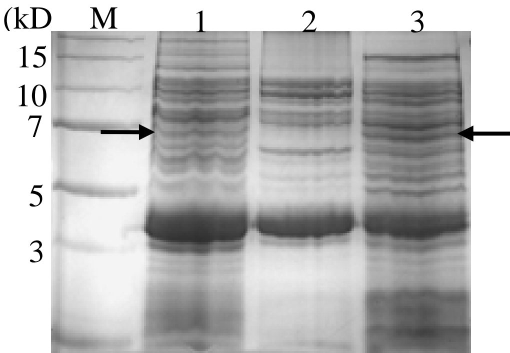

FIGURE 1. Membrane protein profiles.
Membrane proteins were isolated from the wild-type Temecula strain (Lane 1), the PD0528 deletion mutant (Lane 2), and from a strain containing both the PD0528 deletion mutant and pAM61, which carries a wild-type copy of the PD0528 gene (Lane 3). The membrane proteins were separated on an 8% SDS-PAGE gel and stained with Coomassie blue. The sizes of the molecular weight standards (lane M) are indicated on the right. The position of the PD0528 protein on the gel is indicated by the arrows.

We next examined how reintroduction of PD0528 impacted the phenotypic changes observed in the PD0528::Km mutant. As reported in the Symposium Proceedings for 2005, the strain containing the PD0528::Km mutation has a number of distinctive phenotypic properties: it grows faster than wild-type strains, it is impaired in its ability to form cell-to-cell aggregates in liquid culture, and it is able to form a continuous lawn on solid medium. We expected that the reintroduction of PD0528 to the PD0528::Km mutant would restore the wild-type phenotype. However, this was not the case. As summarized in Table 1, the phenotypic properties of the PD0528::Km mutant strain are not altered by reintroduction of the wild-type PD0528 gene on plasmid pAM61. This would suggest that there is a second mutation(s) in the PD0528::Km mutant and that this second

mutation(s) is responsible for the distinctive phenotypic properties of the PD0528::Km mutant. Since the properties of the second mutation(s) could be masking the phenotypic defects caused by the PD0528::Km mutation, we cannot draw any conclusions concerning how the absence of PD0528 impacts *Xf* cell physiology.

Table 1. Summary of the phenotypic comparison

	Wild-type	PD0528::Km (a)	PD0528::Km + pAM61 (b)
Growth in liquid culture	~7-10 days	~4-5 days	~4-5 days
Biofilm	Yes	No	No
Confluent lawns on plate	No	Yes	Yes
Produce PD symptoms	Yes	No	No

(a) These characteristics are present in both a PD0528::Km Temecula strain and a PD0528::Km Travers strain.
(b) After obtaining these results, we extracted pAM61 from the PD0528::Km mutant and resequenced the PD0528 gene on pAM61 to confirm that a functional copy of PD0528 had in fact been introduced into the strain.

We considered two possible explanations, which are not mutually exclusive, for the presence of this second mutation(s). One possibility is that the second mutation(s) is a consequence of the way we generated the PD0528::Km mutation. However, it is also possible that the PD0528 gene is essential. According to this hypothesis, the presence of the second mutation(s) is necessary for *Xf* survival in the absence of PD0528. To determine if either of these explanations is correct, we decided to regenerate the PD0528 null mutation. In one experiment, we introduced the PD0528::Km mutation using the gene replacement plasmid pAM12, but selected for transformants on PD3 plates containing either 5 µg/ml or 10 µg/ml kanamycin. In a second experiment, we introduced a PD0528::Cm mutation using a gene replacement plasmid similar to pAM12, which carried a chloramphenicol resistance marker. As a control, we also generated similar gene replacement plasmids for another *Xf* gene, PD0939, which encodes a phage-related protein. Transposon insertions into this gene have no effect on *Xf* physiology or pathogenicity (Guilhabert and Kirkpatrick, unpublished).

The results from this analysis, which are presented in Table 2, allowed us to draw a number of conclusions. First, when we select for either the PD0528::Km mutation or the PD0939::Km mutation on PD3 medium containing 5µg/ml kanamycin, we obtain fast growing transformants that are unable to form biofilms. We have performed this analysis multiple times and monitored the plates for the appearance of slower growing transformants over time. Nonetheless, in spite of repeated attempts, slow growing transformants were not obtained using PD3 medium containing 5µg/ml kanamycin as the selective medium. Second, we were able to obtain slow growing PD0939::Km transformants when we used PD3 medium containing 10 µg/ml kanamycin and PD0939::Cm transformants when we used PD3 medium containing 5 µg/ml chloramphenicol. Since the phenotypic properties of these transformants are the same as those of the PD0939::EZ-TN KAN-2 mutant, we have concluded that the fast-growing transformants we obtained by selecting on PD3 medium containing 5µg/ml kanamycin arose as a consequence of our selection procedure and that these transformants have probably acquired a second mutation(s). Third, although we made repeated attempts, we have been unable to generate PD0528::Cm transformants or PD0528::Km transformants when selecting on PD3 medium containing 10 µg/ml kanamycin. This would suggest that PD0528 is an essential gene. Experiments are currently underway to test this hypothesis.

Table 2. Summary of the transformation results

	Selection	*Xf* transformants	Growth in liquid	Biofilm	Confluent lawn
PD0939::EZ-TN KAN-2	Km-5µg/ml	Yes	7-10 days	Yes	No
PD0939::Km	Km-5µg/ml	Yes	4-5 days	No	Yes
	Km-10µg/ml	Yes	7-10 days	Yes	No
PD0939::Cm	Cm-5µg/ml	Yes	7-10 days	Yes	No
PD0528::Km	Km-5µg/ml	Yes	4-5 days	No	Yes
	Km-10µg/ml	No	-	-	-
PD0528::Cm	Cm-5µg/ml	No	-	-	-

During the last year, we have also examined the feasibility of using the PD0528 promoter for expressing genes in *Xf*. For this analysis, we generated fusion constructs between the PD0528 promoter and the firefly luciferase gene on plasmid pBBR1MIC-3, which confers tetracycline resistance (Kovach *et al.* 1995). The resulting plasmid was then introduced into the *Escherichia coli* strain DH5α and into the *Xanthomonas campestris pv. campestris* (*Xcc*) strain Xcc8004. Our initial results are very promising. Although the PD0528-*luc* fusion is expressed at a very low level in *E. coli*, it is highly expressed in *Xcc*. Because there is not a homolog to PD0528 in *Xcc*, it might be possible to gain insights into the factors involved in the regulation of PD0528 in *Xf* by examining its regulation in *Xcc*.

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

Project Leader:
Bruce Kirkpatrick
Department of Plant Pathology
University of California
Davis, CA
bckirkpatrick@ucdavis.edu

Cooperators:
Tanja Voegel
Department of Plant Pathology
University of California
Davis, CA
tmvoegel@ucdavis.edu

Jeremy Warren
Department of Plant Pathology
University of California
Davis, CA
jgwarren@ucdavis.edu

Michele Igo
Section of Microbiology
University of California
Davis, CA
mmigo@ucdavis.edu

George Bruening
Department of Plant Pathology
University of California
Davis, CA
gebruening@ucdavis.edu

Paul Feldstein
Department of Plant Pathology
University of California
Davis, CA
pafeldstein@ucdavis.edu

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

ABSTRACT
Xylella fastidiosa (*Xf*) possesses genes for hemagglutinins (HAs), large adhesion proteins involved in cell-cell aggregation and biofilm formation. Mutations in either one of the functional HAs, HxfA (PD2118) or HxfB (PD1792), result in hypervirulent strains that move faster and cause more severe disease in grapevines. Computer analyses of the HA proteins identified several regions that might be possible adhesion domains (ADs) responsible for cell-cell and/or cell-surface binding. We cloned 6 *Xf* HA fragments that may contain potential ADs into protein expression vectors and to date have prepared antibodies against 1 AD protein fragment that is conserved in both HxfA and B. Recombinant proteins from the other 5 ADs are being purified and prepared for injection. Western blot analyses of using *Xf* proteins extracted from *Xf* cells grown in liquid culture showed a very faint reaction with an *Xf* protein of approximately 220kd but this result needs to be confirmed using a higher quality antibody. Recent discoveries in the Bruening lab using phage technology indicate that HA are more abundant in cells grown on solid medium compared to liquid medium and we are now repeating our Western analyses using cells grown on solid medium. Once *Xf* HA cell-cell binding domains are identified they will be expressed in transgenic tobacco and grapevines where we hope the proteins will act as a “molecular glue” to aggregate insect-inoculated *Xf* cells, retard their ability to systemically colonize plants and potentially provide a unique form of resistance against PD.

INTRODUCTION
Xylella fastidiosa (*Xf*) *h*emagglutinins (HAs) are large secreted proteins (200-300kD) that play important roles in mediating cell-cell contact and plant pathogenicity. Mutations were made in both *Xf* HA genes, HxfA (PD2118) and HxfB (PD1792), by transposon mutagenesis and the resulting mutants did not form aggregates in liquid culture and they had reduced biofilm formation *in vitro* and *in planta* (Guilhabert and Kirkpatrick 2005). When inoculated into grapevines the mutant cells showed hypervirulence and more rapid colonization of xylem vessels (Guilhabert and Kirkpatrick 2005). The premise of this research is to determine whether over-expressing *Xf* HA adhesion domains in the xylem, either by transformation of grapevines or inoculation of grapevines with HA expressing endophytes, the HA will act as a “molecular glue” which clumps the *Xf* cells and retards their ability to systemically colonize grapevine and cause Pierce’s disease (PD).

Because of the large size of the HA genes (10kb), we cannot transform grapevines with the whole HA gene. Therefore we are trying to identify the active adhesion domains (ADs) responsible for cell-cell aggregation by dividing the HA genes into several smaller fragments that we believe will contain the cell-cell AD. Recombinant proteins derived from these fragments are being expressed in *E. coli*, purified and injected into rabbits to produce AD specific antisera. The resulting antisera will be used in ELISA, Western blot analysis, immunolocalization studies and cell-cell clumping experiments to determine which of the HA fragment(s) contain functional ADs that can later be transformed into plants.

OBJECTIVES
1a. Use antibodies we have prepared against a conserved, putative binding domain (AD2) that is present in both *Xf* hemagglutinins (HA), which we have named HxfA and HxfB, to determine the native size and location of *Xf* HA in cultured *Xf* cells and PD-affected grapevines.
  b. Determine if these antibodies (Fab fragments) can prevent cell-cell clumping in liquid *Xf* cultures.

c. Prepare an affinity column using HA domain antibodies and isolate native *Xf* HAs from culture cells. Establish the identity of affinity purified, putative HAs by n-terminal sequencing.
d. Determine if native HAs and HA domain fusion proteins can bind to *Xf* cells.
e. Inject affinity purified HA proteins into rabbits and obtain HxfA and B specific-antibodies. Determine if HxfA and B specific antibodies can block cell-cell clumping of *Xf* grown in liquid medium.

2a. PCR-amplify, clone and express as fusion proteins, additional hypothetical adhesion domains of HxfA and B.
b. Prepare rabbit polyclonal antibodies against each Hxf A/B domain fusion protein.
c. Determine if antibodies against various HxfA/B domain fusions can block cell-cell clumping of Xf grown in liquid medium.

3a. Transform bacterial grapevine endophytes with portion(s) of the Xf HA domains that mediate cell-cell clumping. Determine if transformed endophyte cells can bind *Xf* cells in vitro.
b. Transform tobacco, an experimental host of *Xf* and an easily transformable plant, with *Xf* HA binding domains. Use antibodies prepared in Objective 1 to determine if *Xf* HA proteins can be found in tobacco xylem fluid.

4a. Mechanically inoculate grapevines with *Xf* HA-expressing grapevine endophytes.
b. Mechanically inoculate endophyte colonized grapevines and HA-transgenic tobacco with wild type (wt) *Xf* cells. Compare disease progression and severity in endophyte colonized grapevines and transgenic tobacco with non-protected controls.

RESULTS

Objective 1a. Cloning of adhesion domain 2 (AD2) of HxfA and antibody generation

Because of the sequence similarity between AD2 of HxfB and HxfA, a 1133bp fragment from HxfA (Figure 1) was PCR-amplified, cloned in the expression vector pet29b and expressed as His-fusion protein in *E. coli.* The expressed protein was purified by Ni-column chromatography and the identity and integrity of the fusion protein was verified by sequencing. A total of 1mg purified protein was injected into a NZW rabbit after pre-immune serum was taken. Five bleeds were taken and the resulting anti-AD2 antibodies were analyzed by ELISA. Only a 2-fold signal increase occurred in indirect ELISA analyses of post-injection antisera compared to the pre-immune serum; this result indicated the AD2 antigen was not especially antigenic or that insufficient antigen was used to elicit high quality Abs. Although the antiserum was of comparative low titer, we used it in *Xf* cell clumping and Western blot analyses.

Determination of native size and location of *Xf* HA in *Xf* cultured cells and grapevines by Western blot analysis

Several Western blot experiments with a variety of protein samples of wild type Temecula, $HxfA^-$ and $HxfB^-$ mutants were conducted. The cells were grown in liquid PD_3 or XDM_2 (Leite et al. 2003) media for 10 days and 100ml of conditioned medium was concentrated using Centricon plus 70 filters (Millipore). The conditioned medium was purified by dialysis and ReadyPrep 2-D Cleanup Kit Biorad) and protein concentration was determined by using a Bradford assay. Western blots of whole cells and medium proteins were performed using the AD2 antibody. A faint band of ~200kDa could be detected in wild type whole cell protein, but not in proteins in purified medium samples suggesting that HAs are possibly not secreted but associated with the outer membrane of *Xf* cells. A corresponding band was not detected in whole protein extracts of the HxfA and B mutants (Figure 2). However, the signal was weak and there were non-specific reactions between other *Xf* cellular proteins and the AD2 Abs and additional experiments, preferably using higher quality antibodies produced against the other *XF* HA fusion proteins, will need to be done in order to confirm these preliminary results.

Objective 1b. Determine if AD2-antibodies or conditioned media can facilitate clumping of *Xf*.

A mixture of the 4th and 5th bleed AD2-antibodies was used to assess their ability to clump *Xf* cells in liquid culture. If the antibodies bound to HA domains that are responsible for cell-cell clumping, we might expect to see a decrease in clumping if the HAs were not located on the surface of the *Xf* cell which is what would be expected because other bacterial HAs are secreted. If the HA was physically linked to the *Xf* cell we would have expected an increase in clumping due to cross-linking *Xf* cells by the antibody. We observed no significant differences in clumping between antibody-treated and pre-immune serum which suggests that the AD2 domain is not present on the outside of *Xf* cells if the HA is associated with the outer membrane or that the AD2 does not mediate cell-cell interactions. These results indicate that additional putative ADs must be evaluated.

Experiments were also conducted with “conditioned” media. Conditioned media is PD3 medium in which wt *Xf* cells were grown to stationary phase and the cells were removed by centrifugation. Such medium would be expected to contain HA proteins if they were secreted. By adding HA-containing conditioned media in various concentrations to $HxfA^-$ and $HxfB^-$ mutant cells growing in log phase we would expect to complement with soluble HA and restore the clumping phenotype. No clumping was observed which is indirect evidence that *Xf* HA is not secreted into the medium, at least in large amounts.

Objective 2. Identification, cloning and expression of additional ADs for antibody generation

It has been shown for FHA, the filamentous hemagglutinin of *Bordetella pertussis* (Renauld-Mongenie et al. 1996) that the active HA domains are located at the N-terminal half of the protein and that C-terminal deletions have no effect of the HA activity or secretion of the protein. The secretion domain (TPS-domain) was identified at the N-terminal end of HxfA and B (Guilhabert and Kirkpatrick 2005) and additional analysis revealed a FhaB domain located between position 2000-2300 of

HxfA. This conserved domain is found in other bacterial proteins that mediate adhesion and therefore they could be act as a possible adhesion domain. To evaluate this hypothesis, a knockout in that region will be done, and the FhaB domain will be replaced by the kan-cassette. We also conducted hydrophobicity plots of the proteins to determine potential antigenic sites. An RGD (Arg-Gly-Asp) site at position 2780 in HxfA and at position 3062 in HxfB was found. The RGD site in the filamentous HA proteins of *Bordetella pertussis* mediates binding of B. pertussis to lung epithelial cells (Ishibashi et al. 2001).

The *in silico* analyses led us to divide HxfA and HxfB into three regions named AD1-3, AD4 and AD5, each for HxfA and HxfB (Figure 1). All six ADs were cloned into expression plasmid pet30b, the identity and integrity of the amplified fragments was verified by sequencing and the constructs were transformed into *E.-coli*. Vectors containing AD5 of HxfA and AD4 of HxfB and were transformed into the new expression *E. coli* host (ArcticExpress DE3, Stratagene). AD4 of HxfB was expressed to high levels and the integrity of the protein verified by sequencing. AD 4 antigen has been prepared and will be injected into rabbits on October 27. AD1-3 from HxfA and B, as well as AD4 from HxfA were also expressed at high levels and we are now verifying the sequence of these proteins with the anticipation that they will also be ready to inject into rabbits. Recombinant fusion proteins of AD5 of both HxfA and B seemed to be slightly smaller in size than expected so additional characterization of these proteins will be needed before preparing antisera.

Objectives 3 and 4: It will be necessary to identify HA cell-cell binding domains before these fragments can be transformed into plant hosts (tobacco and grapevines) or bacterial endophytes of grapevines. Good progress has been made towards identifying fragments of the *Xf* HAs that mediates cell-cell binding in the three months this project has received funding.

In addition, a collaborative effort between the Bruening and Kirkpatrick labs has identified M13 phage that can specifically bind to HxfA (see Bruening 2006 PD/GWSS report for details). This phage can be used much like the antibodies that are being raised against the various *Xf* HAs ADs described in this report. Our labs are now pursuing collaborative research to use the phage to determine the size and location of native *Xf* HAs and to use the specificity of the HxfA-specific phage to better understand the interactions that mediate *Xf* cell-cell attachment.

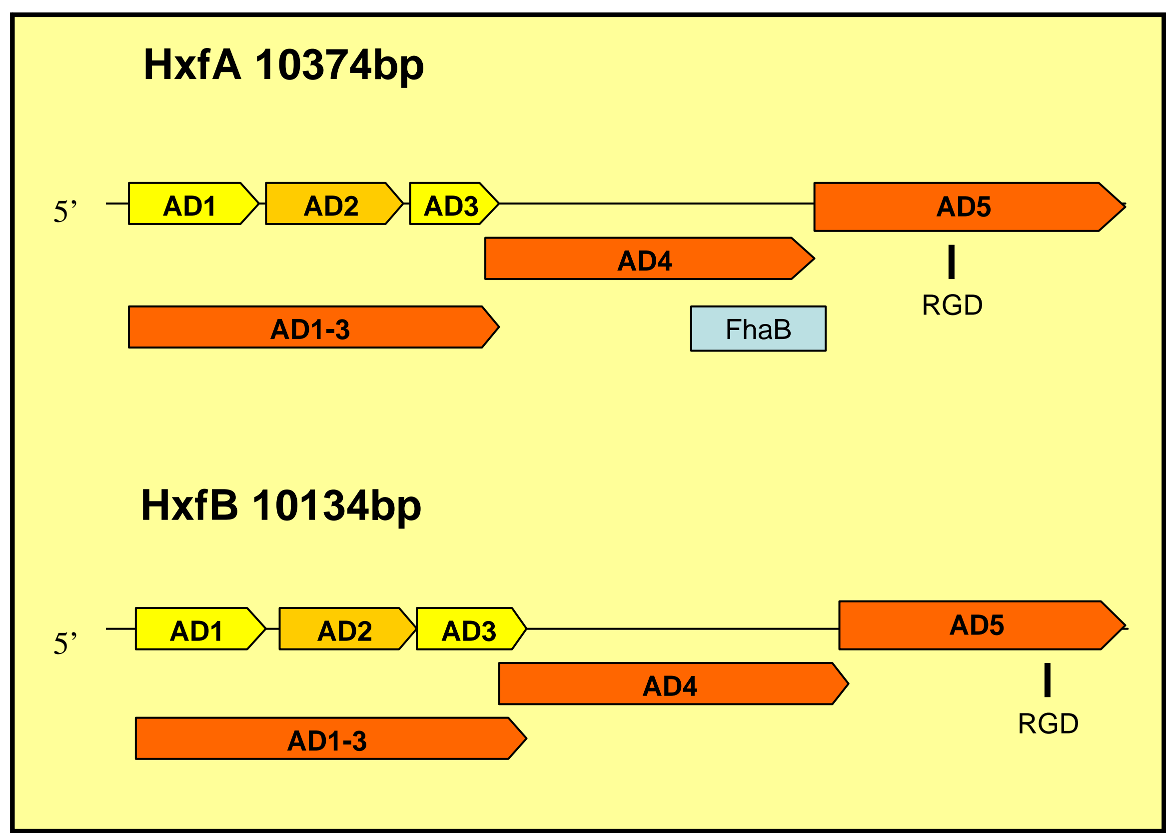

Figure1. Identification putative *Xf* HA adhesion domains (AD) based on data base analysis with other bacterial HAs and hydrophobicity plots. Antibodies made against AD2 of HxfA provided preliminary information regarding the size and locations of native HA. AD1-3, AD4 and AD5 of both HxfA and HxfB were cloned and expressed in *E. coli*. AD4 and AD1-3 from both HxfA and B are ready to be injected into rabbits while further characterization of AD5 from both HAs will be needed.

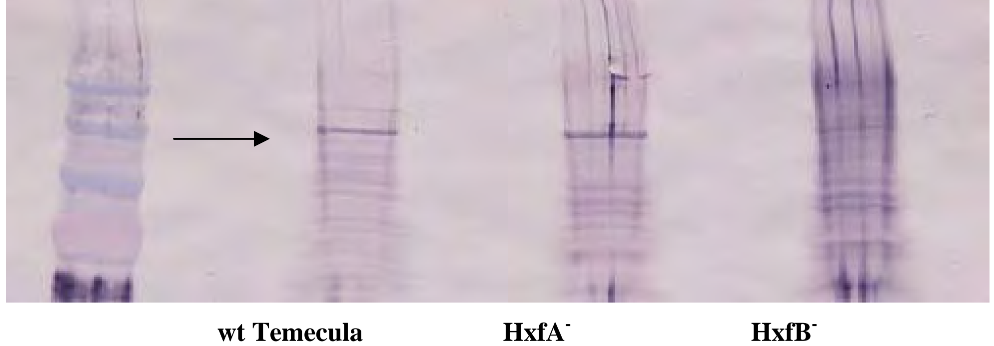

Figure 2. Western Blot analysis of whole cell proteins from wild type Temecula, HxfA and HxfB mutants. Arrow denotes a protein band of ~200kDa that is detectable in the wild type, but not in the mutant cells.

CONCLUSIONS

We have made good progress in our goal of identifying what HA domain(s) mediate cell-cell binding. Changes in the manner in which we express HA fragments in *E. coli*, i.e. using a different expression system (ArcticExpress DE3, Stratagene) which contains a second plasmid expressing chaperones that help fold over-expressed fusion proteins in the cytoplasm and therefore reduce their degradation and the amount of recombinanat protein that ends up in insoluble inclusion bodies, should yield both better antigens and more soluble proteins to perform *Xf* cell agglutination assays. We will also inject HA ADs into 2 rabbits instead of 1 to increase the likelihood of producing higher quality antibodies. The discovery made by Feldstein and Bruening of M13 phage that specifically binding to HxfA should also facilitate the localization and characterization of native HAs associated with *Xf* cells in culture and *in planta*. Once identified, cell-cell HA binding domains will be expressed in bacterial endophytes that will be inoculated into tobacco and grapevine that will be subsequently challenged with *Xf* to determine if these proteins can bind to and retard the systemic movement of *Xf* in plant hosts.

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DETECTION OF SIDEROPHORES IN THE ENDOPHYTIC BACTERIA *METHYLOBACTERIUM* SPP. ASSOCIATED WITH *XYLELLA FASTIDIOSA*

Project Leaders:

Paulo T. Lacava
University of São Paulo/ ES ALQ
Piracicaba, SP 13400-970
ptlacava@esalq.usp.br

Maria E. Silva Stenico
University of São Paulo/CENA
Piracicaba, SP 13400-970

Flávia T. H. Pacheco
University of São Paulo/CENA
Piracicaba, SP 13400-970

Cooperators:

Welington L. Araújo
University of São Paulo/ESALQ
Piracicaba, SP 13400-970

Ana V. C. Simionato
University of São Paulo/IQSC
São Carlos, SP 13560-970

Emanuel Carrilho
University of São Paulo/IQSC
São Carlos, SP 13560-970

Siu Mui Tsai
University of São Paulo/CENA
Piracicaba, SP 13400-970

João Lúcio Azevedo
University of São Paulo/ESALQ
Piracicaba, SP 13400-970

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ABSTRACT

We analyzed the production of siderophores by endophytic bacteria *Methylobacterium* spp., which occupy the same niche as *Xylella fastidiosa* (*Xf)* in citrus plants. All strains of *Methylobacterium* spp. tested were CAS-positive for siderophore production. *Methylobacterium* spp. produced hydroxamate-type, but not catechol-type siderophores. Specific primers for pyoverdin, a hydroxamate type ferrisiderophore receptor gene were used to amplify this gene from *Methylobacterium* strains. The growth of *Xf* was stimulated by the presence of a supernatant-siderophore of endophytic *Methylobacterium mesophilicum*. If *Xf* is able to use heterologous siderophores during its establishment inside the host plant, it may benefit from production of siderophores by endophytic symbionts.

INTRODUCTION

Endophytes colonize the living, internal tissues of plants without causing any immediate, over negative effects (Hallmann et al., 1997; Azevedo et al., 2000). Research has shown that endophytic microorganisms isolated from surface disinfected plant tissues exhibit a potential as biocontrol agents against phytopathogens (Sturz et al., 1998) and insects (Azevedo et al., 2000) as well as increasing plant growth and hastening plant development (Lodewyckx et al., 2002). However, synergistic interactions between endophytes and phytopathogens have not been studied yet.

Bacterial siderophores are low-molecular-weight compounds with high iron (III) chelating affinity (Sharma and Johri, 2003) that are responsible for the solubilization and transport of iron (III) into bacterial cells. Iron is an essential mineral and its sequestration by specific bacterial siderophores may induce the development of plant disease (Nachin et al., 2003; Etchegaray et al., 2004). Acquisition of iron from siderophores produced by other microbial species has already been described for *Escherichia coli*, *Salmonella typhimurium* (Martinez et al., 1990), *Actinobacillus pleuropneumoniae* (Diarra et al., 1996), *Streptomyces* sp. (Imbert et al., 1995), and *Arthrobacter flavescens* (Winkelmann, 1991).

Xylella fastidiosa (*Xf*) is the causal agent of Citrus Variegated Chlorosis (CVC), which is an important disease of citrus species (Hartung et al., 1994). In Brazil, over 70 million sweet orange trees (38%) are affected, and CVC is responsible for losses of US$ 100 million per year to the Brazilian citrus industry, affecting all commercial sweet orange varieties (de Souza et al., 2005). *Xf* was the first plant pathogen to have its genome completely sequenced and putative genes for membrane receptors, including siderophores, were found (Simpson et al., 2000).

The genus *Methylobacterium*, which occupies the same ecological niche as *X. fastidiosa*, was the most frequently isolated endophytic bacterium from CVC-symptomatic citrus plants (*Citrus sinensis*). Recently, an interaction between *Methylobacterium* species and *Xf* was strongly indicated (Araújo et al., 2002; Lacava et al., 2004).

OBJECTIVES

The aim of this study was to evaluate the ability of *Methylobacterium* spp., isolated as citrus endophytic bacteria (Araújo et al., 2002), to produce siderophores and to investigate the capacity of *Xf* to use siderophores produced by *Methylobacterium mesophilicum* (*M. mesophilicum*) for growth and development.

RESULTS

All strains of *Methylobacterium* spp. tested were CAS-positive for siderophores production (Table 1), and the siderophores production tested by the CAS-agar assay revealed that 66% of CVC-symptomatic, 55% of uninfected, 20% of asymptomatic and 10% of tangerine strains of *Methylobacterium* spp., showed very high production. Also, all strains of *Methylobacterium*

spp. were negative in the Arnow assay, which means that, these strains are negative for catechol-type siderophores (Table 1). However, all strains of *Methylobacterium* spp were able to product hydromate-type to varying degrees as shown by the by Csák assay (Table 1).

Only three strains of endophytic *Methylobacterium* (AR5.1/5, AR5.1/6 and AR1.6/2) were PCR positive (Table 1), but these three strains were isolated from plants that showed CVC symptoms (Araújo et al., 2002). The strains AR5.1/5 and AR5.1/6 also produced very high concentrations of siderophore in the CAS-agar assay. These primers were developed to detect pyoverdin, a siderophore hydroxamate-type receptor, in *X. fastidiosa* (Pacheco et al., 2001) and these same primers recognized at least in three strains of *Methylobacterium* the same amplicon detected in *Xf*.

Growth of the *Xf* in PW broth medium was stimulated by the presence of *M. mesophilicum* supernatants that contained siderophores (Figure 1) and inhibition of this same strain was observed in the negative control (PW broth medium without a source of iron) (Figure1).

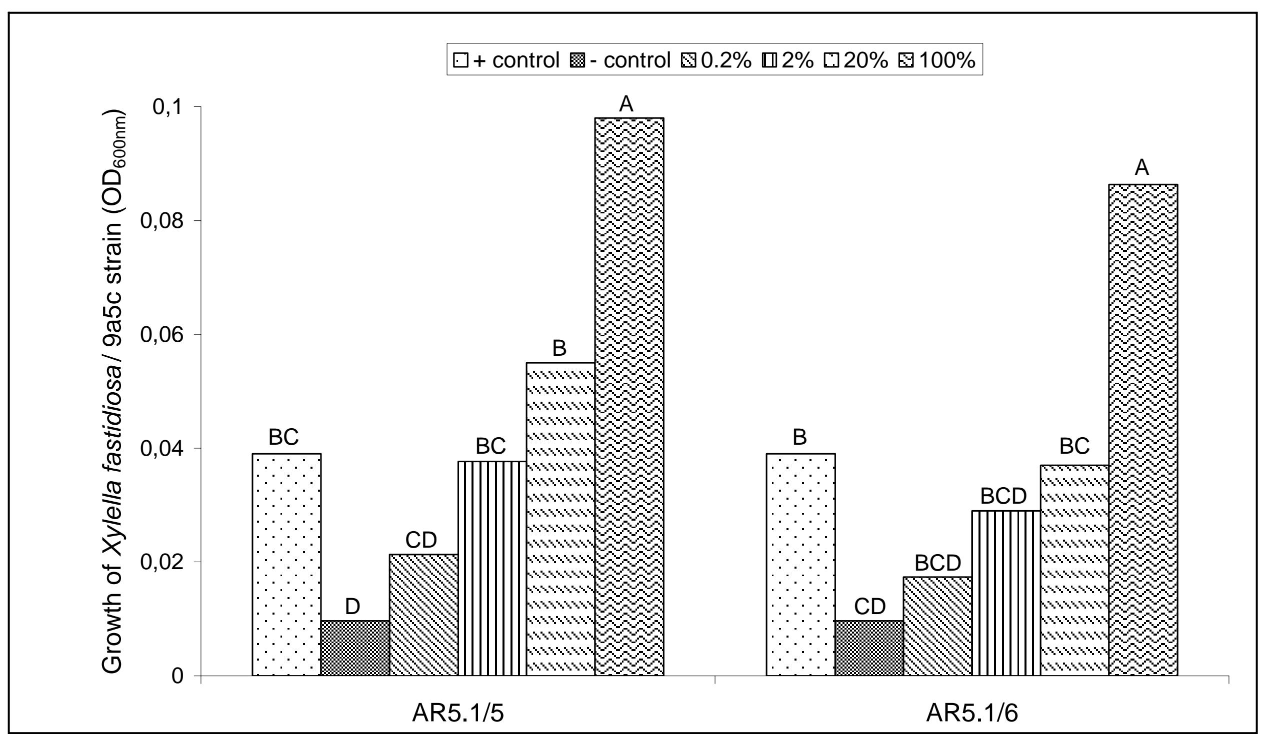

Figure 1. Effect of cell-free supernatants of the endophytic bacteria *M. mesophilicum* (AR5.1/5 and AR5.1/6 strains) with siderophore-production on the growth of *Xf* in PW broth medium. Negative control: PW broth medium without a source of iron and positive control: standard PW broth medium. Different letters on bars for same treatment means statistic difference by Tukey's test at 5% of significance.

Table 1. Siderophore production by endophytic *Methylobacterium* strains.

Strains	CAS-Agar Univesal test*	Csák test* (Hydroxamate-type)	Arnow test* (Catechol-type)	PCR**
AR1.6/1	+	+	-	-
AR1.6/6	+++	++	-	-
AR3/20	++	+	-	-
AR4/19	+	+	-	-
AR5/1	++	+	-	-
AR5.1/4	+++	+	-	-
AR5.1/5	+++	+++	-	+
AR5.1/6	+++	++	-	+
AR1.6/2	+	+	-	+
AR1.6/3	+++	+	-	-
AR1.6/8	+++	+	-	-
AR1.6/11	++	+	-	-
AR1.6/4	+	+	-	-
ER1/21	+	+	-	-
ER1.6/1	+	+	-	-
ER1.6/4	++	++	-	-
ER1.6/5	+++	+	-	-
ER5/2	++	+	-	-
SR1.6/6	+++	+	-	-
SR1.6/13	++	++	-	-
SR3/27	++	++	-	-
SR1.6/1	+++	+	-	-
SR1.6/15	++	+	-	-
SR5/4	++	++	-	-
SR1.4/10	+++	++	-	-
SR1.6/4	+++	+	-	-
SR1.6/2	+++	+	-	-
PR1/3	+	+	-	-
PR1.4/10	+++	+	-	-
PR2/2	+	+	-	-
PR3/5	+	+	-	-
PR3/11	++	++	-	-
PR3/15	+	+	-	-
PR5/4	+	+	-	-
PR5.1/1	++	+	-	-
PR3/8	++	++	-	-
PR3/17	++	+	-	-

* Intensity: -, none; +, low; ++, high; +++, very high
**Presence (+) or absence (-) of PCR product

CONCLUSIONS

The present data corroborates the hypotheses that there is a relationship between *Xf*, causal agent of CVC, and the endophytic bacteria *Methylobacterium* (Araújo et al., 2002; Lacava et al., 2004). In addition, our results indicated that *Xf* was able to use *Methylobacterium* siderophores *in vitro* as a source of iron (Figure 1), and suggested that in some instances *Methylobacterium* could help the growth of *Xf*, particularly under environmental conditions where iron sources are limited. Iron-siderophore complexes are taken up by specific transport systems, but some microorganisms have also developed transport systems for heterologous siderophores produced by other species (Raaijmakers et al., 1995; Howard, 1999).

Endophytes must compete with plant cells for iron supply, and therefore siderophore production may be highly important for endophytic growth (Idris et al., 2004). Additionally, the production of siderophores has been reported to be one of the mechanisms to outcompete pathogens (O'Sullivan and O'Gara, 1992; Schippers et al., 1987) and may have the same function in endophytes. The present study suggested that *Xf* can use molecules produced by endophytic bacteria as siderophores. In this context, as a factor influencing the symptom of CVC (Pacheco et al., 2001; Silva-Stenico et al., 2005), the genus *Methylobacterium* could help *Xf* to survive inside the xylem vessels because competition for iron in the environment has an important role in microbial systems.

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MULTI-LOCUS SIMPLE SEQUENCE REPEATS AND SINGLE NUCLEOTIDE POLYMORPHISM MARKERS FOR GENOTYPING AND ASSESSING GENETIC DIVERSITY OF *XYLELLA FASTIDIOSA* IN CALIFORNIA

Project Leader:
Hong Lin
Crop Disease, Pest, &
Genetics Research Unit
SJVAC, ARS-USDA
Parlier, CA 93648
hlin@fresno.ars.usda.gov

Cooperators:
Harshavardhan Doddapaneni
Dept. of Vitic. & Enology
University of California,
Davis, CA 95616

Andrew Walker
Dept. of Vitic. & Enology
University of California
Davis, CA 95616

Edwin Civerolo
SJVAC, ARS-USDA
Parlier, CA 93648

Russell L. Groves
Department of Entomology
University of Wisconsin
Madison, WI 53706

Reporting Period: The results reported here from work conducted October 2004 to September 2006

ABSTRACT
In California, information regarding the population structure and genetic diversity as well as the genetic evolutionary and epidemiological relationships among *Xylella fastidiosa* (*Xf*) strains in agricultural populations is not clear. To develop effective management strategies, we need to understand pathogen population structure and genetic diversity in the agricultural ecosystem. Here we report development and utilization of two multilocous marker systems, Simple Sequence Repeats (SSR) and single nucleotide polymorphism (SNP) for genotyping and assessing genetic diversity of *Xf* in California. Strain diversity studies using SSRs on samples from different geographic populations and/or from different hosts demonstrated that host selection plays an important role in *Xf* genetic differentiation among agricultural populations in California. Whole-genome comparison of four sequenced strains identified 12,754 potential SNPs in coding sequences and 20,779 SNPs in non-coding regions across four *Xf* strains. Small scale validation (16 loci) tests showed that SNP genotype is tightly linked to the hosts from which the strains were derived. Together, SNP and SSR marker systems appear to be useful tools for pathogen detection and population genetic analyses.

INTRODUCTION
Host plant resistance is a critical component of integrated crop management. However, durability of resistant grape plants depends upon the variability and adaptability of the pathogen population as well as disease resistance gene(s). Population genetics research demonstrates that the evolutionary potential of a pathogen is reflected in its genetic diversity and its genetic structure. Pathogen populations with higher evolutionary potential are more likely to overcome host resistance than pathogen populations with a lower evolutionary potential (MacDonald and Linde, 2002). The resulting changes in population structure or virulence can lead to host resistance breakdown. Therefore, understanding pathogen genetic diversity is critical in developing an effective disease control strategy. To characterize population structures of *Xylella fastidiosa (Xf)* and to understand genetic diversity of *Xf* in agricultural systems, sensitive and accurate marker system(s) are required. The goal of this project is to develop a reliable marker system(s) that unambiguously identifies *Xf* strains from various geographic locations and host plants and further to understand the pathogen dynamics. Previously, we reported the development of multilocus simple sequence repeats (SSR) markers for *Xf* population genetic analysis (Lin et al., 2005). This marker system appeared to be sensitive in detection and powerful in discriminating *Xf* genotypes. This marker system also provides high throughput capability for a large scale population sample analyses. Recently, we developed a new marker system; single nucleotide polymorphism (SNP). We performed whole-genome sequence analysis of CVC, PD, ALS and OLS strains and identified potential SNP loci in both coding and non-coding regions (Doddapaneni et al., 2006). This marker system has proven to be powerful and reliable for distinguishing genetic relatedness. This marker system is very sensitive, has a high degree of specificity, and is quite powerful in detecting genetic polymorphism. Further, adaptability to high a through-put diagnostic platform makes this system an ideal tool for large scale studies of *Xf* population genetics and epidemiological risk assessment analyses.

OBJECTIVES
1. Analyze *Xf* seasonal population dynamics; spatial and temporal disease development and genetic diversity.
2. Comparative whole genome analyses of the *X. fastidiosa* strains to identify SNP loci and develop SNP based marker system for fingerprinting *Xf* strains.

RESULTS AND DISCUSSION
Objective 1.
Previously, we analyzed genetic diversities and geographic population structures of *Xf* in California vineyards (Napa, Sonoma and Kern and Riverside counties). Results based on multi-locus SSR marker systems and hierarchical sampling

showed that a larger proportion of total genetic diversity (68.89 %) was attributed to genetic variation among different host plants. These results suggest that host selection plays an important role in *Xf* genetic differentiation among agricultural populations in California. Using the same SSR marker system, we extended our investigation to *Xf*-induced, almond leaf scorch (ALS) disease in California's San Joaquin Valley (SJV). Survey data for two years of ALS disease incidence was analyzed to characterize the progress of almond leaf scorch disease development within selected orchards and to evaluate the seasonal population dynamics associated with *Xf* adaptation and host selection in almond-associated populations. The seasonal collection and detection studies showed that *Xf* populations were low in early season (March and April), when *Xf* is less likely detectable by PCR. *Xf* populations quickly increased after May which parallels increased plant growth and the activity of *Xf* vectors. Successful *Xf* isolation/culture and PCR detection were comparable after July. To further characterize if the spatial and temporal aspects of disease development associated with *Xf* genetic differentiation among geographic locations and/or with host adaptation, two almond orchards, Richline and McCall, were studied in Kern and Fresno Counties, respectively. In the Richline orchard, two almond cultivars including Sonora and Nonpareil were affected with ALS symptoms while only the cultivar Sonora was *Xf*-infected at the McCall Orchard. ALS samples from these three populations; Richline-Sonora, Richline-Nonpareil and McCall-Sonora were collected from May to October months. Using five SSR markers, allelic types and allelic frequencies of haplotypes among these three populations were analyzed (Table 1). Genetic relationships among three populations showed that *Xf* population derived from Richline-Sonora was closely related to the population of McCall-Sonora even though they are geographically separated while the *Xf* population from the Nonpareil cultivar in Richline orchard was genetically distant from the population derived from Sonora at either location (Figure 1). This result again confirms our earlier findings that suggests host selection plays an important role in determining population differentiation.

Our next experiment is to determine host-pathogen-vector interactions. In this study we will address questions of host selection, pathogen adaptation, and the role of vectors in transmitting *Xf* strains. Specifically, a vineyard adjoining an almond orchard was chosen. We will analyze gene flow between populations resulting from the movement of the vectors. *Xf* strains will be collected from PD-affected vines and ALS affected almond trees. We will also collect samples of adult green sharpshooters which are actively dispersing in these sites. The first sampling was conducted from September to October in 2006 and the early sampling (April-May) and late sampling (August-October) will be conducted in 2007. That way, over-wintering populations will be included for determination the effect of genetic drift or initial inoculum sources.

Table 1. Allele frequencies of three ALS *Xf* populations at five SSR loci.

	Population I (Site = Richline, Host = Sonora)			Population II (Site = Richline, Host = NonPareil)			Population III (Site = McCall, Host = Sonora)		
Locus	Allele (bp)	# of Observations	Allele Frequency	Allele (bp)	# of Observations	Allele Frequency	Allele (bp)	# of Observations	Allele Frequency
ASSR 19	279	16	0.64	279	2	0.08	279	27	0.93
	288	5	0.20	288	21	0.92	288	1	0.03
	294	4	0.16	294	0		294	1	0.03
OSSR 12	279	9	0.36	279	23	1.00	279	2	0.07
	308	16	0.64	308	0		308	27	0.93
CSSR 12	282	9	0.36	282	22	0.95	282	2	0.07
	291	16	0.64	291	1	0.05	291	27	0.93
GSSR 20	304	10	0.40	304	22	0.95	304	2	0.07
	332	4	0.16	332	1	0.05	332	20	0.69
	341	11	0.44	341	0		341	7	0.24
ASSR 4	366	5	0.20	366	22	0.95	366	2	
	372	4	0.16	372	0		372	2	0.07
	393	16	0.64	393	1	0.05	393	22	0.75
	397	0		397	0		397	5	0.17

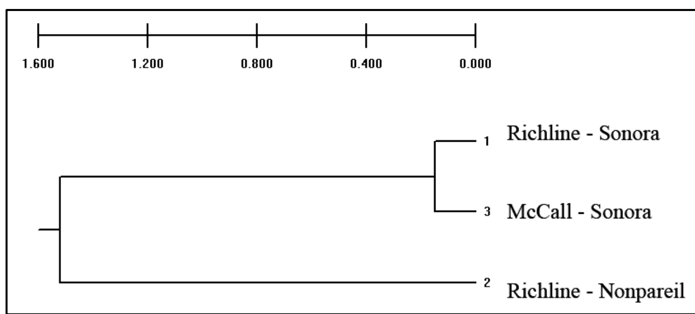

Figure 2. Genetic distances among three ALS *Xf* populations using UPGMA cluster analysis with Nei's coefficient.

Objective 2.

Comparative whole genome analyses of the *X. fastidiosa* strains: *Xf* Temecula-1 (grapevine strain), *Xf* 9a5c (citrus strain) *Xf* Ann1 (oleander strain) and *Xf* Dixon (almond strains) were carried out to identify SNP loci. The analyses identified 1,579 genes and 194 non-coding homologous sequences present in the genomes of all four strains, representing a 76.2% conservation of the entire genomic sequence. Our analysis shows that 51 Ann1 and Dixon genes were present at the end of sequenced contigs (partially sequenced) and were excluded from further analysis. Therefore, SNPs were identified in 1528 conserved gene alignment files. There were 12,754 potential SNPs in coding sequences and 20,779 SNPs in the non-coding sequences in these conserved regions. The average SNP frequency was 1.08×10^{-2} per base pair, which translates to approximately one SNP for every 93 bp of the DNA. SNPs were defined according to their nucleotide types as transversion or transition types. On average, 85.2% of SNPs are transversion type suggesting that transversion was the major type of SNP in *Xf*. In *Xf* 60.33% of the SNPs cause synonymous changes while 39.67% cause non-synonymous changes. For this analysis, those gene pairs that show internal INDELs that cause frame shift mutations were not included. A database called "Xfbase" has been developed under an IIS6.0 server using CGI scripts is available at http://cropdisease.ars.usda.gov/CVC_index.htm. A summary of whole genome SNP analyses is presented (Doddapaneni et al., 2006). We designed SNP detection primers for 16 SNP loci (Table 2) and validated by using 18 strains representing four strains from grape, citrus, almond and oleander plants. Strains were validated as *Xf* using HL5 and HL6 primers (Francis et al., 2006). The results clearly demonstrate that the SNP genotypes are associated with the hosts from which the strains were derived (Table 3). Next, 700-1,000 potential SNP loci located in interesting gene regions will be selected for screening using a set of 5-10 grape, almond, oleander and citrus *Xf* strains to confirm that these loci are truly polymorphic (to eliminate SNP caused by sequence error or isolated mutation event). To increase data throughput and reduce operational costs, we optimized multiplex PCR primers that can amplify up to 5 SNP loci in a single reaction. Our recently designed HTP format protocol allows analysis of up to 10 SNP loci per sequencing run. In the future, genome wide multiplex SNP detection primers based on screening results (~100 loci) will be analyzed. This *Xf* SNP genetic analysis system appears to be a powerful new marker system for *Xf* genetic study.

Table 2. Details of the loci and primers used for SNP validation. Allele frequency information is mentioned in the next table.

Gene ID	Description	Left primer (5'-3')	Right primer (5'-3')
XF0450	Two-component system, regulatory protein	ATTACCGCAACCGATGG	TCGTTCGCTTTTGCTTTTG
XF0677	Type 4 fimbriae assembly protein	GTACGCGCCAGGGTATTCT	CAAGCAACGTCTCAATGC
XF0845	Family 3 glycoside hydrolase	TTCTTCCGTCAAGACAACG	GCCGGAGTTTTTCAAGAGG
XF1267	1,4-beta-cellobiosidase	TTACGAAGAAGGCATAAAATATG	GCAAAACCATTGACACTAGC
XF1532	Oxidative stress transcriptional regulator	GTGCGCGTAGCATTGTTG	ACACGAACGGCTCCTCAA
XF2352	Cold shock protein	ATGCAGAGCGGTACAGTTAAG	TATTGGCGTGATATTCGATG
XF2545	Two-component system, regulatory protein	ACGTATGGGGCTGCGTAT	CGATTGTTCAATTCCAAAGC

CONCLUSIONS

SSR and SNP marker systems appear to be useful for strain identification and for analysis of genetic diversity. The multi-locus SSR marker system is particularly suitable for *Xf* population genetic study due to its powerful and unambiguous discrimination ability of genetically related strains across independent genetic loci. Information generated from SNP markers will advance our knowledge in the understanding of *Xf* variation associated with functional gene sets which can be used to define the genome-wide Linkage Disequilibrium blocks and in linking SNP genotype to the *Xf* phenotype. Results from this project will be used to generate comprehensive SSR allele frequency and SNP association databases. These two databases will be complementary in strengthening the power of SSR marker in strain discrimination and the power of SNP marker for functional-related genotyping which can aid in *Xf* epidemiological and strain virulence studies.

Table 3. Single Nucleotide Polymorphism data at different genomic loci across multiple strains of *Xf*. A total of 16 genes that are conserved in all four sequenced strains and showing SNPs were selected based on their putative function. Primers were designed to amplify the SNP regions in 4-5 host specific strains of grape, citrus, almond and oleander. Target regions were PCR amplified, sequenced and aligned. The data presented here shows SNPs in functional genes that differentiate host-associated strains.

Host	Strain, Location	Polymorphism at selected gene loci								
		XF 1532 I(77bp) *	**XF 1532** II (101bp)	**XF 1267** I (263 bp)	**XF 1267** II (293 p)	**XF 2545** (106 bp)	**XF 2352** (86 bp)	**XF 0845** (258 bp)	**XF 0677** (125 bp)	**XF 0450** (151 bp)
Grape	Stag leap, Napa, CA	C	C	G	A	C	T	A	T	G
	Temecula, CA	C	C	G	A	C	T	A	T	G
	PD-6,Riverside, CA	C	C	G	A	C	T	A	T	G
	PD-7,Riverside, CA	C	C	G	A	C	T	A	T	G
	Xf-10, Kern, CA	C	C	G	A	C	T	A	T	G
Citrus	9a5c, Brazil	T	G	G	A	C	C	C	C	A
	CVC-10, Brazil	T	G	G	A	C	C	C	C	A
	CVC-12, Brazil	T	G	G	A	C	C	C	C	A
	CVC-14, Brazil	T	G	G	A	C	C	C	C	A
	CVC-16, Brazil	T	G	G	A	C	C	C	C	A
Oleander	Cathedral	T	G	G	A	T	T	A	T	G
	TIB	T	G	G	A	T	T	A	T	G
	T1	T	G	G	A	T	T	A	T	G
	T5	T	G	G	A	T	T	A	T	G
Almond	Manteca	T	G	A	G	C	T	A	T	G
	ALS-2, Costa, CA	T	G	A	G	C	T	A	T	G
	ALS-6, Solano, CA	T	G	A	G	C	T	A	T	G
	ALS-7	T	G	A	G	C	T	A	T	G
Allele frequency		5C:14 T	5C:14 G	4A:15G	4G:15A	4T:15C	5C:14 T	5C:14 A	5C:14 T	5A:14G

* Base pair number in parenthesis indicates the location of the target SNP nucleotide in the predicted coding sequence. Details can be obtained from the multiple alignments at our website.

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

Project Leader:
Steve Lindow
Plant and Molecular Biology
University of California
Berkeley, CA
icelab@socrates.berkeley.edu

Cooperators:
Clelia Baccari
Plant and Molecular Biology
University of California
Berkeley, CA
cbaccari@nature.berkeley.edu

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

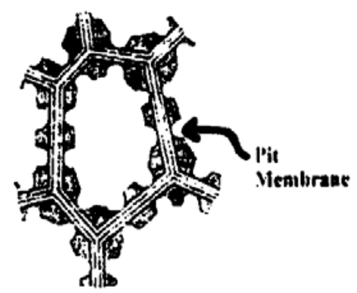
ABSTRACT

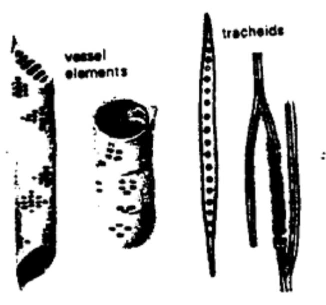
We are studying the process of movement of *Xylella fastidiosa* (*Xf*) cells between xylem vessels and through plants by analyzing the changing proportion of genetically distinct strains, initially introduced into the plants by distance and time from point of inoculation. We are also determining whether bottlenecks in movement of *Xf* cells in plants are more extreme in resistant plants than in susceptible plants, and whether this phenomenon can be exploited as a tool to screen germplasm for resistance to *Xf*. We expect that the process of movement of *Xf* involves a progressive and sequential colonization of a large number of xylem vessels that is limited by anatomical features of plants (nature of pit membranes and other barriers to vessel to vessel movement in the stem). The resulting in bottlenecks practically limit the number of *Xf* cells that can move from one vessel to another, and thus constitute a major factor that confers resistance in plants.

INTRODUCTION

Xylella fastidiosa (Xf) has a rather unique means of colonizing plants and causing symptoms, which make strategies of disease control that are useful in other bacterial diseases ineffective. Many agriculturally important plants besides grapevines, including citrus, almond, alfalfa, and coffee, are susceptible to diseases caused by *Xf* (Hopkins 1989). The bacterium is transmitted to new host plants during xylem sap feeding by sharpshooter vectors and then multiplies and spreads from the site of inoculation to colonize the xylem; a water transport network of vessels composed of dead, lignified cells. Vessels are interconnected by channels, called bordered pits, that allow the passage of xylem sap but block passage of larger objects due to the presence of a pit membrane (Choat et al. 2003, Esau 1977). Bacterial cells attach to the vessel wall and multiply, forming biofilm-like colonies that can, when sufficiently large, occlude xylem vessels, blocking water transport (Alves et al. 2004, Fry and Milholland 1990a, Newman et al. 2003). In susceptible plants, leaf scorching, fruit shriveling and other symptoms result, likely due to the increased stress of xylem blockage as colonization ensues. However, within the majority of host plants, *Xf* behaves as a harmless endophyte (Freitag 1951). The population size of *Xf* in grapevines resistant and susceptible to Pierce's disease (PD) is highly correlated with symptom expression (Alves et al. 2004, Fry and Milholland 1990a, Fry and Milholland 1990b, Hopkins 1981, Newman et al. 2003, Krivanek and Walker 2004). A much higher proportion of vessels are colonized by *Xf* in symptomatic tissues that in non-symptomatic tissues (Newman et al. 2003). However we still lack an understanding of the process of colonization and what specifically about high populations of *Xf* leads to symptom expression. The pathways by which water moves through plants via the xylem are spatially complex. It is simplistic to consider axial water movement in stems via xylem vessels as simple vertical "pipes". Indeed, xylem vessels themselves often follow complicated paths through a tissue with respect to each other (Figure 1). More importantly, the water in a given vessel is in contact with that of different vessels as well as with those in adjacent tracheids via the many pits in the cell walls (Figure 1). Pits are adjacent to one another on either side of the cell wall and thus come in pairs. The pit membrane is composed of the primary cell wall and middle lamella of adjacent cell walls of the pit pairs (Esau 1977). In a bordered pit the secondary cell wall forms a border over the pit membrane leaving a small opening called a pore. While secondary cell walls can be thickened via the intrusion of lignin and other polymers into the cellulosic matrix of the primary cell wall, pits represent local "thinning" of the primary wall with only a minimal amount of cellulose and pectin, which allows relatively free diffusion of water and solutes from one cell to another. Thus instead of a limited number of vertical "pipes' that conduct water through a stem, there are thousands of alternative pathways that water might travel in a tissue. The interconnectivity of the xylem cells is presumably one means by which the plant overcomes injuries or other insults that would disrupt the movement of water via a given xylem element by shunting it to adjacent cells.

In the context of PD it thus becomes obvious that in order for water movement in a stem to be so restricted that disease develops, a large percentage of the xylem pathways must be blocked for disease to occur. Yet, while over 40% of the xylem vessels in a single section of an infected grape stem may be infested with *Xf* (Newman et al. 2003) this alone is unlikely to explain water stress. Serial sections of grape tissue however, demonstrated that different xylem vessels are blocked in different cross sections; the percentage of occluded vessels in one of several sections along 5 mm of petiole was five times that of a single cross section. Given that inoculation of grape with *Xf* must occur in a relatively few sites on a vine, it is clear that the pathogen has the ability to move both axially and radially in xylem tissues. Such extensive movement must take some time, explaining why the disease is "progressive" and appears only several weeks after inoculation.





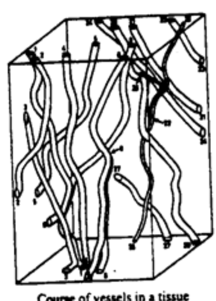


Figure 1. Structure and arrangement of xylem vessels.

Dispersion of *Xf* through the xylem network likely involves following the natural course of the xylem stream through the plant, but would be expected to require a mechanism for accessing vessels connected only by bordered pits because pit membranes do not readily allow passage of objects 20 nm or larger (Choat et al. 2003). Considerable evidence suggests that *Xf* degrades pit membranes to traverse bordered pits. *Xf* has been shown to express genes predicted to encode pit membrane degrading enzymes such as cellulases and pectinases *in vitro*. Furthermore, a mutant blocked in production of polygalacturase (pectinase) was unable to move within grape and was avirulent. In addition, transgenic grapes expressing a pear polygalcturonase inhibiting protein (PGIP) exhibited more resistance to *Xf* than did untransformed plants (Aguero et al. 2005). Work from our lab, using a gfp-marked strain of *Xf* reveals that it could be seen transiting the pit of grape xylem (Newman et al. 2003) (Figure 2).

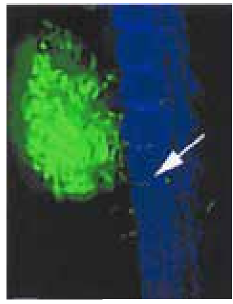


Figure 2. Image of xylem vessel.

While the movement of *Xf* has been recognized as an important trait necessary for disease, the process is still poorly understood. It is generally agreed that symptoms of PD do not occur until a large number of vessels are colonized by *Xf*. Studies by Newman et al. (2003) found a very high correlation between incidence of highly colonized vessels and symptom development in grapes. Thus, *Xf* must move through many (perhaps hundreds of) different xylem cells if such high levels of colonization are to occur. *Xf* attains higher population in susceptible cultivars than in more resistant species and cultivars of grapevines. More recent studies have shown that systemic infections occur in both susceptible and tolerant genotypes of grape. However, susceptible genotypes were characterized by higher cell populations especially in the stem internodes (Krivanek and Walker 2004). It is also known that in resistant grapes varieties, as well as other plant species, *Xf* can have a systemic infection with relatively low populations in greenhouse conditions (Fry and Milholland 1990a, Fry and Milholland 1990b). Krivanek and Walker (2004) note that the mechanism of resistance to *Xf* is localized within the stem xylem and is not fully functional or absent in the xylem of petioles and leaf blades. They observed little difference in the colonization of these tissues as opposed to those of the stem xylem. They speculate that a more constitutive resistance mechanism is present in stem xylem based on nutritional or structural differences between resistant and susceptible stem xylems.

OBJECTIVES

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

RESULTS

In these past few months, we have been propagating plant material from naturally resistant species such as *Vitis rotundifolia* and the highly susceptible *Vitis vinifera* species to conduct comparative experiments. The representative resistant cultivars used are Tampa and Roucaneuf, both of which are field resistant. The susceptible cultivars we are working with include Cabernet Sauvignon, also used in our initial study. In a preliminary experiment, we inoculated a large number of Cabernet Sauvignon plants with a mixture of two isogenic and highly virulent strains of *Xf* strains (Temecula and KLN61) via petiole needle inoculation. These isogenic strains could be distinguished by the fact that KLN61, but not Temecula, was resistant to kanamycin. When plants were sampled 50 cm from the inoculation point several weeks after inoculation, 46% were found to be infected with only strain KLN61 and 20% were infected only with strain Temecula while the remaining 34% of the plants were not infected with either strain. While both strains were initially found at the site of inoculation, in none of the plants were both of the strains found at distal sampling sites. We interpret these results to suggest that anatomical features of the plant greatly limit the number of cells of *Xf* that can move from one infected xylem vessel to an adjacent uninfected vessel,

and that sequential passage of *Xf* cells through such a series of physical "bottlenecks" characterizes the process of plant colonization. If only a few cells were transferred to adjacent xylem vessels when moving to an adjacent vessel as suggested by the microscopy analyses of Newman et al. (2003) (Figure 2), then with time it is likely that only one genotype of an originally mixed genotype inoculum would be present after many such "bottlenecks" that are encountered during movement in the plant. A cartoon illustrating this process is shown in Figure 3.

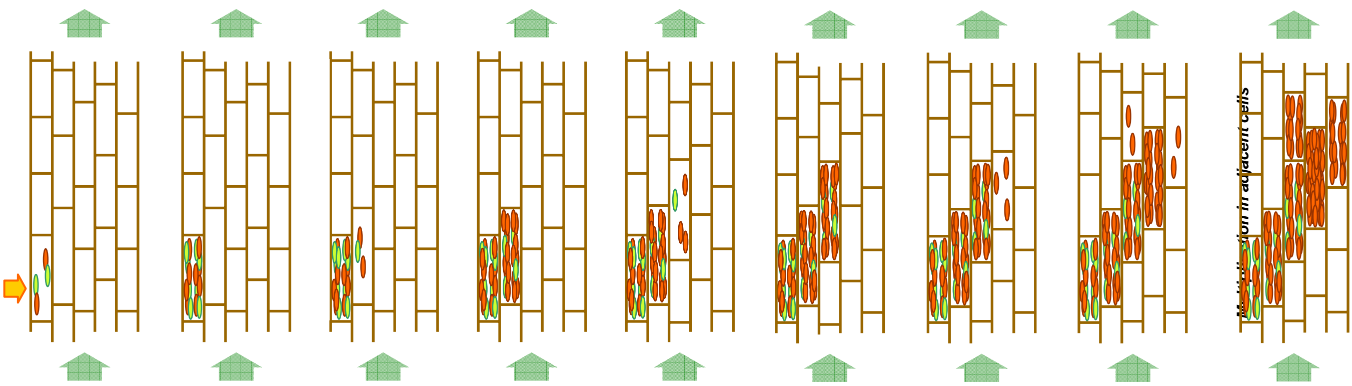


Figure 3. *Xf* movement in the plant.

In another preliminary experiment, ten rooted cuttings of a susceptible Cabernet Sauvignon variety were stem-inoculated with a suspension (10^7 cells/ml) of equal populations of Temecula and KLN61. Ten rooted cuttings were inoculated only with Temecula and ten with only KLN61. Inoculums of suspensions were prepared from two-week old plates of *Xf* in SCP buffer. Concentrations of *Xf* cells were estimated with a spectrometer and adjusted to approximately equal cell densities. The population of each *Xf* strain in suspension was estimated following inoculation by dilution plating on PWG media, followed by counting colonies on PWG and PWG+Kan media. A stem of each vine was inoculated with 5μl of suspension. The inoculation site was marked with tape. Each plant was sampled at five internodes location: 0 (point of inoculation), 10, 20, 30, 60 cm from the inoculation site plus a proximal petiole to the inoculation site. Six plants were sampled at week one, two, four, for a total of 18 plants, and 12 plants were sampled at week ten. The proportion of Temecula and KLN61in the plant part sampled was calculated for each sample as mentioned above. Twenty-seven out of the thirty plants (90%) at 10 weeks were showing symptoms and proved to be infected when assayed by culturing. At week 10, out of the four plants co-inoculated with both strains (plants numbered seven, eight, nine, ten) one plant, number seven, had nearly all Tem strain when cultured at the sampling location, and plant number nine had nearly only KLN61. The other two plants still had a mixture of the two strains in different ratios, indicating that it takes more than a 10 week period for a single strain to completely occupy the plant stem. This was also confirmed from the first experiment where culturing was done at 14 to 18 week post-inoculation. Over time, only one of the two strains ultimately dominates in infecting the test grapes. We found that both strains were still present in the petioles proximal to the point of inoculation at week 10 in plant numbers seven and eight (petiole samples from plant numbers nine and 10 were contaminated), while just one of the two strains continued to invade the rest of the stem, as the infection and resulting symptoms (disease) progressed.

CONCLUSIONS

Our preliminary results indicate that the segregation of the mixed inoculums initiate within two to four weeks of inoculation in susceptible grape varieties and is evident in samples within 10 to 20 cm of the point of introduction. Thus it would appear that the process of movement of *Xf* through plants is a stochastic one, which is characterized by growth in a given xylem vessel where it is introduced, followed by "active escape" of at most a few cells into adjacent uncolonized vessels, and then further multiplication of the cells which starts the process anew.

In this study we are exploiting the use of mixtures of phenotypically identical but genetically distinct strains of *Xf* to better understand the process of progressive movement of *Xf* through plants. We hypothesize that anatomical features of plants (nature of pit membranes and other barriers to vessel to vessel movement in the stem) limit the number of *Xf* cells that can transit from one vessel to another and represent important factors conferring resistance in plants. It would is expected that the stochastic processes that tend to segregate cells of one strain from another during progressive movement would increase the degree of segregation with distance from the point of inoculation (with increasing numbers of vessels the cells had to traverse to get from one part of the stem to another given that each vessel in grape is an average of only about 10 cm long). Thus, for a given plant inoculated with a mixture of cells, the proportion of one strain compared to the other would either increase or decrease along a predictable trajectory given the stringency of the "bottleneck" that it faced while moving from one vessel to another. This is depicted in Figure 4. While at the point of inoculation of an equal mixture of cells into the stem, the ratio of the two strains would be 1.0, the proportion of strain A in a mixture with strain B would decrease to 0 for some plants or increase to 1.0 in others. The departure from a ratio of 1.0 should increase with distance for a given plant, and when considered over many plants the variance in the proportion of the strains in a mixture should increase with distance.

This is depicted in Figure 5. Furthermore, we hypothesize (as speculated by Walker) that resistant grape varieties harbor anatomical differences from susceptible varieties that limit the movement of *Xf* from vessel to vessel. Such plant would thus present a more extreme “bottleneck” to *Xf* at each movement event and hence we would expect a more rapid segregation of mixtures of *Xf* at a given point away from inoculation.

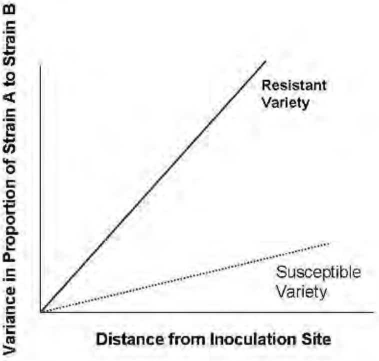


Figure 4. The proportion of one strain compared to the other would either increase or decrease along a predictable trajectory given the stringency of the “bottleneck” that it faced while moving from one vessel to another

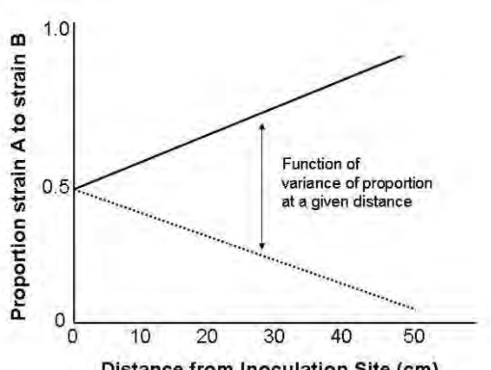


Figure 5. The departure from a ratio of 1.0 should increase with distance for a given plant, and when considered over many plants the variance in the proportion of the strains in a mixture should increase with distance.

We expect to test this model of movement of *Xf* in grapevines of varying susceptibility to PD. This study will provide considerable insight into the process of bacterial movement, which is central to the disease process though it remains poorly understood. Moreover, it will provide new and necessary tools for screening grape germplasm for resistance to *Xf*. As Walker (Krivanek and Walker 2004) has noted, since “resistant” grape varieties still support the growth and movement of *Xf*, albeit at a lower level than susceptible varieties, qualitative measures of *Xf* presence, such as by PCR, are not suitable for screening germplasm. Furthermore, difficult quantitative measures, such as culture plating or optimized ELISA, are required to distinguish resistant varieties. We expect that the segregation of mixed inoculums in “resistant” varieties will be rapid as suggested by the relationships described in figures 4 and 5, and that a simple estimation of the presence or absence of segregation of mixed inoculums near the site of inoculation would provide an accurate yet quick and easy method for assessing resistance levels.

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CONTRIBUTION OF *XYLELLA FASTIDIOSA* GENES UNIQUE TO GRAPE STRAINS TO ITS VIRULENCE TO GRAPE AND UTILITY IN SPECIFIC DETECTION OF GRAPE STRAINS BY DNA-BASED METHODS

Project Leader:
Steven E. Lindow
Dept. of Plant and Molecular Biology
University of California
Berkeley, 94720-3112
icelab@socrates.berkeley.edu

Cooperator:
William S. Feil
Dept. of Plant and Molecular Biology
University of California
Berkeley, 94720-3112
bhfeil@nature.berkeley.edu

Reporting Period: The results reported here are from work conducted September 2005 to September 2006.

ABSTRACT

Xylella fastidiosa (*Xf*) is a group of genetically similar strains that infect a wide range of plants. We hypothesized that discrete genetic factors among the very similar strains determine the ability of a strain to infect a particular host plant. To better understand what makes grape a good host for all grape strains but not for strains such as oleander and almond that cannot colonize grape, we conducted experiments to look for host specific genes of the grape strain. Through our microarray and *in silico* genomic studies, we have identified 20 potential *Xf* grape strain virulence genes. Of these, we have focused on 10 genes. We excluded 10 genes based on criteria such as they are phage related, DNA modification genes, part of a repeated gene complex, or are predicted house keeping genes, and thus not likely to have a role in plant virulence. It was clear from our studies that the microarray studies have produced fewer unique genes (genes present in one strain and lacking in another) to grape strains than expected, indicating that the identity between *Xf* 'Temecula' and other non-grape strains must be closer than expected. Our *in silico* comparisons also revealed a high level of identity between grape and non-grape strains of *Xylella*. Because of this, we are now using dual labeling with our microarray studies to determine even small differences in gene sequence rather than simple lack of a particular gene. This is a more sensitive way to determine qualitative differences between the strains. We have now made knock-out mutants for seven of the 10 genes unique to grape strains that we expect to be most likely involved in virulence to grape. We used constructs that have a Kanamycin gene inserted near the 5' end of the gene for optimum efficiency in knocking out a given gene while preventing partial transcripts to be made in such knockout strains. Inoculation studies with grape have shown that several of these genes confer the ability to move within grape and thus to incite disease at sites away from the point of inoculation. The growth of these mutant strains in grape near the point of inoculation was not usually impaired, suggesting that such traits are involved specifically in other aspects of movement and symptom development in grape.

INTRODUCTION

Xylella fastidiosa (Xf) is a group of genetically similar strains that infect a wide range of plants. A particular strain often has a relatively small and distinct host range when compared to other strains. Some strains of *Xf* originating from host plants other than grape do not sustain viable populations or are not virulent in grape. In particular, many of the strains of *Xf* isolated from almond do not infect grape (Almeida and Purcell 2003). This strongly suggests that differing genetic factors among the strains determine the ability of a strain to infect a particular host plant. Other studies provide evidence for host specificity among the *Xf* strains (Chen et al. 1992; Chen et al. 1995; Pooler and Hartung 1995;Hendson et al. 2001; Bhattacharyya et al. 2002a, 2002b; Doddapaneni et al. 2006). Cross inoculations in greenhouse studies showed that the oleander and grape strains of *Xf* were not pathogenic to citrus and that the almond strain was not pathogenic to oleander (Feil et al. *unpublished*). In California, three distinct strains of *Xf* as designated by their host range are recognized; the grape strain, the almond strain, and the oleander strain.

To better understand the underlying genetics of *Xf* as it relates to pathogenesis, several strains have been sequenced. The *Xf* '9a5c', a citrus strain, was fully sequenced in Brazil (Simpson, 2000). The draft-genome sequences of the almond and oleander strains of *Xf*, 'Dixon' and 'Ann1', respectively, are also publicly available. We used this information to identify a list of genes present in the grape strain genome but missing in other strains that do not sustain viable colonies in grape. We also developed a DNA microarray based on the sequence of the Temecula grape strain to interrogate the genomes of other strains by a process of DNA-DNA hybridization. We tested the ability of target DNA from non-grape strains to hybridize to probes designed from the reference strain, *Xf* 'Temecula', which were affixed to epoxy slides. During this process, we determined that most strains are highly identical to each other, having genes that are at least similar in sequence to reference genes in strain Temecula; very few genes were found in the Temecula strain that were lacking in other *Xf* strains. We thus have used a more sensitive approach to identify unique genes of the grape strain that is based on competitive hybridization of mixed DNA samples to the DNA microarray. Using this method, as well as *in silico* and other single strain hybridization results we have now obtained a very short list of genes that were found in all grape strains of *Xf* but are lacking, or substantially divergent in non-grape strains of *Xf*. The goals of this project thus was to determine the role of such genes in the virulence of *Xf* to grape and other plants, and to determine if such genes would be useful in distinguishing grape strains of *Xf* from all other strains in PCR-based detection schemes.

OBJECTIVES
1. Determine the relative contribution of grape strain-specific genes to growth and virulence of *Xf* in grape.
2. Design primers selective for grape-strains of *Xf* to enable the selective detection of grape strains in host plants.

RESULTS
Objective 1
Our studies to narrow the list of potential virulence genes unique to grape strains of *Xf* has resulted in a list of 20 genes (Table 1), 10 of which (in **Bold** print) are considered most likely by us to be potential virulence genes. As mentioned above, our microarray studies in which DNA from non-grape strains was hybridized alone to a DNA microarray populated with genes from *Xf* strain Temecula resulted in fewer than the expected differences in gene content between grape strains of *Xf* and other non-grape strains. This suggested that the differences in the strains was not due only to differences in gene content, but also to variations in the genes that might be in common to most, if not all, *Xf* strains. The higher sensitivity of the dual labeled arrays has allowed us to reveal those genes that vary substantially in sequence between *Xf* strains. These genes are included in the list of strain-specific genes listed in Table 1.

Table 1. List of 20 genes unique to grape strains of *Xf*.

Gene ID (a)	Predicted function	Size (bp)	In an operon? (b)	If yes, neighbor(s)
PD0028	**Unknown function**	**354**	No	
PD0105	Unknown function	468	Yes	valS, holC, pepA (house keeping genes)
RXFZ00317	Type I restriction-modification system specificity subunit	366	Yes	Type I restriction enzyme subunit hsdR
PD0370	Unknown function (phage)	303	?	Other phage
PD0371	DNA binding protein (phage)	402	?	Other phage
PD0515	**Unknown function**	**399**	No	
PD0540	**Unknown function**	**441**	No	
PD0829	**Unknown function**	**507**	No	
PD0872	**Iron-sulfur flavoprotein**	**654**	No	
PD1242	**Hemolysin**	**1140**	Yes	Hypothetical cystolic protein
PD1426	Unknown function	618	Yes	A series of six hypothetical cystolic proteins interdispersed among six hypothetical proteins followed by a RTX family calcium-binding cytotoxin or bacteriocin (frpC) (PD1415 – PD1427)
PD1434	**Unknown function**	**363**	No	
PD1510	**Unknown function**	**417**	No	
PD1511	**Unknown function**	**567**	No	
RXFZ02076	**Unknown function**	**345**	Yes	RXFZ02077 Unknown function
PD1606	Unknown function	795	No	Phage remnants nearby
PD1607	Modification methylase NspV	1455	Yes	PD1608
PD1608	Type II restriction enzyme NspV	663	Yes	PD1607
PD2071	Type I restriction-modification system specificity determinant	1335	Yes	PD2070 – PD2076 Type I restriction system
PD2075	Type I restriction-modification system specificity subunit	1218	Yes	Same as above

(a) Locus tags starting with PD are genes called by FAPESP, Brazil (http://aeg.lbi.ic.unicamp.br/world/xfpd/), those starting with RXFZ were called by Integrated Genomics (http://ergo.integratedgenomics.com/ERGO/), Chicago, Ill.
(b) A transcriptional operon was determined by the size of the intergenic region(s) (<≈50bp) and the absence of a terminator.

We produced knock-out mutants for 10 potential virulence genes identified in Table 1. These mutants were made by inserting a *kan* gene within the target gene to both disrupt the gene and to enable selection of chromosomal gene replacements. The target genes were chosen because of their size (>300 bp which would indicate that they are sufficiently large to be a functional gene and not a non-transcribed open reading frame) as well as because they had predicted functions that would plausibly be linked to virulence and/or host specificity. We eliminated some genes based on the fact that they resembled remnant phage genes or conferred expression of some other function that could not be logically thought to be associated with virulence. We then compared the identity of these genes to the genes present in the sequenced almond or oleander strains. While some of these identified genes from *Xf* 'Temecula' had at least high partial identity with a gene in an

almond or oleander strain, they were chosen because of differences in the location of the start or stop codon which would have yielded proteins of substantially difference size, or there were major differences in regions within the gene which likely would have altered its function. These differences almost certainly would have yielded highly different protein products. We have completed tests on knock-out mutants of seven of the ten grape strain-specific genes so far. The relative contribution of each of these unique genes on growth and virulence were studied by inoculating gene knock-out mutants into grape host plants (Figure 1). The incidence of infection of grape was much lower in several mutant strains of *Xf* (mutations in genes PD0105, PD0540, or PD1434). These mutants also exhibited much less lower levels of disease severity (# of leaves/plant symptomatic) in those plants in which infection occurred (compare Figures 1A and 1B). In addition, mutants with knockouts in genes PD872 and PD1510 also had reduced disease severity compared to wild-type strains, although the incidence of infection with these strains was similar to that attained by the wild-type strain. In all cases, the lower disease severity appeared to be due to a reduced progress of disease in the grapevines distal to the point of inoculation; while disease was noted up to 150 cm away from the point of inoculation in the wild-type strain, disease was restricted to sites much closer to the point of inoculation in many of the mutants (Figure 1B). In contrast, growth, measured as CFUs per gram of petiole tissue at or near the inoculation site was not significantly reduced in any mutant compared to that of the wild-type strain. The population sizes attained in petioles near the point of inoculation was quite high, suggesting that all of the mutants had similar ability to the wild-type strain to multiply in grape at least near where inoculated into grape, and that deficiencies in virulence are associated with reduced abilities to move throughout the plant.

Genes PD1007 and PD1608, genes unique to grape strains but not selected by us as among the 10 most likely genes involved in grape virulence, were included in these tests as "negative controls". These genes are apparently genes introduced by phage infection and associated with phage biology and thus we did not expect them to be involved in grape virulence. As expected, mutants of these two genes did not exhibit any significant different in the ability to infect or multiply in grape compared to wild-type strains (Figure 1). While a limited sample size of genes, it appears that our decision to disregard phage genes as virulence determinants in *Xf* was justified.

Objective 2

As this was a new objective for this project, work has only recently begun on developing and testing DNA sequences as primers for selective detection of grape strains of *Xf*. In silico studies to test the specificity of oligonucleotides based on the novel genes noted in Table 1 are being assessed prior to actual testing of them as PCR primers against a collection of *Xf* strains of different origins and host ranges.

CONCLUSIONS

The results of the tests of our knockout mutants provide us with optimism that the novel genes associated with grape strains of *Xf* are in fact contributing to the unique behaviors of such strains to cause Pierce's disease (PD). The identification of the unique genes to grape strains of *Xf* and the understanding of how these unique genes confer host specificity and virulence to grape will help researchers with their breeding programs for resistance to PD. These genes could also be studied to find targets for chemical or other types of control. Knowing those unique genes necessary for grape virulence should also prove valuable for the design of specific primers for the detection of all *Xf* grape strains. We are in the process of design such primers now.

Figure 1. Incidence (A), movement (B), and growth (C) of *Xf* following inoculation with either mutant or wildtype *Xf* strains. Observations were made 12 weeks after inoculation.

Since there are only a few sequenced strains available for a direct comparison, finding the unique genes in grape required us to examine hybridization profiles from other non-sequenced strains and determine the absences of genes in those genomes. All grape strains of *Xf* should carry the same suite of genes for growth and virulence in grape. However, the grape strain has other hosts than grape. Some of the unique genes we find may be used for other reasons than just grape related virulence. If we determine those genes uniquely needed for virulence in grape, we will also determine what constitutes a grape strain. Knowing what every grape strain processes genetically will allow us to develop better molecular screens, especially for strains collected from non-grape hosts, and may allow us to work towards the discovery of more specific remedies to PD.

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IDENTIFICATION OF TRAITS OF *XYLELLA FASTIDIOSA* CONFERRING VIRULENCE TO GRAPE AND INSECT TRANSMISSION BY ANALYSIS OF GLOBAL GENE EXPRESSION USING DNA MICROARRAYS

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

Cooperators:
Nian Wang
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Bill Feil
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

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ABSTRACT

Xylella fastidiosa (*Xf*) regulates virulence factors important in both virulence to grape as well as colonization of sharpshooter vectors via its production of a fatty acid molecule (known as DSF) whose production is encoded by *rpfF*. The RpfF homologue of *Xf* strains that cause Pierce's disease (PD), synthesizes a fatty acid cell-cell signal (DSF) that is apparently similar to that produced by *Xanthomonas campestris* pv. *campestris (Xcc)*. *Xf rpfF* mutants exhibit increased virulence to plants, however, they are unable to be spread from plant to plant by their insect vectors. While we have identified a key regulator of virulence and insect transmission in *Xf* we lack an understanding of the traits that are regulated by this pathogen in response to the DSF signal molecule. We thus are initiating studies to determine the rpf-regulation in *Xf*. We are exploiting a DNA microarray developed in another project that addresses host specificity genes in *Xf* to assess gene expression differences in isogenic RpfF^{+} and RpfF^{-} strains of *Xf* strain Temecula. The microarray contains 2555 gene-specific 70 bp oligodeoxynucleotides including negative and positive controls. Microarray analysis was performed to identify genes that are controlled by DSF and/or RpfC. DSF bioassay with reporter strain *Xcc* 8523 (pKLN55) indicated that DSF production is most abundant 10 days after inoculation when *rpfF* expression is most active. Preliminary results reveal that at least 124 genes are controlled in response to *rpfF* in *Xf*, including those encoding gum production, type IV pili and hemagglutinin. Clearly this regulator has a large effect on the physiological function of *Xf*. Microarray analysis revealed that more than 300 genes are also controlled by RpfC, including some of the same genes regulated by rpfF as well as genes such as *tonB*. Microarray-based gene expression results are being verified using quantitative Reverse Transcriptase-PCR. Comparison of the RpfF and RpfC regulons reveals that a complex pattern of expression of potential virulence genes contribute to the virulence of *Xf* and explains the hypervirulence of *rpfF* mutants and the reduced virulence of *rpfC* mutants. Work is also underway to determine the subset of *Xf* genes that is plant-inducible and the identity of those whose plant-inducible genes whose expression is also dependent on DSF production.

INTRODUCTION

PD of grape, a chronic problem in the grape industry in California now promises to be a far more devastating disease due to the introduction of the glassy-winged sharpshooter, which is a far more effective vector of the pathogen *Xf* (Purcell 1997). *Xf* apparently causes disease by multiplying within, and thus blocking, xylem vessels (Hill and Purcell 1995, Hopkins 1989). The colonization by *Xf* in grape and sharpshooters shows great similarities to microbial biofilms that form in other aquatic systems. Biofilms of bacteria develop on solid surfaces that are exposed to a continuous flow of nutrients to form thick layers. These structures consist primarily of an EPS matrix in which the bacteria are embedded. Cells in biofilms are inherently more resistant to many stresses such as antimicrobial compounds, viruses, and predators. The EPS matrix aids in the nutrition on the cells by accumulating various types of nutrients in a way analogous to an ion exchange column (Wolfaardt et al. 1994). Thus, cells in such aggregates are much more able to grow and survive than planktonic cells, which might be thought of as "scouts" for other colonization sites. Small molecules such as *N*-acyl homoserine lactones (AHLs), small peptides, butyrolactone derivatives or a fatty acid (known as DSF), play key roles as signals (Bassler 2002,Whitehead et al. 2001) in biofilm formation in numerous species of bacteria. The signals, which increase in concentration with population density, typically coordinate the expression of genes involved in exploitation of a host organism. The virulence of many pathogens is usually greatly reduced when the ability to produce signaling compounds is disrupted by mutation.

Much evidence now indicates that *Xf* regulates virulence factors via its production of a fatty acid molecule (known as DSF) whose production is encoded by *rpfF*. *Xf rpfF* mutants exhibit dramatically increased virulence to plants, however, the *rpfC* mutant showed decreased virulence to plants (Newman et al. 2004). Numerous genes with various functions were identified to be controlled by *rpfF* in the plant pathogens *Xanthomonas campestris* pv *campestris (Xcc)*, and *Xanthomonas oryzae* pv *oryzae (Xoo)* (Chatterjee and Sonti 2002). The RpfF homologue of *Xf* strains that cause PD, synthesizes a fatty acid cell-cell signal (DSF) similar to that of *Xcc* (Newman et al. 2004, Scarpari et al. 2003). Once DSF concentration reaches a threshold level, *Xf* senses DSF by RpfC, a two-component regulator containing both sensor kinase and response regulator domains, which then controls downstream genes. While DSF levels clearly are involved in regulation of virulence and behavior of *Xf*, we still do not understand what virulence factors are, nor how they are regulated in this pathogen. We thus are investigating the regulon dependent on DSF (rpfF regulon) as well as those genes dependent on rpfC both in culture and in plants to determine those factors that influence the interactions of the pathogen with plants and insects.

OBJECTIVES

1. Determine those genes in *Xf* whose transcription is controlled by *rpfF*, the regulator of virulence and insect transmission, by assessing global gene expression using DNA microarrays.
2. Determine the number and identity of genes in *Xf* that are expressed in grape plants but not in culture by assessing global gene expression using DNA microarrays.
3. Assess the contribution of individual genes of *Xf* whose transcription is dependent on *rpfF* in its virulence and insect transmissibility.

RESULTS

Dynamics of DSF production by *Xf* Temecula

To investigate the dynamic production of DSF by *Xf* on PWG medium, wild type strain Temecula was inoculated onto PWG medium and cell number and rpfF expression was measured with time. *Xf* reaches its stationary phase (maximum growth) around 10 days after inoculation (DAI) (Figure 1). Expression of *rpfF* reaches its peak in the early stationary phase and began to decline in late stationary phase (Figure 1). Likewise, the accumulation of DSF also was maximum at about eight days. This indicated that DSF production was most abundant in the early stationary phase.

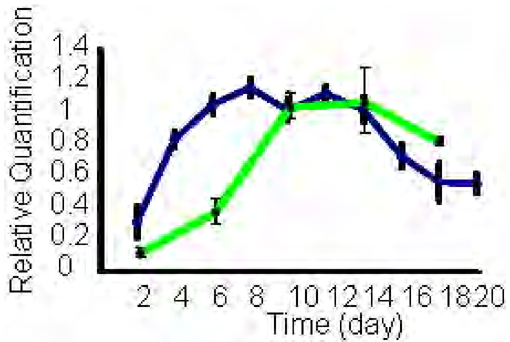

Figure 1. Dynamic of rpfF expression in *Xf*. *Xf* were cultured on PWG medium at 28°C. Green line indicates the growth curve of *Xf*; blue line indicates the expression level of rpfF measured by quantitative real time PCR.

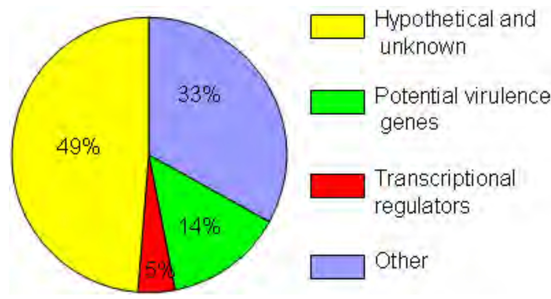

Figure 2. Categories of genes controlled by DSF in *Xf*.

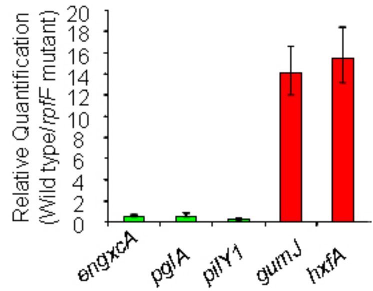

Figure 3. Virulence genes controlled by *rpfF* in planta identified with QRT-PCR. *engxcA*: endo-1,4-beta-glucanase, *pglA*: polygalacturonase, *pilY1*: type IV pili assembly protein, *gumJ*: EPS biosynthesis protein, *hxfA*: Hemagglutinin adhesions.

DSF controls the expression of many genes of various functions

Microarray analysis was used to identify genes controlled by DSF by comparison of expression profiles of wild type strain Temecula and *rpfF* mutant, which is DSF deficient. Considering the timing of DSF production in the wild type strain, samples of RNA were obtained at 10 DAI to maximize DSF-dependent gene expression. In total, 124 genes were identified to be dependent on *rpfF* for expression. Around 49% of the *rpf*F-dependent genes are hypothetical or unknown genes, 14% are potential virulence genes, 5% are regulatory genes and 33% belong to other categories including house keeping genes (Figure 2). Some of the most interesting genes, including most of the genes normally considered to be putative virulence genes in *Xf* are listed in Table 1. Such virulence genes include gum genes, hemagglutinin genes involved in attachment, and genes involved in secretion of extracellular products. The genes for hemagglutinins, and gum as well as several others were up-regulated in the presence of DSF (Table 1). In contrast, type IV pili genes, and cell wall degrading genes are downregulated in the presence of DSF (Table 1). These data are consistent with the apparently lowered production of GUM and high motility of the *rpfF* mutant as well as the fact that the RpfF mutant is much less adherent to surfaces in culture studies. The fact that DSF affects expression of some virulence genes indicates that DSF play key roles in regulation of virulence of *Xf*.

QRT-PCR was performed to confirm the expression profile of several key virulence genes. Both *gumJ* and *hexA* are upregulated by *rpfF* (Figure 3). Type IV pili gene *pilY1* and cell wall degrading enzyme genes *engxcA* and *pglA* are all downregulated by *rpfF* (Figure 3). These are consistent with the microarray data.

Expression of virulence genes are induced in planta

In order to understand the expression profile of *Xf* in planta, QRT-PCR was used to compare the expression level of several key virulence genes in culture and in planta. All the virulence genes tested are induced 2-23-fold in planta including *gumJ*, *hexA*, *pilY1*, *engxcA* and *pglA* (Figure 4). Thus *Xf* is clearly responding to chemical or physical signals in the plant to regulate gene expression. The rather artificial conditions in culture media commonly used to grow *Xf* also may not be conducive to expression of traits that normally might be expected to be expressed in plants. Thus better defining those genes that are expressed in plants will be a major goal in our continuing work since it should provide a better insight into the behavior of *Xf* in plants. We thus will strive to define those genes that are expressed in plants in a DSF-dependent fashion. Since they should be most directly related to those involved in virulence to grape.

Identification of genes controlled by RpfC with microarray analysis

Microarray analysis was also performed to identify genes that belong to RpfC regulon. Initial results indicate that at least 300 genes are controlled by RpfC. Potential virulence genes including IV pili genes, GUM genes, and TonB and TonB-dependent transporter genes are mostly up-regulated by RpfC. In contrast, hemagglutinin genes are downregulated by RpfC. QRT-PCR also confirmed the regulation of GUM and hemagglutinin genes by RpfC (Figure 5).

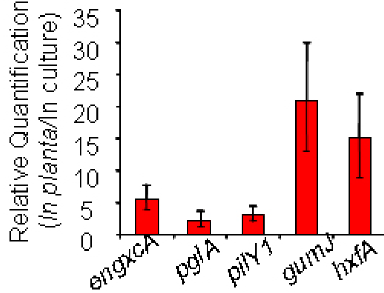

Figure 4. Comparison of gene expression of *Xf* in culture vs. in planta. *engxcA*: endo-1,4-beta-glucanase, *pglA*: polygalacturonase, *pilY1*: type IV pili assembly protein, *gumJ*: EPS biosynthesis protein, *hxfA*: Hemagglutinin adhesions.

Figure 5. Effect of *rpfC* mutations on gene expression in *Xf* as measured by QRT-PCR. A: PD1851 (*engxcA*), B: PD1856 (*engxcA*), C: *fimA*, D: *gumB*, E: *gumJ*, F: *gumD*, G: *hxfA*, H: *hxfB*, I: *pglA*, J: *pilB*, K: *pilY1*.

Table 1. RpfF regulon of *Xf* (partial). Ratio of the abundance of RNA corresponding to a given gene in the wild-type strain to that recovered from an RpfF mutant is shown. Values less than 1.0 represent genes repressed in presence of DSF while values greater than 1.0 represent genes induced in the presence of DSF.

Gene Name	Wild type / *rpfF* mutant	Gene product
RXFZ02125	0.579	PilF protein
RXFZ02230	0.352	PilM
RXFZ02111	0.658	PilY1
RXFZ00028	0.281	PilT
RXFZ02547	0.374	PilA2
RXFZ00412	0.481	Extracellular serine protease precursor
RXFZ00730	0.288	ABC transporter ATP-binding protein
RXFZ00407	0.399	Pathogenicity-related protein
RXFZ02551	0.655	Sensor protein pilS
RXFZ00951	0.62	TonB-dependent receptor
RXFZ00320	0.687	Type I restriction-modification system
RXFZ01803	5.466	GumJ
RXFZ01637	3.485	Amino acid permease
RXFZ02199	2.01	Sensory transduction protein kinase
RXFZ02793	1.9	Hemagglutinin-like protein
RXFZ01626	1.658	Hemagglutinin-like protein
RXFZ02541	1.623	Two component system histidine kinase
RXFZ01123	1.74	Type III restriction modification system

Table 2. Comparison of the RpfF and RpfC regulons.

Gene categories	RpfF	RpfC
Type IV pili	Repressed (mostly)	Upregulated (mostly)
GUM gene (EPS)	Upregulated	Upregulated
Cell wall degrading enzymes	Repressed	Upregulated/Repressed
TonB and TonB dependent transporter	/	Upregulated
Hemagglutinin	Upregulated	Repressed

CONCLUSION

Microarray analysis and QRT-PCR have been successfully to identify genes that are differentially controlled by RpfF and RpfC. Comparison of the RpfF and RpfC regulons reveals that a complex pattern of expression of potential virulence genes contribute to the virulence of *Xf* and explains the hypervirulence of *rpfF* mutants and the reduced virulence of *rpfC* mutants (Table 2). Down-regulation of type IV pili and cell wall degrading enzymes genes and up-regulation of GUM and hemagglutinin genes may explain the lower attachment capability of the *rpfF* mutant and its high motility in planta. This leads to more blockage of the xylem, thus more virulence to grape infected by *rpfF* mutant compared to wild type strain. The up-regulation of type IV pili genes, GUM genes and down-regulation of hemagglutinin gene are probably responsible for the higher attachment capability of *rpfC* mutant compared to wild type strain. The up-regulation of some of the cell wall degrading enzyme genes might contribute to its less virulence on grapevines. The up-regulation of TonB and TonB dependent transporter by *rpfC* may also contribute to tolerance of toxic preformed plant defense chemicals in the xylem and its transportation of iron. The *rpfC* mutant should be much more susceptible to some toxic plant metabolites than the wild type strain. The damage to its iron transportation might also contribute to its weak growth of the *rpfC* mutant in the xylem, which is iron limited. Considering the induction of virulence genes in planta as identified with QRT-PCR, it is expected that more intriguing results will be done by studying its expression profiles of the wild type strain Temecula and the *rpfF* and *rpfC* mutants in planta. Together our expression profiling is providing much insight into the behaviors of the pathogen within plants and insects and should help develop methods to alter pathogen behavior for disease control.

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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
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Cooperators:
Subhadeep Chatterjee
Dept. of Plant and Microbial Biology
University of California
Berkeley, CA 94720

Alexander Purcell
Dept of Env. Sci. Policy & Mgmt.
University of California
Berkeley, CA 94720

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT
Xylella fastidiosa (*Xf*) produces an unsaturated fatty acid signal molecule called DSF that changes its gene expression in cells as they reach high numbers in plants. We have investigated DSF-mediated cell-cell signaling in *Xf* with the aim of developing cell-cell signaling disruption as a means of controlling Pierce's disease (PD). We have extensively investigated both the role of DFS-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-cell signaling, performed genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control. *Xf* strains that overproduce DSF cause disease symptoms in grape, but only at the site of inoculation and the cells do not move within the plant as do wild-type strains. Thus elevating DSF levels in plants should reduce movement of *Xf* in the plant and also reduce the likelihood of transmission by sharpshooters. We identified bacterial strains that can interfere with *Xf* signaling both by producing large amounts of DSF or by degrading DSF. We have identified the genes needed to degrade DSF and when they were transferred to and over-expressed in other strains they conferred the ability of these strains to degrade DSF. When co-inoculated into grape with *Xf*, both DSF-producing strains and DFS-degrading strains greatly reduced the incidence and severity of disease in grape. Non-endophytic bacterial species were also established in high numbers inside grape leaves and petioles following spray application to plants with a high concentration of a silicon-based surfactant with a low surface tension. PD was reduced in plants after topical application of a DSF-producing strain of *Erwinia herbicola*. To verify that disease control is due to DSF interference, we have constructed mutants of these strains blocked in their ability to produce or degrade DSF and showed that the mutants are deficient in disease control. Given that DSF overabundance appears to mediate an attenuation of virulence in *Xf* we have transformed grape with the *rpfF* gene of *Xf* to enable DSF production in plants. Initial results indicate that plants produce at least some DSF and are much less susceptible to disease.

INTRODUCTION
Endophytic bacteria such as *Xf* colonize the internal tissues of the host, forming a biofilm inside the plant. A key determinant of success for an endophyte is the ability to move within the plant. We expect activities required for movement to be most successful when carried out by a community of cells since individual cells may be incapable of completing the feat on their own. Cells assess the size of their local population via cell-cell communication and coordinately regulate the expression of genes required for such processes. Our study aims to determine the role of cell-cell communication in *Xf* in colonization and pathogenicity in grapevines and transmission by the insect vector. *Xf* shares sequence similarity with the plant pathogen *Xanthomonas campestris* pathovar *campestris (Xcc).* 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. Two of the Rpf proteins, RpfB and RpfF, work to produce a diffusible signal factor (DSF) which has recently been described as an alpha,beta unsaturated fatty acid.

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. We now have shown that *Xf* makes a molecule that is recognized by *Xcc* but probably slightly different than the DSF of *Xcc*. Based on our knowledge of density-dependent gene regulation in other species, we predict the targets of Rpf regulation would be genes encoding extracellular polysaccharides, cellulases, proteases and pectinases necessary for colonizing the xylem and spreading from vessel to vessel and perhaps adhesins that modulate movement.

Given that the DSF signal molecule greatly influences the behavior of *Xf* including both its virulence to grape and ability to be vectored by sharpshooters we have investigated various ways by which this pathogen can be "confused" by altering the local concentration of the signal molecule in plants to disrupt disease and/or transmission. Our preliminary work revealed that several other bacterial species can both positively and negatively interact with the DSF-mediated cell-cell signaling in *Xf*, but until this study we did not know of the manner in which the interaction occurred nor whether such strains had the potential to affect the virulence of *Xf* in grape. In this period we have extensively investigated both the role of DFS-production by *Xf* on its behavior within plants, the manner in which other bacterial strains affect such cell signaling, the extent to which other endophytes could modulate density-dependent behaviors and virulence in *Xf* by interfering with cell-

cell signaling, obtained genetic transformation of grape to express DSF, and explored other means to alter DSF abundance in plants to achieve PD control.

OBJECTIVES

1. Identify bacteria that interfere with DSF-mediated cell-cell signaling in *Xf*, and conduct pathogenicity tests on grapevines colonized by DSF-interfering bacteria to determine potential for PD control
2. Isolation of mutant strains of DSF-degrading and DSF-producing bacteria that can no longer interfere in cell-cell signaling to verify that disease control is linked to cell-cell signal interference
3. Molecular identification of genes conferring DSF-degrading activity
4. Engineer the grapevine endophytes to express genes conferring DSF-degradation and DSF-synthesis activities and test whether the resulting transgenic endophytes are capable of disease control
5. Creation of grapevines expressing genes conferring DSF-degradation and DSF-synthesis activity to test for PD resistance
6. Evaluate topical application of DSF-degrading and DSF-producing bacteria with penetrating surfactants for PD control

RESULTS

Objective 1. Disease control with DSF-interfering strains

We have previously isolated a variety of bacteria and tested them for their ability to interfere with cell-cell signaling in *Xf*. We found several strains that negatively affected signaling in *Xcc* while several strains were found to produce DSF. When co-inoculated into grapevines with *Xf,* strains that either inhibit or activate cell-cell signaling in greenhouse studies both reduced PD; DSF-producing strains tended to be most effective in blocking disease. To better understand the contribution of DSF production by *Xf* on virulence to grape we varied DSF in *Xf* in several ways. As we observed earlier, RpfF- mutants of *Xf* are hypervirulent to grape, producing more than twice the level of disease symptoms and producing symptoms earlier than a wild-type strain (Figure 1). In contrast, when DSF is over-produced in Xf by expressing *rpfF* under the control of the highly active and constitutive *kan* promoter, such strain are greatly reduced in virulence (Figure 1). While DS-overproducing strains caused infection at the site of inoculation, the pathogen and diseased leaves were not seen away from that site. A similar reduced virulence phenotype was observed in an RpfC- mutant of *Xf*. Such a mutant over-expresses DSF compared to a wild-type strain, suggesting that RpfC negatively regulates DSF production in *Xf*. The RpfC- mutant caused infection only at the site of inoculation, and hence was much less virulent than the wild-type strain (Figure 2). The population size of the RpfC- mutant in the plant was always much lower than that of the wild-type or RpfF- mutant and was undetectable further than about 30 cm from the site of inoculation, unlike the wild-type strain (Figure 2). In contrast, the population size of the RpfF- mutant was higher at all points away from the site of inoculation than the wild-type strain (Figure 2). These results all support our model that DFS regulates genes required for movement of *Xf* from colonized vessels. We hypothesize that *rpfF*-deficient mutants may be causing increased vessel blockage in the grapevine, leading to increased symptom expression.

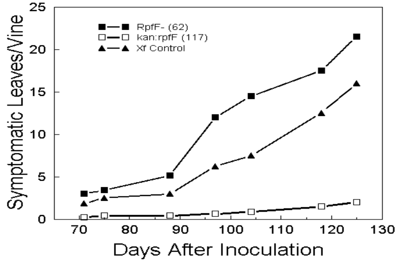


Figure 1. Severity of PD in grape inoculated with a DSF-over-producing strain of *Xf* or with an RpfF- mutant compared to plants inoculated with wild-type Temecula.

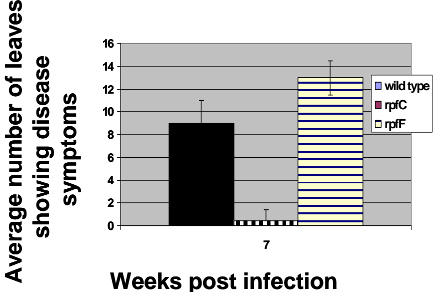


Figure 2A. Severity of PD on grape inoculated with the *rpfC* mutant of *Xf* which overproduce DSF or the RpfF- mutant, which is unable to produce DSF and the wild type Temecula.

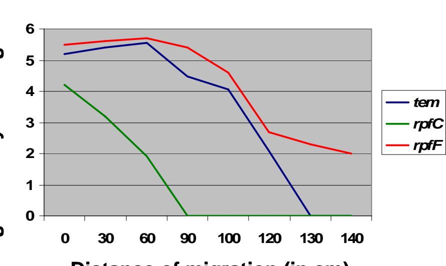
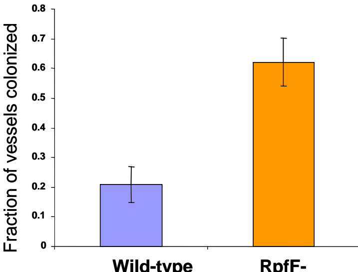


Figure 2B. Migration assay of wild type, RpfC and RpfF- mutants in the grapevines. Bacteria were isolated from the petioles at different distances from the point of inoculation 14 weeks after inoculation.

To test the model that DSF controls the process of colonization of grape we evaluated the process of colonization of grape by wild-type and RpfF- mutants of *Xf*. This was done using a gfp-marked strain of *Xf* that could be visualized within sections of inoculated grape by confocal laser microscopy. The RpfF- mutant colonized many more vessels than did the wild-type strain (Figure 3). Furthermore, this mutant blocked many more vessels by growing to larger population sizes in the xylem vessels (Figure 4). Thus RpfF, by synthesizing DSF appears to down-regulate virulence in *Xf*. These results explain why an RpfF- mutant is more virulent; by spreading much more extensively, and especially by blocking vessels it will cause more disease symptoms. These results all suggest that altering DSF levels in the xylem will alter the behavior of *Xf* and reduce disease.



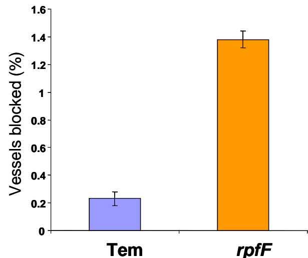


Figure 3. Fraction of grape xylem vessels colonized by wild type *Xf* or an RpfF- mutant 10 weeks after inoculation. The more extensive movement of the RpfF- mutant in grape suggests that RpfF suppresses extracellular enzymes presumably involved in movement of *Xf* between xylem cells.

Figure 4. Percent of grape xylem vessels blocked by wild type *Xf* or an RpfF- mutant 10 weeks after inoculation as determined by confocal microscopy. The more extensive growth of the RpfF- mutant in the xylem suggests that virulence traits such as extracellular enzymes may contribute to growth of cells in the plant.

Objective 2. Linking biocontrol of PD to DSF interference

To establish a rigorous connection between DSF production and disease control, we have constructed mutant strains of those DSF-producing bacteria that perform best in the disease control assays that no longer could produce DSF. These mutants were then compared to their parent strains in disease control assays. We also made mutants of DSF-degrading strains that no longer could degrade DSF. We expected that if DSF interference can provide disease control, then strains no longer able to interfere with DSF signaling will also no longer be able to control disease. All mutants unable to produce DFS (such mutant V134-1 of strain V in Figure 5) were diminished in ability to reduce PD when co-inoculated with *Xf* compared to their DSF-producing wild-type strain. Likewise, mutant strain G741, a mutant of DSF-degrading parental strain G that no longer could degrade DSF also was greatly reduced in ability to control PD when co-inoculated with *Xf* compared to its parental strain (Figure 6). These results suggest strongly that it is the production of, or degradation of DSF in plants by these antagonistic bacteria that makes a large contribution to their ability to reduce PD. The results thus strongly suggest that any method that either increases or decreased DSF abundance in *Xf*-infected plants will have a large effect on the incidence and/or severity of PD.

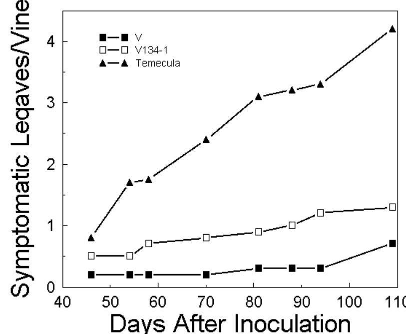


Figure 5. Severity of PD on grape co-inoculated with DSF-producing strain V or mutant V134-1 that is unable to produce DSF and *Xf* strain Temecula. The mutant is less effective in reducing disease compared to the parental strain.

Objective 3. Characterizing DFS degradation pathways

To better exploit DSF degradation as a means of disease control we need to understand how DSF degradation occurs and what genes are involved. We thus molecularly identified the genes conferring the DSF-interference phenotype in *Pseudomonas* strain G, one of the most active DSF degrading bacteria. We have inactivated the genes for interference in *Pseudomonas* strain G individually by random Tn5 mutagenesis and cloned the disrupted loci. We obtained many individual transposon mutants that were completely blocked in DSF degradation. Interestingly, all of the mutations were in the *carAB* genes, encoding carbamoyl-phosphate synthetase activity degradation suggesting that this locus was particularly (solely?) responsible for DSF degradation. These genes are involved in purine and pyrimidine biosythessis. We speculate that the cells require UDP-galactose for the breakdown of DSF, which is an alpha, beta fatty acid. The *carAB* genes have been cloned and shown to restore DSF-degradation in strain G mutants, verifying that these genes were indeed responsible for DSF degradation (Figure 7). Importantly, over-expression of *carAB* in a mutant of strain G that was previously unable to degrade DSF conferred upon it the ability to degrade DSF that is greater than the original strain G (Figure 7). Also, over-expression of *carAB* in *E. coli*, a bacterium with little ability to degrade DS confers upon it the ability to rapidly degrade DSF (Figure 7). This exciting result suggests that not only can we impart DSF degradation activity upon other endophytic bacteria that are good colonizers of grape, but that we can produce bacteria with superior abilities to degrade DSF by over-expressing *carAB*. In very exciting results, we find that the ability of *Pseudomonas* strain G to reduce PD of grape when co-inoculated with *Xf* is greatly increased when the cloned *carAB* is over-expressed in this strain (Figure 8). We thus will be exploring the possibilities of enhancing control of PD by introducing cloned *carAB* genes into better endophytes of grape.

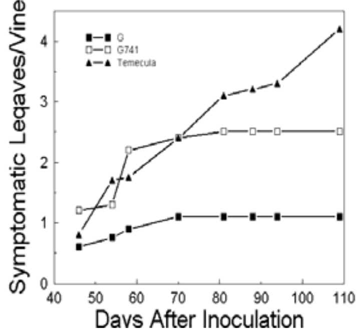


Figure 6. Severity of PD on grape co-inoculated with DSF-degrading *Pseudomonas* strain G or mutant G741 that is unable to degrade DSF and *Xf* strain Temecula. The mutant is less effective in reducing disease compared to the parental strain.

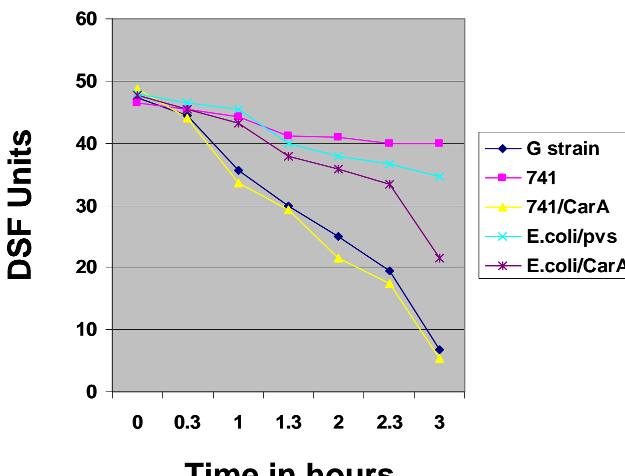


Figure 7. DSF degradation kinetics of bacteria harboring carAB. DSF was added to an overnight grown culture of *Pseudomonas* strain G, mutant G741 (G Mutant), G741/Pcar(G- mutant with the complementing clone), *E.coli* with the vector alone and *E.coli* with pCAB (cloned *carAB* gene). DSF was extracted at different time points and was assayed with an indicator strain.

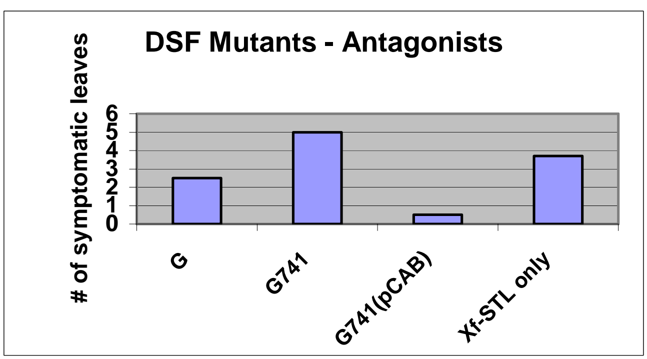


Figure 8. Severity of PD on grape co-inoculated with *Pseudomonas* strain G that can degrade DSF, mutant G741 that can not degrade DSF, or with mutant G741 over-expressing the cloned *carAB* genes on plasmid pCAB compared with plants inoculated with *Xf* strain STL alone. Note that mutant G741 did not reduce disease but over-expression of *carAB* resulted in great disease control.

Objective 4. Expressing DSF degradation and production in grape endophytes
Disease control by DSF-interfering strains will be optimized if they are good colonists of grapevine. To maximize disease control we are expressing the various genes conferring DSF interference in effective non-pathogenic endophytic colonists of grapevine such as *Erwinia herbicola*. We expect that this strategy will deliver the disease control agent directly to the site of the pathogen and result in highly effective control. Since the *rpfF* gene of *Xf* is sufficient to confer expression of DSF in other bacteria we are introducing it into these two species. We have also examined ways of over-expressing *rpfF* in various bacteria to enhance their production of DSF. We have constructed DSF over-producing strains of *E.coli* and 299R (*Erwinia herbicola*) expressing the DSF biosynthetic gene *rpfF* from *Xcc* and X*f*. Dramatic increases in production of extracellular DSF is observed specially with the *Xf* DSF relative to the parental strain (Table 1). We are further studying the ability of the purified DSF from these overproducing strains as well as the strains itself in controlling PD by spraying them on infected grape vines.

Objective 5. Production of DSF in transgenic plants for disease control
We have expressed the *rpfF* gene in grape and tomato transformed at the Ralph M. Parsons Foundation Plant Transformation Facility at the University of California, Davis. Initially, we submitted a tested but un-optimized *rpfF* construct to the facility. We transformed both tomato and grape since we could more quickly obtain evidence of DSF production in tomato since it is much quicker and easier to transform than grape, and since it is susceptible to *Xanthomonas campestris pv.vesicatoria (Xcv)*, a pathogen that produces a DSF similar to that of *Xf* we could more quickly evaluate the effect of *in planta* production of DSF on disease control. The first transformed plants are now mature and have been tested for DFS production. Initial assays reveal that DSF is rapidly degraded by damaged plant tissue during extraction procedures, making it hard to estimate the abundance of DSF within the plants. Therefore different assays are being developed to avoid this complication in assessing DSF abundance. Mature grape plants have now been rooted to produce large numbers of clonal plants that were inoculated with *Xf*. Transformed tomato plants have also been grown to maturity and selfed to produce sufficient seed for inoculation experiments. Our initial results from inoculation of tomato transformed with the *rpfF* gene of *Xcc* reveals that they are much more susceptible to infection from topical applications of *Xcv* (Figure 9). Since RpfF- mutants of *Xcv* are less virulent to tomato unlike RpfF- mutants of *Xf* that are more virulent, DSF increases the virulence of *Xcv* (the opposite of its affect in *Xf*) and hence our finding that the *rpfF*-transformed tomato are more susceptible to *Xcv* suggests that DSF was produced by these plants. Large numbers of clonal rpfF-expressing grapes have

Table 1. Production of DSF by *E. coli* and *Erwinia herbicola* haboring cloned *rpfF* genes from *Xf* and *Xcc*.

Strains	Relative DSF production (Units)
Xcc	100
X. fastidiosa Temecula	4-5
E. coli DH10B (ptrpXccrpfF)	3000
E. coli DH10B (ptrpXfrpfF)	100
E. herbicola (ptrpXccrpfF)	6000
E. herbicola (ptrpXfrpfF)	200

now been produced and inoculated with *Xf* to test for susceptibility to PD. The rpfF-expressing grape are much less susceptible to PD (Figure 10). The severity of disease was reduced over 10-fold compared to non-transformed plants. While *Xf* spread throughout non-transformed plants causing disease on petioles located great distances from the point of inoculation, disease was observed only very close to the point of inoculation in *rpfF*-expressing plants. We thus expect to find that *Xf* is limited in its movement in plants having high indigenous levels of DSF due to the expression of *rpfF*, in a manner similar to what we have observed in DFS-overproducing strains of *Xf*. We are thus very excited about the prospects of enhancing DFS levels in plants as a means of reducing disease. This might best be done in transgenic plants or perhaps in topical applications of DSF or analogs or also by expression in plants by other endophytic bacteria. We are transforming additional plants with both *rpfF* and *rpfB* genes that have been modified to direct the protein product to the chloroplast where fatty acid synthesis (and DSF synthesis) should be much enhanced compared to its production in the cytosol, the presumed location of RpfF in the current transformed plants.

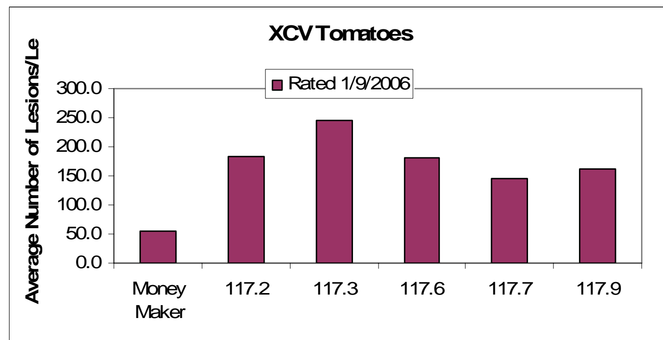


Figure 9. Average number of lesion spots per tomato leaflet.caused by *Xanthomonas campestris* pv. *vesicatoria* on wild type tomato (Money maker) and five transgenic lines expressing the *Xcc rpfF* gene.

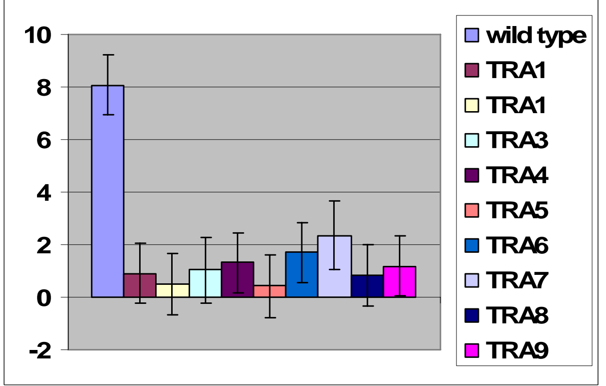


Figure 10. Disease severity (# symptomatic leaves/plant) on Freedom grape transformed with the r*pfF* gene encoding DSF production and inoculated with *Xf*.

Objective 6. Topical application of DFS-interfering bacteria for disease control

We have found that it is possible to establish large populations of bacteria within grape leaves, stems and petioles by simple topical applications of bacterial suspensions to plants in solutions of organosilicon surfactants having very low surface tensions. A variety of bacteria were found to colonize grape for at very high population sizes (> 10^6 cells/petiole) for extended periods of time following topical application (Figure 11). While these bacteria apparently do not spread throughout the plant after inoculation as does *Xf*, by introducing them into the intercellular spaces and perhaps even the xylem of the plant by use of the surfactants that stimulate spontaneous infiltration of the plant, we can inoculate the bacteria into all sites within the plant. Initial studies have shown that topical applications of an *Erwinia herbicola* strain harboring the *Xf rfpF* gene can provide some control of PD (Figures 12 and 13). As noted above, we now can produce much more DSF in surrogate hosts such as *E. herbicola* and will be testing these new stains for disease control. In addition, we have isolated large amounts of DFS from such over-producing strains and have applied it topically with surfactants to determine if it will be taken into the plant and alter pathogen behavior. These inoculated plants will be rated for disease within a few more weeks.

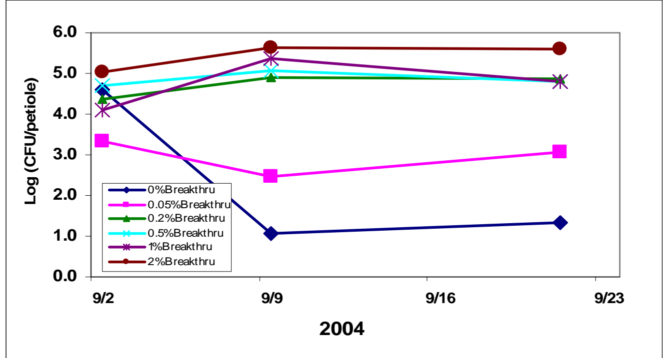
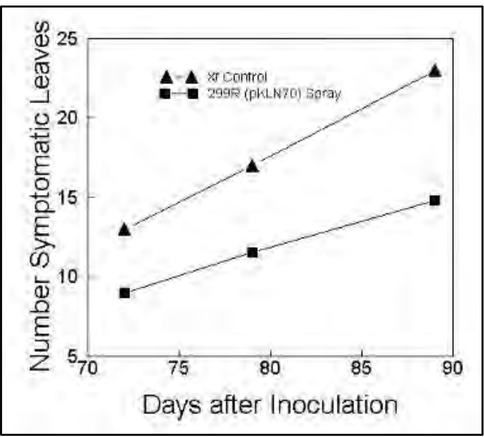
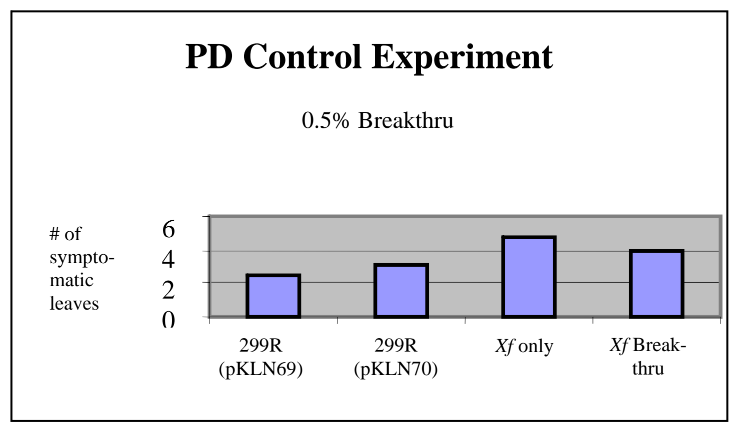


Figure 11. Population size of *E. herbicola* strain 299R in petioles at different times after spray inoculation with different concentrations of Breakthru.





Figures 12 and 13. Severity of PD of grape sprayed with *E. herbicola* harboring plasmids pKLN69 or pKLN70 encoding RpfF and thus DSF production compared to plants inoculated with *Xf* Temecula alone.

CONCLUSIONS

Substantial data now show that cell-cell signaling plays a major role in the epidemiology and virulence of *Xf* and that disruption of cell signaling is a promising means of controlling PD. Cell-cell signaling strongly controls movement and hence disease since *Xf* mutants unable to signal are hypervirulent while strains that overproduce DSF have low virulence and do not move within grape. This suggests that, it will be more efficient to elucidate and target *Xf*'s regulation of colonization strategies rather than individual traits predicted to contribute to virulence based. We have identified bacterial strains that can interfere with *Xf* signaling. These strains proved very effective as protective agents for grapevines when co-inoculated with *Xf*. Both positive and negative interference with DSF signaling reduced disease in grape suggesting that signaling is normally finely balanced in the disease process; such a finely balanced process might be readily disrupted. Since in bacteria *rpfF* is sufficient to encode a synthase capable of DSF production, expression of DFS directly in plants is an attractive approach for disease control. Preliminary results are very encouraging that DSF can be made in plants and will dramatically reduce PD. Alternatively, the use of various bacteria to express DSF in plants may prove equally effective in altering *Xf* behavior and hence disease control. Our observation that large numbers of bacteria could be introduced into grape plants by simple topical applications of cell suspensions in a penetrating surfactant has enabled us to pursue a new strategy of disease control that will enable us to efficiently test those strains that are found to be effective in PD control by a method that should prove practical for commercial use. Thus our investigation of the fundamental issues associated with interactions of *Xf* with grape has led to several very practical possible control measures of PD that can be evaluated over the short term.

FUNDING AGENCIES

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

Project Leader:
Alexander H. Purcell
ESPM-Division of Insect Biology
University of California
Berkeley CA 94720
purcell@nature.berkeley.edu

Researcher:
Clytia Montllor Curley
ESPM-Division of Insect Biology
University of California
Berkeley CA 94720
montllor@nature.berkeley.edu

Cooperators:
Steven E. Lindow
Dept. of Plant and Microbial Biology
University of California
Berkeley CA 94720
icelab@socrates.berkeley.edu

Harvey Hoch
Dept. of Plant Pathology, NYSAES
Barton Laboratory, Cornell University
Geneva, NY 14456
hch1@nysaes.cornell.edu

Thomas Burr
Dept. of Plant Pathology, NYSAES
Barton Laboratory, Cornell University
Geneva, NY 14456

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ABSTRACT

An *rpfF* DSF-deficient mutant that does not adhere to the inside of insect vector mouthparts formed a reduced biofilm *in vitro*, but contributed to biofilm formation when combined with a wild-type *Xylella fastidiosa* (*Xf*)strain. Growth of the DSF-deficient mutant in media PW was comparable to that of the wild type *in vitro* and neither strain appeared to outcompete the other when grown in PW liquid media over two weeks. However the mutant strain showed reduced growth alone, and in co-culture with the wild-type, in a second medium (BHF) that promoted biofilm formation in the wild-type strain. A fluorescent (*gfp*) mutant of Temecula was also used on occasion, but was dropped when it became clear that the mutation reduced growth ability in media and plants. Competitive behavior of the various strains *in planta* is also being investigated in our laboratory. Studies were also undertaken to try to enhance uptake and delivery of *Xf* from artificial feeding sachets to plants. A wild-type and a DSF-overproducing *rpfC* mutant strain of *Xf* were added to sachets and fed to leafhopper vectors. Only one out of 85 insects tested positive for *Xf* after sachet feeding, and no test grape plants have been infected by *Xf* from insects fed this way. Wild-type *Xf* (strain STL), grown on solid media, was also presented for sachet feeding to sharpshooter vectors; one of 24 insects that acquired *Xf* this way was able to transmit to a healthy seedling.

INTRODUCTION

Studies from this lab (R.P.P. Almeida and A.H. Purcell, unpublished) showed that sharpshooters could acquire cultured *Xylella fastidiosa* (*Xf*) cells added to expressed xylem sap in an artificial feeding apparatus (sachet), but did not transmit these bacteria to grapevines, as measured by subsequent symptom development. The assumption was that these cells did not attach to the foregut of the feeding insects. This sort of circumstantial evidence points up the complexity of the bacterial / insect / plant factors necessary for transmission (uptake and delivery) to occur. The importance of attachment and subsequent biofilm formation in this process is also indicated by the behavior of mutants that do not attach to the vector mouthparts and are not insect transmissible (Newman et al. 2004). Studies of *Xf* biofilm formation *in vitro* indicate that chemical make-up of media, substrate, and bacterial genotype/phenotype all play roles in aggregation behavior of *Xf* (e.g., Marques et al. 2002, Leite et al. 2004, Feil et al. 2003, Hoch et al. 2004). Our ultimate objective is to understand the factors that affect the process of attachment and biofilm formation of *Xf* under different environmental conditions. Investigating the conditions, be they environmental or genetic, that promote attachment and subsequent detachment from insect mouthparts is crucial to understanding transmission from insects to plants.

OBJECTIVES

1. Determine whether vector retention (and subsequent delivery) of *Xf* is related to the chemical and physical environment from which the bacteria are grown or acquired.
2. Investigate how *Xf* cells attach (and detach) to specific foregut regions of sharpshooter vectors. *NB: this objective being carried out in collaboration with the Hoch/Burr labs.*

RESULTS

In six experiments in which the *rpfF* mutant KLN61 was grown alone, it made much reduced or no discernible biofilms in glass flasks compared to the wild type Temecula, or the *gfp*-Temecula strain. In an experiment in which biofilms were quantified by sonication of the film after rinsing, and subsequent plating, KLN61 was estimated to make only 6% of the biofilm made by *gfp*-Temecula under identical circumstances. However, both qualitative and quantitative measurement of biofilms created by KLN61 grown together with Temecula (or *gfp*-Temecula) indicated that when the two strains were grown together they were consistently as thick or thicker than those created by Temecula alone.

Furthermore, in four experiments in which we quantified planktonic compared to attached (biofilm) cells of the *rpfF* mutant KLN61 grown together with Temecula or STL wild type *Xf*, the proportion of KLN61 in biofilm was either equal to or greater than the proportion of this strain found in the broth from which the film developed (Table 1). This was determined by sampling broth and a scraping of biofilm from the same flask, and plating on PWG (on which both strains grew) and on selective media (on which only the kanamycin-resistant KLN61 grew). When grown together with the wild-type Temecula, the ability to form biofilm appears to have been restored for the *rpfF* mutant compared to when it was grown alone. The only exception was an early experiment in which we paired KLN61 with the *gfp*-Temecula strain, and in which we found no live KLN61 in the biofilm.

Table 1. Proportion of KLN61 in broth or biofilm in co-cultures with Temecula. Data are from two separate experiments, designated A and B.

	A	Tem+KLN61	KLN61	B	Tem+KLN61	KLN61
Broth- Mean cfu/mL	n=4	1.6 X 10^8	4.2 X 10^7 (27%)	n=3	1.4 X 10^7	2.8 x 10^6 (21%)
Sample film- Mean cfu	n=7	233.8	57.5 (25%)	n=6	67.2	69.8 (100%)

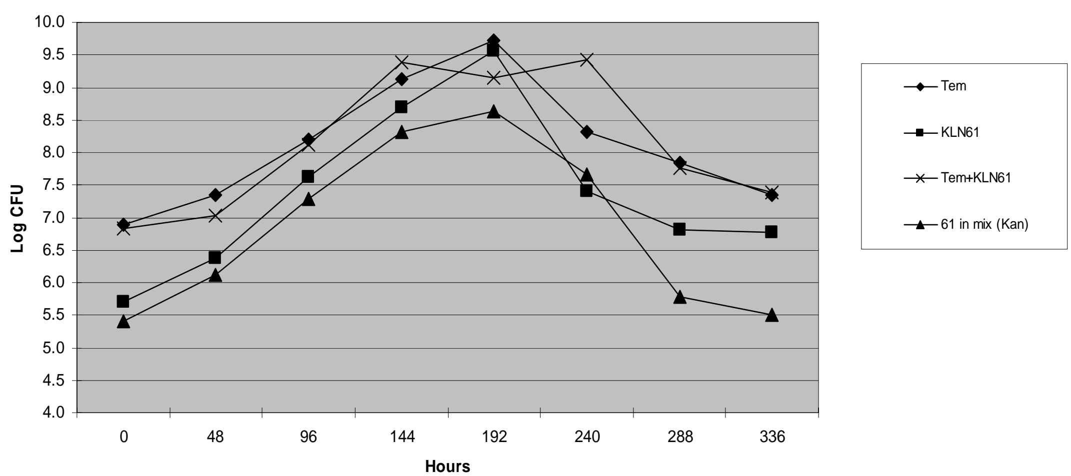

Figure 1. Growth of KLN61 and Temecula strains alone and together in PW broth.

Experiments, done in conjunction with C. Wistrom and C. Baccari in this laboratory, focused on *in vivo* and *in vitro* behavior and population growth of the *rfpF* mutants (KLN61 and KLN62) and the wild-type Temecula strains co-inoculated into grapevines or grown in liquid culture. In the first *in vitro* assay with KLN61 we were not able to determine the proportion of each strain in the co-inoculated media (both strains grew on the "selective media," due probably to an error while making the media). These assays were repeated with PW and a second broth, designated "BHF," to determine whether chemical constituents of the culture medium affect competition potential. BHF is a variant of PW, with added fructose and no BSA that promoted biofilm formation in a wild-type strain of *Xf* (B.H.Feil, unpublished).

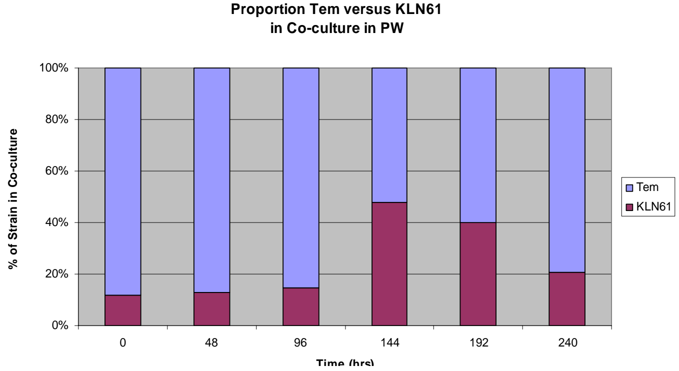

Figure 2. Proportion of the two different *Xf* strains grown together in PW broth.

In PW, growth of the wild-type strain Temecula and KLN61 were comparable (Figure1), though in a previous experiment the population size of Temecula was two orders of magnitude greater than that of KLN61after eight days. When grown together in PW media the proportion of the population made up of KLN61 fluctuated: the proportion approached 50% when the populations were in log phase at 144 hours (even though the initial mixture had a higher proportion of Temecula), and declined as the populations declined (Figure 2).

In one assay using BHF broth, KLN61 did not grow as well as the Temecula strain (Figure 3), and did not fare well in co-culture (Figure 4). Because of variability between assays, additional replicates are needed to determine whether the media (PW vs. BHF) influence the growth and competitiveness of the DSF-deficient compared to the wild-type strain. *In vivo* experiments with these strains are in progress as part of a different project in this laboratory (A.H. Purcell and C.Baccari).

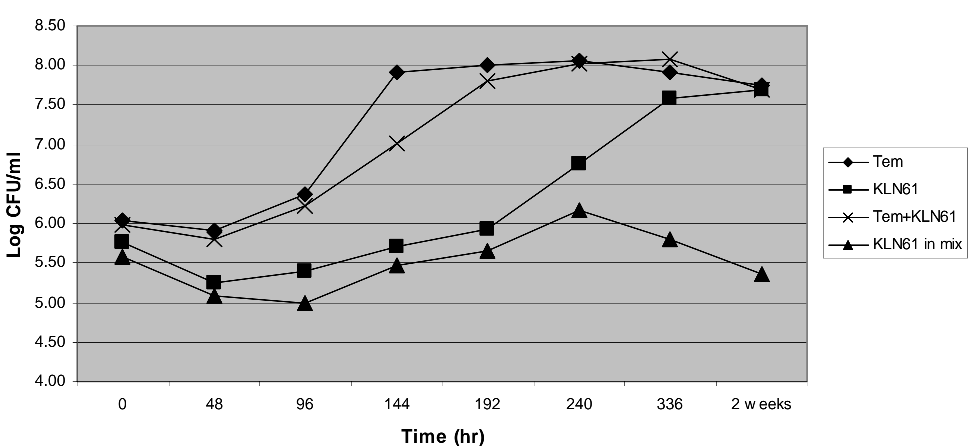

Figure 3. Growth of KLN61 and Temecula strains alone and together in BHF broth.

In vitro Competition of Tem and KLN61 in BHF

% of Strain in Co-Culture

100 80 60 40 20 0

0 48 96 144 192 240 336 2 weeks

Time (hrs)

Tem
KLN61

Figure 4. Proportion of the two different *Xf* strains grown together in BHF broth.

We have begun experiments to see how an environmentally-induced phenotype of *Xf* affects acquisition and retention of bacteria by vectors feeding on experimentally manipulated solutions in feeding sachets. Previous work with *Xf* grown on solid media and added to artificial feeding systems showed that vectors acquired but did not transmit bacteria. Several other presentations of *Xf* in sachets were made. *Xf* Temecula strain and a DSF-over-expressing mutant, *rpfC*, from biofilms formed in liquid media were added to sachets of xylem sap; no insects fed on these sachets transmitted to test plants. We have also fed vectors on xylem expressed from symptomatic plants using a pressure bomb or on extracted *Xf* from petioles of symptomatic plants, added to sachets. Heads of these vectors were cultured, but *Xf* was not recovered from them, and insects fed this way did not transmit bacteria to plants. Finally, we fed insects on sachets of *Xf* (strain STL) grown on solid media. One of 24 insects fed this way transmitted to a healthy grapevine. This was encouraging, given that control insects fed on an STL-infected source plant, had very low transmission as well (one of 17 transmitted).

CONCLUSIONS

Our overall objective is to understand the role of aggregation phenomena in acquisition, retention and delivery of *Xf* by vectors. By manipulating the environment in which *Xf* is cultured, we have found differences in the propensity for different strains to form biofilms *in vitro*. The use of *Xf* mutants with impaired or enhanced ability to perform some part of the aggregation behavior will be important to understanding the interaction between environment and bacterial behavior affecting vector retention and delivery. We have been particularly interested in documenting the behavior of *rpfF* mutants and wild-type bacteria alone and together in different liquid media. We have begun to test how some of these same factors affect acquisition and retention of bacteria by vectors feeding on sachets. Our findings may reveal currently unanticipated ways of interfering with vector transmission and elucidate features of *Xf* biofilms applicable to this bacterium in plants.

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

Project Leaders:
Alexander Purcell
ESPM-Division of Insect Biology
University of California
Berkeley, CA 94720-3114
purcell@nature.berkeley.edu

Steve Lindow
Plant and Microbial Biology
University of California
Berkeley, CA 94720-3102
icelab@socrates.berkeley.edu

Researcher:
Clelia Baccari
ESPM-Division of Insect Biology
University of California
Berkeley, CA 94720-3114
CBaccari@nature.berkeley.edu

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ABSTRACT
Strain KLN61 is an *rpfF* mutant strain of *Xylella fastidiosa* (*Xf*) that cannot be transmitted to plants by the sharpshooter leafhopper, *Graphocephala atropunctata* (BGSS), an efficient vector of the wild-type strain of this bacterium. This mutant was not retained and did not form biofilm in the vector mouthparts, however was hypervirulent and formed biofilm when mechanically inoculated into grapevines (Newman et al. 2004). We created additional mutants of *Xf rpfF* mutant strain, KLN61 using a transposome-mediated mutagenesis technique (Streptomycin EZ::TN custom transposome mutagenesis system). Screening of mutants for restoration of vector transmissibility revealed that strains competed during systemic colonization of grapevines, with only a single strain eventually colonizing most of the plant. This prevented our further pursuit of using vector acquisition to screen for mutants that restored vector transmissibility. We are testing whether production of a signaling molecule in the plant by the wild-type *Xf* strain would restore the transmissibility of the mutant. Two transmission experiments in which BGSS were fed sequentially on the mutant KLN61 (not vector transmissible), then on the parent wild type strain Temecula, or the reverse order, showed that either exposure sequence reduced vector transmission by 35% to over 90% compared to a single exposure to the Temecula strain alone. These unexpected results imply that feeding exposure of vectors to a mutant *Xf* strain that does not produce cell signal can reduce later transmission of normally transmissible strains, as well as reducing transmission by vectors already infectious with a transmissible strain.

INTRODUCTION
In *Xylella fastidiosa (Xf)* the *rpf* gene system for regulation of pathogenicity factors likely regulates genes that are important for colonization and transmission by insect vectors. The *rpfF* gene is one of the essential genes of the rpf cell-cell signaling system. KLN61 which is an *rpfF* knockout, could not perform cell-cell signaling. The *RpfF* gene catalyzes the synthesis of the signaling system molecule, diffusible signal factor (DSF) (Newman et al. 2004). Importantly, while still pathogenic to grape, such strains do not colonize and hence are not vectored by sharpshooters.

The first year, our research objective was to construct a library of *Xf* transposon-disrupting mutants through transposome-mediated mutagenesis in an *Xf rpfF* mutant background. Our first approach involved creating a library in strain KLN61which could be screened for restoration of transmissibility by inoculating mutants into plants on which vectors could subsequently feed. Any mutant strains of the non-transmissible KLN61, which could be transmitted to healthy grape plants by insect vector, would have incorporated genome changes implicated in the transmission of *Xf*. The isolation and identification of these mutants would have allowed us to better understand what *Xf* genes are involved in vector transmissibility. We created a mutant library compatible with the KLN61 mutant background in order to identify other *Xf* genes involved in the complex process of transmission. We designed and constructed a Streptomycin EZ::TN custom transposome mutagenesis system in order to further mutate the *Xf rpfF* mutant strain, KLN61. While the focus of our first grant year was to create the mutant library compatible with the KLN61 mutant strain, in this last past year our focus was on screening mutants. Since the mutants are screened by placing the insect vectors in contact with the source plants that retain the mutant library we faced the question of how multiple strains coexistence would effect *Xf* movement and transmission. To our surprise this part of the research became more interesting then we anticipated (Objective 5).

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

RESULTS

Objective 1

The commercially available transposome system that confers Kan^R was not compatible with our KLN61 strain. In our studies we could not use this vector and had to construct a novel transposon in order for it to be compatible with our Kan^R *rpfF-* mutant. Our laboratory designed and constructed a Streptomycin resistant EZ::TN transposome mutagenesis system in order to further mutate the *Xf rpfF* mutant strain. We introduced our Streptomycin resistant EZ::TN transposome in the strains KLN61 by electroporation techniques to create mutants. Electroporation with our Streptomycin resistant EZ::TN transposome yielded 5 X 10^3 mutants per µg of DNA in *Xf* strain KLN61.

Objective 2

Because of efforts to create the disrupting transposon and the high yield of mutants produced with this disrupting transposon, we focused on screening the existing library, thereby postponing the construction of an activating transposon library of mutants.

Objective 3

To accomplish Objective 3 we designed a library of the DSF mutant transposon-generated mutants to screen using insect vector transmission. The approach was to restore transmissibility through mutagenesis by disrupting genes normally down-regulated by DSF with a disrupting transposon. Because the mutant types that we sought are those in *Xf* that restore transmissibility in the non-transmissible rpfF mutant, the screen should be effectively carried out by the insect vectors. The mutant library was needle-inoculated into the source plants. We were unable to retrieve mutants when source plants were cultured and plated on the Streptomycin selective media. This is likely due to the process of multi-strain competition during the colonization of the plants (as stated in more details in Objective 5) or to the possibility that *rpfF* Streptomycin resistant mutants were not stable in grapevines.

Objective 4

We couldn't carry on with Objective 4 since we were unable to retrieve mutants from source plants. However, our results in investigating strain competition as an explanation for the lack of diversity of mutants recovered gave us an interesting insight into possible explanations for strain competition among *Xf* strains in grape, which we describe below in Objective 5 (new).

Objective 5 (new)

In Objective 5, we are addressing the process by which the DSF signal molecule is produced and recognized by cell populations of *Xf* in plants. If the DFS signal molecule is excreted into the plant after production, it should functionally complement an *rpfF* mutant in *planta*. An understanding of how DSF is perceived by bacteria in *planta* is central to our understanding of how it affects both plant virulence factors, presumably in a density-dependent fashion, and affects insect transmission. To test this model we are interested in how RpfF+ and RpfF- strains coexist in plants and how they might affect vector transmission. Mutants of PD strains of *Xf* are relatively new and not much is known regarding their behavior with regard to transmission and coexistence in *planta*. In *vitro* experiments on solid media have shown that coexistence with wild-type can restore DSF signaling production in *Xanthomonas campestris* (Barber C. E., 1997). The purpose of these experiments was to see if the wild-type strain DSF signal is able to restore the mutant biofilm formation in the mouthparts of the vector and therefore promote transmission. To test this model, we designed transmission experiments to study insect colonization when insects were fed sequentially: first on source plants containing *Xf* Tem, and then onto plants containing the *Xf rpfF* mutant to see if the acquisition of one strain impeded or stimulated the transmission of a subsequently acquired strain.

A total of 90 BGSS free of *Xf,* were divided into two groups of 15 insects each for use as negative controls and two groups of 30 insects each for the experimental treatments. The groups which held 30 BGSS were placed in contact with grapevines infected with Tem containing high population of bacteria for three days to allow the BGSS to acquire *Xf*. We then transferred the insects for another three days feeding period on a symptomatic vine infected by KLN61 and containing high population of the mutant. At the same time we also transferred the insects fed first on KLN61 vines for another three days feeding period on a symptomatic vine infected by Tem. After the BGSS had fed sequentially for three days on each source plant, they were transferred to healthy seedlings, which were tested for confirmation of transmission after eight weeks in the greenhouse. After seven days on healthy grape test plants, the BGSS were removed from the plants, the head of the insects were severed, cultured and plated on PWG media and PWG+Kan media. (Figure 1).

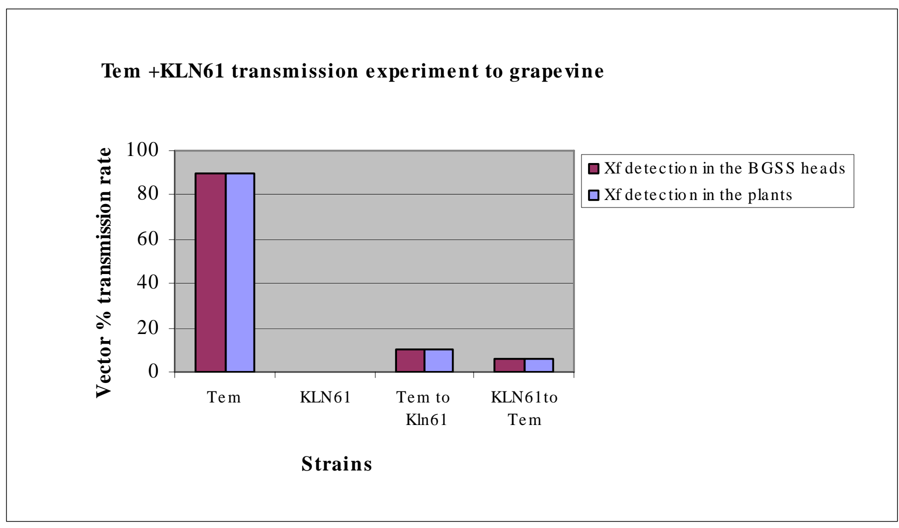

Figure 1. *Xf* Tem and KLN61 transmission to grapevines.

In the first experiment the BGSS groups had been exposed to different source plants, even though all the source plants were infected with the same *Xf* strain. In order to avoid the possibility that transmission results were due to differences among individual source plants in their efficiency as sources for vector acquisition, in the second experiment, we exposed the insects at the same time on the same plants. This ensured that the results were due only to the bacterial strains used and not to variations among the source plants. A total of 60 greenhouse reared *Xf*-free BGSS individuals were divided into four groups of 15 insects. The insects were pre-screened as in the first experiment, divided into groups of five, and caged on different parts of the symptomatic source plants. There were a total of four source plants, two infected with KLN61 and two infected with the Temecula strain. After a three day acquisition feeding period, a group of five BGSS (a total of 20) were removed from each of the source plants, representing the positive controls. The remaining insects were switched to the opposite strain source plants for a three-day period (total of six days of acquisition time). The BGSS were then individually transferred to healthy seedlings for a seven-day IAP. After eight weeks in the greenhouse, the seedlings were tested by culturing to confirm transmission. After seven days on healthy test vines, the insects were removed from the plants and prepared as in the first experiment. The heads were cultured and plated on PWG media and on PWG+Kan media (Figure 2).

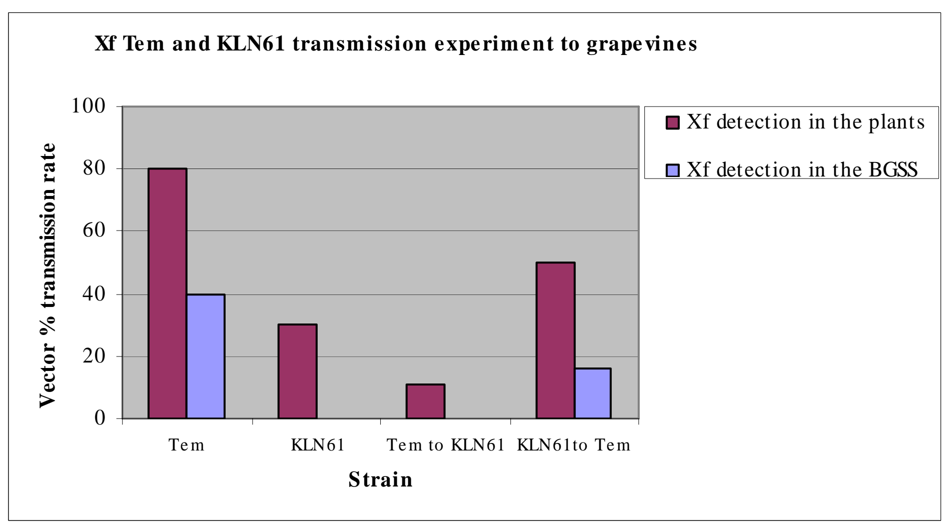

Figure 2. *Xf* Tem and KLN61 transmission to grapevines.

It was surprising that none of the strain combination exposures restored the mutant bacteria transmission. More importantly, when BGSS were exposed to source plants containing the wild type strain first and then source plants containing KLN61, they were unable to transmit the wild type. More experiments are undergoing in our laboratory to further study these interesting phenomena.

CONCLUSIONS

The results of vector transmission experiments to test strain competition between the wild type *Xf* Tem and the non-transmissible *rpfF* mutant were very interesting. We found that one strain inhibited the vector acquisition/transmission of a second strain. These results should have implications for understanding the epidemiological consequences of strain

competition in *Xf*. Because these results are still preliminary and contrary to our expectations, we are repeating them and expanding experiments based on the continuing results.

The implications of our findings relative to strain competition could be useful in better understanding how *Xf* colonizes grapevines. This has broad implications for many other physiological and anatomical studies of PD. Strain competition could also in part explain the effects of biological control (or cross-protection) of PD with *Xf* strains that are not pathogenic in grape (Hopkins 2005). We have begun greenhouse experiments that may elucidate this phenomenon using *Xf* strains from California (Almeida and Purcell 2004) that multiply in grape without causing PD symptoms to see if their prior colonization of plants can prevent infection by typically virulent PD strains.

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

Project Leaders:

Frank Wong
Dept. of Plant Pathology
University of California
Riverside, CA 92521
frank.wong@ucr.edu

Donald A. Cooksey
Dept. of Plant Pathology
University of California
Riverside, CA 92521
donald.cooksey@ucr.edu

Heather S. Costa
Dept. of Entomology
University of California
Riverside, CA 92521
heather.costa@ucr.edu

Rufina Hernandez
Dept. of Plant Pathology
University of California
Riverside, CA 92521
rufinah@ucr.edu

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ABSTRACT

To document the incidence of *Xylella. fastidiosa* (*Xf*) in landscape ornamental hosts, in 2003 and 2004 a survey of plants showing symptoms of scorch or dieback in urban locations in southern California was done. A total of 1,670 samples, representing 161 plant species were taken and analyzed at five locations: Fillmore, San Diego, Redlands, Riverside, and Tustin. From the total, 35% of plants tested (591), representing 102 identified species, gave positive results by *Xf*-specific enzyme linked immunosorbent assay (ELISA). Isolation of bacteria from ELISA-positive plants provided 39 isolates form 14 non-previously reported as *Xf*-hosts species: almond, crapemyrtle, daylily, gingko, jacaranda, grapevine (both *labrusca* and *vinifera*), magnolia, mulberry, oleander, cherry, purple-leaved plum, heavenly bamboo, olive, sweetgum, plum and western redbud. Random amplified polymorphic (RAPD)- polymerase chain reaction (PCR) and sequence analysis of the 16S-23S rDNA intergenic spacer regions (ISR) was used to genetically characterize the strains. Strains isolated from daylily, jacaranda and magnolia grouped with members of *Xf* subsp. *sandyi*. Some strains isolated from cherry, and one strain isolated from western redbud, grouped with *Xf* subsp. *fastidiosa* members and strains isolated from purple-leaved plum, olive, peach, plum, sweet gum, maidenhair tree, crape myrtle and another western redbud strain, clustered with members of the *Xf* subsp *multiplex*. All strains isolated from mulberry and one from heavenly bamboo formed a cluster that has not yet been defined as a subspecies. Koch's postulates were successfully tested for the strains isolated from sweet gum, purple-leaved plum, western redbud and mulberry. Cross-infectivity of those strains to grapevine, almond and oleander was also tested. This information contributed to better understand the role of these different strains in causing disease on plants in urban landscapes. However, the impact of *Xf* infections in landscape hosts and the diversity of strains still are far from being fully understood.

INTRODUCTION

Xylella fastidiosa (Xf) is a xylem-limited, insect-vectored, plant pathogen that can cause severe damage to a wide range of host plants including grape, almond and oleander. In addition to causing Pierce's disease (PD), Almond leaf scorch (ALS) and Oleander leaf scorch (OLS), *Xf* has been implicated in causing bacterial leaf scorch in a number of ornamentals and trees in the mid-Atlantic and southeastern U.S. Affected plants include oak, sycamore, elm, mulberry, maple and other shade trees in the landscape and urban forests (Sherald and Kostka 1992, McGovern and Hopkins 1994, McElrone et al. 1999). Multiple strains of *Xf* with different host ranges have been identified (Chen et al. 1992, da Costa et al. 2000, Hendson et al. 2001), but little is known about the diversity of these populations in the urban landscape and their ability to cause loss in plants of horticultural and agronomic importance. The arrival of a highly efficient vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*) in California has resulted in the rapid spread of this pathogen amongst both agricultural crops and landscape plants. Both PD and OLS are present in this area and recently, disease symptoms have been associated with the presence of partially characterized and potentially new strains of *Xf* in a number of landscape ornamentals including olive, liquidambar and purple-leaved plum. The broad host range of *Xylella* and its ability to hide inside unaffected hosts make it a constant menace for agricultural crops. Very little was known previously about the fate of *Xylella* in ornamentals, the strains they are harboring and their ability to cause disease losses in plants of agronomic importance. To find some information in this subject, we isolate and characterized strains from ornamental hosts. Our studies identified new hosts for the *Xf* subspecies *fastidiosa*, *Xf* subspecies *multiplex*, *Xf* subspecies *sandyi*, and for the mulberry leaf scorch type strains. Some strains appear to have a very limited host range and some have a broader range of hosts, but for most strains the possible host-strain combination has not been extensively tested.

OBJECTIVES

1. Identification of landscape host species infected with *Xf.*
2. Genetic characterization of the strains of *Xf* isolated from landscape plant species.
3. Confirmation of pathogenic infection through inoculation studies with specific isolates.
4. Test ability of new strains to infect established host plants of *Xf* including grape, oleander and almond.

RESULTS

Objective 1. Identification of landscape host species infected with *Xf*.

In 2003 and 2004, a survey that expanded an area of approximately 15,000 km^2 in size was done. Typically, a single sampling run covered an approximate area of 10 km^2. Five urban locations were included: Fillmore (Ventura County), Redlands (San Bernardino County), Riverside (Riverside County), San Diego (San Diego County) and Tustin (Orange County). Starting from a central location in these cities, all plants with typical *Xf* symptoms (scorch, stunt, dieback, wilt, etc) were sampled. Samples were processed for ELISA using the PathoScreen Kit (Agdia Inc. Elkhart, IN). From plants testing ELISA positive attempts to isolate *Xf* were done in two media PD3 and PW and the identity of putative *Xf* colonies was confirmed by PCR using the RST31 and RST33 primer pair (Minsavage et al. 1994). Isolation of bacteria from ELISA-positive plants rendered 39 isolates obtained from almond, crapemyrtle, daylily, gingko, jacaranda, grapevine (both *labrusca* and *vinifera*), magnolia, mulberry, oleander, cherry, purple-leaved plum, heavenly bamboo, olive, sweetgum, plum and western redbud, 14 non-previously reported as *Xf*-hosts species in southern California (Tables 1 and 2)

Table 1. Strains isolated from novel landscape hosts in southern California and their genetic identity.

Host scientific name	Host common name	Isolate designation	County of CA from which strain was isolated	Genetic Identification
Cercis occidentalis	Western redbud	Cercis050	Riverside	*X. fastidiosa* subsp. *fastidiosa*
Cercis occidentalis	Western redbud	Cercis001	Riverside	*X. fastidiosa* subsp. *fastidiosa*
Cercis occidentalis	Western redbud	Cercis049	Riverside	*X. fastidiosa* subsp. *multiplex*
Ginkgo biloba	Maidenhair tree	GB100	Riverside	*X. fastidiosa* subsp. *multiplex*
Hemerocallis sp.	Daylily	HEM034	Riverside	*X. fastidiosa* subsp. *sandyi*
Jacaranda mimosifolia	Jacaranda	JM028	Riverside	*X. fastidiosa* subsp. *sandyi*
Lagerstroemia indica	Crape Myrtle	LI021	San Bernardino	*X. fastidiosa* subsp. *multiplex*
Liquidambar styraciflua	Sweet gum	LS020	San Bernadino	*X. fastidiosa* subsp. *multiplex*
Liquidambar styraciflua	Sweet gum	LS022	San Bernadino	*X. fastidiosa* subsp. *multiplex*
Liquidambar styraciflua	Sweet gum	LS043	San Bernadino	*X. fastidiosa* subsp. *multiplex*
Magnolia grandiflora	Magnolia	MG038	San Bernadino	*X. fastidiosa* subsp. *sandyi*
Magnolia grandiflora	Magnolia	MG038	San Bernadino	*X. fastidiosa* subsp. *multiplex*
Morus alba	White mulberry	MLS063	San Bernardino	*X. fastidiosa* subsp. *?*
Morus alba	White mulberry	MLS059	San Bernardino	*X. fastidiosa* subsp. *?*
Morus alba	White mulberry	MLS012	San Bernadino	*X. fastidiosa* subsp. *?*
Morus alba	White mulberry	MLS024	Riverside	*X. fastidiosa* subsp. *?*
Nandina domestica	Heavenly bamboo	NI065	San Bernardino	*X. fastidiosa* subsp. *multiplex*
Olea europaea L.	Olive	G12	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC057	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC086	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC045	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC052	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC053	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PC076	San Bernardino	*X. fastidiosa* subsp. *multiplex*
Prunus cerasifera	Purple leaved-plum	PCAcl12	Riverside	*X. fastidiosa* subsp. *multiplex*
Prunus spp	Cherry	cherry018	San Bernardino	*X. fastidiosa* subsp. *fastidiosa*
Prunus spp	Cherry	cherry019	San Bernardino	*X. fastidiosa* subsp. *fastidiosa*
Prunus spp	Cherry	23Bing	Riverside	*X. fastidiosa* subsp. *fastidiosa*
Prunus spp	Cherry	37Rainier	Riverside	*X. fastidiosa* subsp. *fastidiosa*
Prunus spp	Cherry	17Bing	Riverside	*X. fastidiosa* subsp. *fastidiosa*
Prunus spp	Cherry	24Tulare	Riverside	*X. fastidiosa* subsp. *multiplex*
Unknown	Bush	UK005	Riverside	*X. fastidiosa* subsp. *multiplex*

Note: strain ID performed by sequence analysis of 16S-23S rDNA ISR sequences and RAPD-DNA (Hernandez-Martinez et al. 2006a, Hernandez-Martinez et al. 2006b)

Objective 2. Genetic characterization of the strains of *Xf* isolated from landscape plant species.
RAPD-PCR and sequence analysis of the 16S-23S rDNA ISR was used to genetically characterize the strains. Strains isolated from daylily, jacaranda and magnolia grouped with members of *Xf* subsp. *sandyi*. One strain isolated from western redbud, and one strain isolated from cherry grouped with *Xf* subsp. *fastidiosa* members. Strains isolated from purple-leaved plum, olive, peach, plum, sweet gum, maidenhair tree, crape myrtle and another western redbud strain, and three cherry strains clustered with members of the *Xf* subsp *multiplex*. Thus, the strains showed considerable diversity but belonged to previously described groups and subspecies and some hosts also can be infected with more than one subspecies, as shown here for the cherry and redbud strains (Tables 1 and 2).

Table 2. Current status of the identification of *Xf* strains isolated from landscape hosts.

Plant species	Common name of the host	Genetic Identification	Host symptoms	Current status
Ginkgo biloba	Maidenhair Tree or Ginkgo	*Xf* subsp. *multiplex*		Koch's postulates not completed using mechanical inoculation, but bacteria consistently associated with symptomatic plants by ELISA, PCR and direct culturing.
Lagerstroemia indica	Crape Myrtle	*Xf* subsp. *multiplex*		
Olea europaea	Olive	*Xf* subsp. *multiplex*		
Prunus cerasifera	Cherry plum	*Xf* subsp. *multiplex*		Koch's postulates completed. We called the disease purple-leaved scorch. A strain PC045, infected almond but not grape or oleander.
Liquidambar styraciflua	Sweet gum	*Xf* subsp. *multiplex*		Koch's postulates completed. We called the disease sweet gum dieback. A strain LS022, did not infect almond, grape or oleander.
Cercis occidentalis	Western Redbud	Cercis049 strain of *Xf* subsp. *multiplex*		Koch's postulates completed for two genotypically different strains. Cercis049 does not infect grape, almond or oleander and Cercis001 infected almond and grape but not oleander.
		Cercis001 strain *Xf* subsp. *fastidiosa*		
Morus alba	White Mulberry	Mulberry leaf scorch (maybe a new subspecies)		Koch's postulates completed; MLS definitely found in California. It does not infect oleander, grape or almond (Hernandez-Martinez et al. 2006b).
Nandina domestica	Heavenly Bamboo	Mulberry leaf scorch (maybe a new subspecies)		Koch's postulates not completed using mechanical inoculation, but bacteria consistently associated with symptomatic plants by ELISA, PCR and direct culturing.

Table 2. (continued).

Plant species	Common name of the host	Genetic Identification	Host symptoms	Current status
Hemerocallis	Day Lily	*Xf* subsp. *sandyi*		Koch's postulates not completed using mechanical inoculation, but bacteria consistently associated with symptomatic plants by ELISA, PCR and direct culturing. Isolates from Day Lily, Jacaranda and Magnolia caused scorch symptoms when inoculated into Oleander test plants.
Jacaranda mimosifolia	Jacaranda	*Xf* subsp. *sandyi*		
Magnolia grandiflora	Southern Magnolia	*Xf* subsp. *sandyi*		
		Xf subsp. *multiplex*		
Nerium oleander	Oleander	*Xf* subsp. *sandyi*		Previously established as a host.
Prunus spp.	Cherry	*Xf* subsp. *fastidiosa*		Koch's postulates not completed using mechanical inoculation. But two strains (17Bing and cherry018) diseased grape and almond plants. Another strain (24Tulare) produce mild disease symptoms in almond but do not disease grape.
		Xf subsp. *multiplex*		
Prunus	Plum	*Xf* subsp. *multiplex*		Koch's postulates not completed using mechanical inoculation.

Note: strain ID performed by sequence analysis of 16S-23S rDNA ISR sequences and RAPD-DNA (Hernandez-Martinez et al. 2006a, Hernandez-Martinez et al. 2006b).

Objectives 2 and 3. Mechanical incoculation of novel strains into ornamental hosts.
In 2005, selected isolates of *Xf* from landscape host plants *Liquidambar styraciflua*, *Nandina domestica*, *Olea europea*, *Prunus cerasfiera, lagerstroemia indica* and *Prunus sp.* were inoculated into their respective hosts of origin, grape, almond and oleander to confirm pathogenicty and to see if any were also known PD, ALS or OLS genotypes. Plants were tested at three month intervals by ELISA and for plants testing positive (at least two-times background), direct culturing of the pathogen was attempted. Mechanical inoculation technique (Hill and Purcell 1995) worked on grape, oleander and almond, as well as for the new hosts, liquidambar, mulberry, redbud, and purple-leaved plum. Bacteria were able to cause systemic infections and produce disease symptoms.

Mechanical inoculations of a strain isolated from liquidambar.
Xf was isolated from trees showing progressive dieback and decline in southern California. Three isolates were recovered from trees testing positive by ELISA and confirmed as *Xf* using the specific PCR primer set RST31-33. Isolated strains were further characterized as members of the *Xf* subsp. *multiplex* by sequencing of their 16S-23S rDNA ISR and random amplified polymorphic DNA-PCR analysis. The pathogenicity of one strain, LS022, was confirmed by inoculating glasshouse-grown sweetgum plants. Nine months after inoculation, the pathogen was recovered from five of 25 inoculated plants showing dieback symptoms. Inoculation of grapevines, oleanders and almonds with the LS022 strain or inoculation of sweetgum plants with PD, OLS, or ALS-strains did not result in any disease or recovery of the pathogen up to one year later.

Inoculation results are shown in Table 3 and the aspect of diseased plants are seen in Figure 1. These experiments completed Koch's postulates for this disease and indicate that this strain lacks cross-infectivity to grapevine, almond or oleander.

Table 3. Evaluation of sweet gum, almond, grape and oleander plants inoculated with *Xf* isolated from sweet gum.

Xf strain/	Inoculum source plant	Tested plant	Number inoculated	No. of plants positive (a)		
				ELISA	Culture	PCR
LS022	Sweetgum	Sweetgum	25	7	3	3
LS022	Sweetgum	Almond	15	0	0	0
LS022	Sweetgum	Grape	15	0	0	0
LS022	Sweetgum	Oleander	15	0	0	0
A05	Grape	Grape	15	15	15	15
Riverside3	Oleander	Oleander	15	15	15	15
276	Almond	Almond	15	10	10	10
A05	Grape	Sweetgum	10	0	0	0
276	Almond	Sweetgum	10	0	0	0
Riverside3	Oleander	Sweetgum	10	0	0	0

(a) Number of plants tested positive for the presence of *Xf* based on the number of plants inoculated using commercial ELISA kits, media culturing methods, and RST31-33 primers for PCR analysis (Minsavage et al. 1994).

Figure 1. Sweet gum plants mechanically inoculated with *Xf* strain LS022 showing chlorosis and tip dieback (leftt) as compared to a healthy non-diseased plant (right).

Mechanical inoculations of *Xf* strain PC045 isolated from Purple leaved plum (*Prunus cerasifera*) into the original host grape, oleander and oleander plants.

The pathogenicity of one strain, PC045 was tested inoculating glasshouse grown purple-leaved plum, oleander, grapevine and almond plants. Three months after inoculation, purple-leaved plum and almonds started showing typical leaf scorch symptoms and the pathogen was recovered from all inoculated plants. Inoculation of grapevine and oleander plants with the same strain did not result in any disease or recovery of the pathogen up to six months later. This indicates that this strain was cross-infective to almond but not to oleander or grape. Inoculation results are shown in Table 4 and the aspect of diseased plants are seen in Figure 2. The fulfillment of Koch's postulates established that *Xf* caused purple-leaved plum leaf scorch increasing the host range for this bacterium.

Table 4. Evaluation of purple-leaved plum, almond, grape and oleander plants inoculated with *Xf* isolated from purple-leaved plum.

Xf strain	Inoculum source plant	Tested Plant	Number inoculated	No. of plants positive (a)			No of sick plants
				ELISA	Culture	PCR	
PC045	Purple leafed-plum	Purple leafed-plum	15	15	14	14	15
PC045	Purple leafed-plum	Almond	15	15	15	15	15
PC045	Purple leafed-plum	Grape	15	0	0	0	0
PC045	Purple leafed-plum	Oleander	15	0	0	0	0
STL	Grape	Grape	15	15	15	0	15
Riverside3	Oleander	Oleander	15	15	15	0	15

(a) Number of plants tested positive for the presence of *Xf* based on the number of plants inoculated using commercial ELISA kits, media culturing methods, and RST31-33 primers for PCR analysis (Minsavage et al. 1994).

Mechanical inoculations two strains: cercis049 and cercis001 of *Xf* isolated from redbud (*Cercis occidentallis*).

The pathogenicity of two strains isolated from redbud was tested inoculating glasshouse redbud, oleander, grapevine and almond plants. Six months after inoculation, plants are starting to show leaf scorch and stunting symptoms and the pathogen has been recovered from few plants. The strain cercis001 inoculated in grapevines and almonds produced typical PD and ALS symptoms respectively and bacteria have been recovered from diseased plants. The strain cercis049 did not produce disease symptoms in grape but it seems to infect almonds producing mild symptoms. Inoculation of oleander plants with both strains did not result in any disease or recovery of the pathogen up to six months later (Table 5, Figure 3). This indicates that the strain cercis001 is a PD strain or a member of the *Xf* subsp. *fastidiosa*, able to infect almond and grape. A PD strain, STL was able to disease redbud plants, which indicated cross-infectivity of the strains. The infectivity of the strain cercis049 remains under evaluation.

Figure 2. Purple-leafed plum (left) and almond (right) plants mechanically inoculated with *Xf* strain PC045 showing leaf scorch as compared to a healthy non-diseased plant.

Table 5. Evaluation of grape, almond and oleander plants inoculated with *Xf* isolated from Redbud (*Cercis occidentallis*).

Xf strain/ subspecies	Inoculum source plant	Tested plant	Number inoculated	No. of plants positive (a)	
				ELISA	Culture*
Cercis049	*C. occidentalis*	Red bud	20	4	0
Cercis001	*C. occidentalis*	Red bud	20	3	3
Cercis049	*C. occidentalis*	Oleander	10	0	0
Cercis001	*C. occidentalis*	Oleander	10	0	0
Cercis049	*C. occidentalis*	Almond	7	4	0
Cercis001	*C. occidentalis*	Almond	10	4	3
Cercis049	*C. occidentalis*	Grape	10	0	0
Cercis001	*C. occidentalis*	Grape	15	11	3
STL	Grape	Red bud	10	6	0
Buffer		Red bud	10	0	0

(a) Number of plants tested positive for the presence of *Xf* based on the number of plants inoculated using commercial ELISA kits, media culturing methods.

CONCLUSIONS

Ornamental hosts harbor different strains of *Xf*. Members of four groups of *Xf* were isolated. *Xf* subsp. *fastidiosa* from cherry and western redbud; *Xf* subsp *multiplex* from crape myrtle, maidenhair tree, olive, sweetgum, purple-leaved plum and western redbud; *Xf* subsp *sandyi* from daylily, magnolia and jacaranda; and the mulberry leaf scorch group from heavenly bamboo and mulberry. We have the first report of Mulberry leaf scorch (MLS) in California (Hernandez-Martinez et al. 2006b), expanding the number of strains present in this state, we also found evidences that MLS strains are likely non-pathogenic to grape or oleander. We showed that strains isolated from jacaranda, daylily, and magnolia are able to produce disease in oleander but not in grape. We tested the Koch's postulates for purple-leaved plum and found that a strain (PC045), cross-infected almond but not grape or oleander. On the other hand, strains isolated from sweetgum seems to form a new pathovar since a strain inoculated in grape, oleander and almond did not produce disease symptoms. Two different strains were isolated from redbud, one a *Xf* subsp. *fastidiosa* member (cercis001) infected redbud, almond and grape while cercis049,a member of the *Xf* subsp. *multiplex* does not seem to be pathogenic towards almond or grape. We found out that cherries can be affected by two genetically different strains of *Xf* , however Koch's postulates has not been successfully

Figure 3. Redbud (*Cercis occidentallis*) plants mechanically inoculated with *Xf* strain cercis001 showing leaf scorch and stunting (left) as compared to a healthy non-diseased plant (right).

tested. Some studies still are underway to fulfill the Koch's postulates as well as to reveal their fate on grape, almond and oleander. Since knowledge of the source of inoculum is essential in developing effective disease management strategies, additional studies must be done to elucidate the full host range of *Xf*. For now, the results of this work increased our information about the hosts range spectrum of the pathogen and their latent risk in ornamentals.

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

Project Leaders:
Glenn M. Young
Dept. of Food Science and Technology
University of California
Davis, CA 95616

Michele Igo
Section of Microbiology
University of California
Davis, CA 95616

Cooperators:
Ayumi Matsumoto
Dept. of Food Science and Technology
University of California
Davis, CA 95616

Bruce Kirkpatrick
Dept. of Plant Pathology
University of California
Davis, CA 95616

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ABSTRACT
The lack of genetic and molecular tools that can be used to study the biology of *Xylella fastidiosa* (*Xf*) has made it extremely difficult for researchers to use genetic methods to establish the importance of a particular gene in the development of Pierce's disease (PD). During the period under review, we have focused on developing plasmid vectors that are stably maintained in *Xf* throughout the infection cycle. To increase the stability of autonomously replicating plasmid vectors, we have introduced two different types of stabilizing elements into plasmid vectors pXF004, pRL1342, and pBBR1MCS-5. These stabilizing elements include the plasmid addiction systems, *hok/sok* and *parDE*, and the active partitioning system, *parA*. We are currently examining how addition of these stability elements affects plasmid maintenance both *in vitro* and *en planta*. We have also developed two integration vectors, which will allow researchers to introduce genes into two different nonessential regions of the *Xf* chromosome. We are currently evaluating the properties of the insertion strains *en planta* to make sure that these strains still exhibit the normal PD infectious cycle and have begun to examine the usefulness of both of these vectors for complementation analysis in *Xf*.

INTRODUCTION
Xylella fastidiosa (Xf) is the causative agent of numerous economically important plant diseases, including Pierce's disease (PD) of grapevine (Hopkins and Purcell 2002). An important feature of the *Xf* infectious cycle is the ability of this pathogen to colonize and interact with the xylem tissue of plants and the foregut of insect vectors. Successful colonization of these hosts is dependent on the ability of *Xf* to subvert host defense networks and to acquire essential nutrients. The virulence determinants of *Xf* include proteins involved in adhesion and biofilm formation, extracellular enzymes, and toxins.

A fundamental strategy for investigating virulence in bacterial pathogens is to generate mutations and examine the impact of the absence of these gene products on pathogenicity. Over the past five years, many research laboratories have been generating insertion mutations in specific *Xf* genes and examining the impact of these mutations on the development of PD (Guilhabert and Kirkpatrick 2003, Feil *et al*. 2003, Reddy *et al*. 2004, Roper *et al*. 2004, Meng *et al*. 2005, Hernandez-Martinez *et al*. 2006). These studies have led to the identification of a number of mutant strains that do not show the normal PD infection cycle. Although the simplest explanation for these phenotypes is that the gene containing the insertion mutation is required for the normal development of PD, it is also possible that a secondary mutation was acquired during the construction of the original mutation and that the secondary mutation is responsible for the phenotype.

The classic approach to overcoming this type of objection is to perform complementation analysis. If the reintroduction of a wild-type copy of the gene into the mutant strain restores the normal PD infection cycle *en planta*, the researcher can conclude that the specific gene is important for the development of PD. One common strategy used to reintroduce the wild-type copy of a gene in Gram-negative bacteria involves the use of autonomously replicating plasmid vectors that carry antibiotic resistance genes and multiple cloning sites. Plasmid vectors with these features have been developed that are capable of replicating in *Xf* and that are stably maintained in the presence of antibiotics. These plasmids have been extremely useful for introducing genes into *Xf* and for *in vitro* complementation studies. Unfortunately, most of these plasmids are quickly lost from *Xf* in the absence of selective pressure, which limits the usefulness of these plasmids for studies *en planta*. Therefore, a major goal of this study is to develop a set of plasmid vectors that will allow researchers to perform complementation analysis *en planta*.

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

RESULTS

Develop a stable plasmid vector for *Xf*

A number of plasmid vectors have been developed that are capable of replicating in *Xf* (Qin and Hartung 2001, Vanamala *et al.* 2002, Guilhabert and Kirkpatrick 2003, Guilhabert *et al.* 2006). These plasmids have different origins of replication and belong to different incompatibility groups. However, in the absence of direct antibiotic selection, none of these plasmids are maintained in *Xf*. One set of plasmids that has been successfully introduced into *Xf* are derivatives of the IncQ broad host-range plasmid RSF1010. Three of the most useful RSF1010 derivatives are pXF004, pXF005 and pRL1342 (Guilhabert and Kirkpatrick 2003, P. Wolk, unpublished). Another plasmid that has been successfully introduced into *Xf* is pBBR1MCS-5 (Gabriel 2005). This plasmid contains the origin of replication from the broad host range plasmid pBBR1 from *Bordetella bronchiseptica* (Kovach *et al*. 1995). Significant to this proposed project is the fact that the only real problem with these vectors is that they are not maintained in *Xf* in the absence of antibiotic selection.

To circumvent this problem, we are evaluating whether stability can be achieved by introducing plasmid-addiction systems and plasmid partitioning elements into existing *Xf* vectors. A plasmid addiction system is a two-component stable toxin-unstable antitoxin system. Examples of these systems include the *hok/sok* system of plasmid R1 and the *parDE* system of plasmid RK2 (Zielenkiewicz and Ceglowski 2000). When a bacterium loses the plasmid harboring either of these addiction systems, the cured cells loose the ability to produce the unstable antitoxin and the lethal effect of the stable toxin quickly kills the bacterium. Thus, a plasmid addiction system guarantees that all living bacteria maintain the plasmid throughout infectious cycle. The plasmid partitioning system, *parA*, consists of a centromere-like region adjacent to two co-regulated genes that encode an ATPase and a centromere specific DNA-binding protein, which is required for faithful plasmid segregation at cell division (Gerdes *et al*. 2000). Addition of these systems to unstable plasmids has been demonstrated to increase plasmid stability in many Gram-negative bacteria (Zielenkiewicz and Ceglowski 2000, Gerdes *et al*. 2000).

The first set of plasmids we constructed contained both an RSF1010 replicon and an *ori*15A replicon We found that the presence of the *ori*15A replicon made the pXF-derived vectors extremely unstable in *E. coli*. To overcome this problem, we generated a second set of pXF-derived plasmids that only carried the origin of replication from RSF1010 in combination with the *hok/sok* locus or other stability elements. The removal of *ori*15A greatly increased the stability of these plasmids in *E. coli*. Therefore, we decided to use this second set of plasmids in our *Xf* stability experiments. In addition to plasmids based on the pXF vectors, we have also generated a series of plasmids based on pRL1342, which carries a chloramphenicol resistance gene (P. Wolk, unpublished). Like pXF004 and pXF005, pRL1342 has the origin of replication from RSF1010 and is not stable in *Xf* in the absence of antibiotic selection. Since the pRL1342-derived series of plasmid vectors confer resistance to chloramphenicol, they might be particularly useful for genetic complementation analysis using *Xf* mutants that are resistant to kanamycin.

After each plasmid was constructed, we evaluated its long term inheritance properties. The individual plasmids, along with other representative control plasmids, were transferred into *Xf* by electroporation (Guilhabert and Kirkpatrick 2001). We then evaluated the effect of the addiction modules on plasmid stability in *Xf* using previously established methods (Guilhabert and Kirkpatrick 2003). As shown in Table 1, the parental plasmids pXF004 and pRL1342 were lost almost immediately. In contrast, the new plasmids, which carried the *hok/sok* stability element, were still present after generation 10. This suggests that the presence of *hok/sok* is having an impact on plasmid maintenance. However, the new plasmid vectors were eventually lost in the absence of selective pressure by generation 20. Thus, although plasmids containing *hok/sok* were not lost as quickly as the starting vectors, the increase in stability was not sufficient to make the series of vectors containing an RSF10101 replicon useful for complementation *en planta*.

Table 1. Successful plasmids in introducing into *Xf*.

	Replicon	Antibiotic marker	Addiction system	Partitioning system	Stability in *Xf*	
					10 generations	20 generations
pXF004 [a]	RSF1010	Km	-	-	-	-
pAM18	RSF1010	Km	*hok/sok, parDE*	-	+	-
pAM24	RSF1010	Km	*hok/sok*	-	+	-
pRL1342 [b]	RSF1010	Cm	-	-	-	-
pLLC005	RSF1010	Cm	*hok/sok*	-	+	-
pAM59	RSF1010	Cm	*hok/sok*	*parA* [d]	+	-
pBBR1MCS-5 [c]	pBBR1	Gm	-	-	in progress	in progress
pAM89	pBBR1	Gm	*hok/sok*	*parA* [d]	in progress	in progress
pAM90	pBBR1	Gm	-	*parA* [e]	in progress	in progress

[a] Guilhabert and Kirkpatrick, 2003., [b] Peter C. Wolk (unpublished), [c] Kovach et al., 1995., [d] *parA* from pR1, [e] *parA* from *Agrobacterium* pTAR

Another set of plasmids we constructed is based on plasmid pBBR1MCS-5 (Kovach *et al*. 1995). This plasmid was successfully used by Gabriel and his colleagues for complementation of the *Xf tolC* gene *en planta* (Gabriel 2005). Due to the severity of the *tolC* mutation on grapevine colonization, the presence of the wildtype *tolC* gene may be providing the selective pressure necessary for plasmid maintenance *en planta*. Since pBBR1MCS-5 is not stably maintained in many bacterial species in the absence of selective pressure, pBBR1MCS-5 in its current might not be as useful for complementation analysis of other *Xf* genes that have a less severe effect on *Xf* growth *en planta*. However, given the promising nature of the *tolC* studies, we decided to include pBBR1MCS-5 as one of the plasmids in our stability studies.

As shown in Table 1, we have successfully introduced pBBR1MCS-5 derivatives containing different stability elements into *Xf* and are currently examining their *in vitro* stability. We have also found that it is much easier to isolate pBBR1MCS-5 plasmid DNA from *Xf* than it is to isolate plasmid DNA containing RSF1010 origins of replication. This would suggest that pBBR1MCS-5-derived plasmids are present in *Xf* at a higher copy number than RSF1010–derived plasmids. It is our hope that the higher copy number of the pBBR1MCS-5 derivatives in *Xf* in combination with the increased stability provided by *hok/sok* will result in a plasmid that can be stably maintained in *Xf* in the absence of selective pressure.

Development of integration vectors for complementation analysis *en planta*

Another method commonly used for complementation in Gram negative bacteria is to use plasmid vectors that are capable of autonomous replication in *E. coli*, but are unable to replicate in host bacteria. In most integration vectors, the gene of interest and an antibiotic cassette are flanked by DNA sequences from a nonessential region of the bacterial chromosome. Recombination between the homologous regions of the plasmid and the bacterial chromosome results in the integration of the gene of interest and antibiotic resistance gene into the chromosome at the nonessential region. The antibiotic resistance cassette is included on these vectors to facilitate the identification of strains containing the integrated vector. Although it is not easy to reisolate the introduced genetic markers from the bacteria following integration, insertion plasmids are extremely useful for certain types of genetic studies, particularly complementation analysis. One major advantage of using this type of vector is that once the gene of interest is integrated into the host bacterial chromosome, it will be maintained without antibiotics selection.

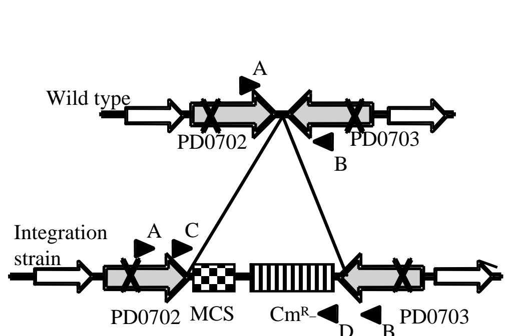

Figure 2A. Strategy of integration
▶ : primers

Primer pairs

A+B B+C C+D

M 1 2 3 4 5 6 M

(kb)

4.0

3.0

2.0

1.5

1.0

Figure 2B. Confirmation of integration
Lanes 1, 3, and 5: Wild type
Lanes 2, 4, and 6: Integration strain

The first step in developing integration vectors for use in *Xf* was to identify regions of the chromosome that could serve as targets for the integration event. One important characteristic of the target is that insertions into this location do not impact *Xf* physiology or its ability to cause PD. We have selected two places on *Xf* chromosome as potential targets of our integration vectors. The first integration target is PD0939, which encodes a phage-related protein. PD0939 was chosen because transposon insertions into this gene do not impact the development of PD in grapevine (Guilhabert and Kirkpatrick, personal communication). The integration vector that targets the PD0939 gene confers chloramphenicol resistance and is named pLLC021. The second target is the intergenic region between PD0702 and PD0703. Based on the genomic sequence of *Xf-PD*, both of these genes are predicted to contain frameshift mutations (Van Sluys *et al*. 2003). The integration vector that targets this intergenic region also confers chloramphenicol resistance and is named pLLC018. The relative orientation of PD0702 and PD0703 with respect to the targeted intergenic region is shown in Figure 2A.

The next step was to introduce our integration vectors into *Xf* and to select for chloramphenicol resistant transformants. Because these vectors are unable to replicate in *Xf*, the chloramphenicol resistant transformants must have arisen as the result of a recombination event(s) between the integration vector and the *Xf* chromosome. An example of the recombination events that led to one of these chloramphenicol resistant transformants is illustrated in Figure 2A. The PCR experiment, which confirmed that the recombination event had occurred at the appropriate chromosomal location for this transformant are shown in Figure 2B.

Finally, we examined the impact of insertions at PD0939 and within the intergenic region between PD0702 and PD0703 on the growth phenotypes of the two strains. These experiments indicated that the insertion containing strains have growth properties that are similar to a wildtype strain in both liquid culture and on solid medium. They also exhibit normal biofilm formation. We are currently evaluating the properties of the insertion strains *en planta* to make sure that these strains still exhibit the normal PD infectious cycle and have begun to examine the usefulness of both of these vectors for complementation analysis in *Xf*.

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GREENHOUSE RESPONSES OF *VITIS VINIFERA* 'CHARDONNAY,' *AMBROSIA TRIFIDA* VAR. *TEXANA*, AND *IVA ANNUA* WITH *XYLELLA FASTIDIOSA* ISOLATES FROM TEXAS HOST PLANTS

Project Leaders:

Mark C. Black
Dept. of Plant Pathol. & Microbiology
Texas A&M University
Agric. Research and Extension Center
Uvalde, TX 78802
m-black@tamu.edu

James S. Kamas
Dept. of Horticultural Sciences
Texas A&M University
Fredericksburg, TX
j-kamas@tamu.edu

Alfred M. Sanchez
Dept. of Plant Pathol. & Microbiology
Texas A&M University
Agric. Research and Extension Center
Uvalde, TX 78802

Penny S. Adams
Department of Horticultural Sciences
Texas A&M University
Fredericksburg, TX

James L. Davis
Dept. of Plant Pathol. & Microbiology
Texas A&M University
Agric. Research and Extension Center
Uvalde, TX 78802

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ABSTRACT

Sixty isolates of *Xylella fastidiosa* (*Xf*) from species or cultivars of Vitaceae (33), Asteraceae (23), Platanaceae (2), Moraceae (1), and Sapindaceae (1) were twice inoculated (8May to 1Jun06, seven greenhouse experiments) into two adjacent internodes of own-rooted 'Chardonnay' grape. Each RCBD five-replication experiment had eight to twelve treatments that included at least one winegrape isolate and one SCP buffer check. Leaf scorch symptoms on 8Aug were compared with *Xf*-serology (DAS-ELISA OD and proportion OD>0.3) on petioles collected 8Aug to 1Sep06. Some grape isolates had consistently caused Pierce's disease (PD) symptoms at 10 to 12 weeks after inoculation. A few 'Chardonnay' plants inoculated with certain *Xf* isolates from *Vitis vinifera*, *Helianthus annuus*, *Iva annua*, *Ambrosia trifida* var. *texana*, and *Platanus occidentalis* had mild PD symptoms and positive ELISA reactions. Some isolates did not cause symptoms. Evaluations will be repeated in late 2006. Twenty-one *Xf* isolates from Vitaceae (7), Asteraceae (12), Platanaceae (1), and Moraceae (1) were twice inoculated (10Jul to 20Jul06, one greenhouse experiments per host) into two adjacent internodes of *A. trifida* var. *texana* or *I. annua* grown from seed. Each RCBD six-replication experiment had twenty-three treatments that included six isolates from *Vitis* spp. and two SCP buffer checks. Symptoms were not detected. Two internode samples (inoculated zone, one internode above inoculation zone) collected 13,21,25,26Sep06 (9 to 10 weeks after inoculation) as plants senesced were assayed using *Xf*-serology (DAS-ELISA OD and proportion OD>0.3). One of six isolates from *Vitis*, one isolate from *Platanas*, and 12 isolates from spp. in Asteraceae colonized *A. trifida* var. *texana.* Three of six isolates from *Vitis*, one isolate from *Morus*, and 11 isolates from spp. in Asteraceae colonized *I. annua.*

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A PROPOSED NEW STANDARD PROTOCOL FOR DIAGNOSIS OF *XYLELLA FASTIDIOSA*

Project Leaders:

M. Francis
Department. of Plant Pathology
University of California
Davis, CA 95616
mfrancis@fresno.ars.usda.gov

Edwin L. Civerolo
USDA-ARS
SJVASC
Parlier, CA 93648
eciverolo@fresno.ars.usda.gov

Cooperators:

H. Doddapaneni
Department of Vitic. and Enology
University of California
Davis, CA 95616

Hong Lin
USDA, ARS
SJVASC
Parlier, CA 93648

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

The Interim Commission on Phytosanitary Measures of the International Plant Protection Convention (IPPC) adopted recommendations on the publication of International Standards for Phytosanitary Measures (ISPM). This guideline produces standardized documents describing procedures and methods for the detection and identification of pests of quarantine significance. The documents are reviewed by a panel of experts which also includes members from the regional plant protection organizations (i.e. NAPPO, EPPO, COSAVE, etc). These protocols describe procedures and methods for detection and identification of pests that are regulated by contracting parties and relevant for international trade. These are addressed to diagnosticians/diagnostic laboratories performing official tests as part of phytosanitary measures and provide reliable diagnostic protocol(s) for relevant pests. There is a need to develop the protocol for detection of *Xylella fastidosa* (Xf) in several hosts. We drafted such a document for Xf detection in 2005. Here we propose to update that protocol in the light of recently developed Xf diagnostic procedures and genomics data. The proposed protocol also includes the recently developed bioassay for Xf in the model plant *Nicotiana tabacum* cv. SR-1. This highly sensitive host is an excellent indicator plant to test the pathogenicity of Pierce's disease and almond leaf scorch disease strains of Xf. The procedure includes the use of *in vitro*-propagated tobacco plants grown in controlled environment (i.e., light and temperature) room. The SR-1 plants are grown in small pots to reduce space requirements, and symptoms appear in only 6-8 weeks. Xf strains from different plant hosts induce distinct symptoms in SR-1 tobacco. The protocol is applicable for disease surveys, and for quarantine and certification programs.

Section 4:
Pathogen and Disease Management

ENABLING TECHNOLOGIES FOR GRAPE TRANSFORMATION

Project Leader:
Alan B. Bennett
PIPRA
University of California
Davis, CA 95616
abbennett@ucdavis.edu

Cooperators:

Cecilia Chi-Ham
PIPRA
University of California
Davis, CA 95616
(clchiham@ucdavis.edu)

Sara Boettiger
PIPRA
University of California
Davis, CA 95616
(sboettiger@ucdavis.edu)

David Tricoli
PIPRA
University of California
Davis, CA 95616
(dmtricoli@ucdavis.edu)

Tamara Holst PIPRA
University of California
Davis, CA 95616
(|tlpholst@ucdavis.edu)

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ABSTRACT
Patenting of agricultural biotechnologies has expanded dramatically over the last 25 years and can represent a significant barrier to new crop development. Thus, navigating the intellectual property (IP) rights of commonly used research tools is essential to prevent downstream legal or regulatory obstacles for deployment of promising new technologies. The research proposed here seeks to develop and test a grape-specific transformation system for developing genetically engineered *Vitis* that addresses legal IP issues, meets high technical standards and is designed with attention to the emerging regulatory framework. The proposed plant transformation system can serve as a platform tool for the practical deployment of transgenic Pierce's disease (PD) control strategies.

INTRODUCTION
PIPRA, the Public Intellectual Property Resource for Agriculture, is a public sector multi-institutional program designed to provide the framework to manage IP and develop tools that will facilitate humanitarian or commercial development of promising agricultural innovations. In research to control PD, several transgenic strategies have been tested and show long-term promise. However, the gene transfer tools utilized for the research are, in general, proprietary and do not provide features that are likely to be compatible with evolving regulatory frameworks. As a consequence, promising research conducted today may need to be replicated with different tools and technologies if transgenic plants are ever to be deployed for commercial field production. The objective of the research proposed here is to design and test a plant transformation system that addresses IP and regulatory issues and that could be used for research and commercial deployment of transgenic PD control strategies in grapes.

OBJECTIVES
1. Design, develop, and validate a grape-specific transformation system that addresses legal IP, technical and regulatory considerations.
2. Develop alternatives to *Agrobacterium*-mediated transformation for California wine grapes and/or cultivars suitable for generating root stocks.
3. Develop strategies to disseminate biological resources under appropriate licensing agreements for the PD community.
4. Explore collaborative opportunities with researchers developing PD control strategies to link the developed transformation technologies with specific PD resistance technologies.

RESULTS
PIPRA has proposed to identify a suite of complimentary technologies that are scientifically functional and legally deployable for public research and potential commercial uses. Described below are technologies believed to meet these needs.

Plant Transformation
Of a limited number of high efficiency plant transformation methods, the method of choice for essentially all researchers is *Agrobacterium tumefaciens*-mediated transformation. In this process, genes are delivered to plant cells via contact with *Agrobacterium* that harbor plant transformation vectors containing a DNA cassette flanked by *Agrobacterium* T-DNA borders. The T-DNA sequences facilitate transfer and integration of the desired transgene into the plant genome. Patent coverage for *Agrobacterium*-mediated transformation in the U.S. is uncertain because of a long interference which has delayed issuance of the primary patent for over 20 years. By comparison to its European counterpart we can reasonably conclude that when the US patent issues, it will contain methods claims to the use of *Agrobacterium* and T-DNA border

sequences (Fraley et al. 1991). PIPRA's transformation strategy will thus seek to identify alternate strategies to the use of both *Agrobacterium* and T-DNA borders as components of the gene transfer vehicle.

Agrobacterium Alternatives
Rhizobium trifolii, Rhizobium, *Sinorhizobium meliloti*, and *Mesorhizobium loti* species have all been demonstrated to introduce new genetic material into plants (Schilperoort et al. 1986, Broothaerts et al. 2005). Although transformation rates are reduced, experimental data indicates these bacterial species can provide an alternative to *Agrobacterium*-mediated transformation (Schilperoort et al. 1986, Broothaerts et al. 2005, Jefferson 2005). PIPRA is currently assessing the legal landscape surrounding the use of these strains for plant transformation.

P-DNA Technology
PIPRA proposes to employ plant-derived "P-DNA" borders that can functionally substitute for *Agrobacterium*-derived T-DNA border sequences. The J. R. Simplot Company discovered and patented P-DNA sequences that are functionally comparable to those from *Agrobacterium* (Rommens 2004, Rommens et al. 2004, Rommens et al. 2005). While P-DNA borders from *Vitis* have not been reported, we propose to use degenerate primers to isolate putative functionally equivalent sequences from grape. Additionally, we have made arrangements to search for P-DNA border sequences in a Pinot noir cultivar that has recently had its genome sequenced through a collaboration between the Italian Istituto Agrario di San Michele and Myriads Genetics Inc. P-DNA borders are attractive as they allow the creation of transformation vectors in which the entire transferred DNA is plant-derived.

Selectable markers
Genetic engineering of plants typically requires the co-integration of trait-conferring genes with genes that confer positive or negative selection to facilitate identification of genetically modified cells. The most common marker used for research and commercial production is the bacterial neomycin phosphotransferase II (*NptII*) gene that grants resistance to several antibiotics (Miki and McHugh 2004). However, in spite of the fact that *NptII* has been determined to be safe by numerous regulatory agencies, consumers express concern over residual non-plant antibiotic resistance genes in genetically modified crops. Furthermore, broad issued patents and new patent claims covering the use of antibiotic resistance genes for plant transformant selection are in place in the U.S. and not generally available for license. A number of new selectable markers have recently been described (Miki and McHugh 2004) and notably, two plant-derived markers have been reported (Dirk et al. 2001, 2002, Mentewab and Stewart 2005). The plant peptide deformylase (*DEF*) from *Arabidopsis* confers tolerance, when overexpressed, to *DEF*-specific inhibitors which are otherwise lethal to plants (Dirk et al. 2001, 2002). The *Arabidopsis* ABC transporter, *Atwbc19*, provides kanamycin resistance levels comparable to the bacterial-*NptII* gene when overexpressed (Mentewab and Stewart 2005). In contrast to the bacterial-*NptII* gene and bacterial homolog of *Atwbc19*, which provide tolerance to a broader spectrum of antibiotics, the plant transporter appears to provide tolerance only to kanamycin. These two markers have the advantage that, because they are plant-derived genes, risk of horizontal gene transfer resulting in bacterial chemical resistance is greatly reduced. PIPRA has engaged in productive discussions to include these technologies in the transformation vector system.

Marker-free technology
Although excision and removal of selectable markers has been accomplished in many plant species that can be subjected to subsequent rounds of breeding, this approach is not feasible in grape cultivars because of the inability to engage in subsequent rounds of breeding. Here we proposed a strategy that has been demonstrated in several model systems and uses recombinase-mediated gene excision to remove the selectable marker from the genome, after selection of transformed plants, by a mechanism which does not support re-integration (Dale and Ow 1991, Russell et al. 1992, Gleave et al. 1999, Sugita et al. 1999, Sugita et al. 2000, Hohn et al. 2001, Zuo et al. 2001, Schaart et al. 2004). The recombinase-based transformation cassette is designed to incorporate three distinct functionalities: selection for cells that are initially transformed, an inducible recombinase gene that can be transiently activated to excise the selectable marker cassette and a second negative selectable marker (Perera et al. 1993, Gleave et al. 1999) to kill cells in which recombinase-mediated excision does not occur. This approach can achieve removal of the selectable marker during the first generation plant tissue culture stage. Although recombinase-mediated gene excision systems have been filed for patent protection (Moller et al. 2004), preliminary evaluation indicates these technologies are available for non-exclusive licensing.

Promoters
Regulatory elements that control the expression of desirable traits or selectable markers in specific plant or tissue organs and developmental stages are desirable when developing biotechnology products. PIPRA has created a database of promoters with technical and legal information. This database is populated with over 700 promoters and has been valuable in analyzing the IP availability of regulatory elements. A wide array of these promoters is patented; however PIPRA staff and a team of patent attorneys have worked to identify a subset of promoters that are either freely available as public domain resources or owned by PIPRA members. Of particular interest to grape research are the PD responsive grape promoters identified by Dr. Cook and colleagues at the University of California, Davis. A selection of these promoters will be included in the vector system and will accommodate varied expression pattern needs (i.e. constitutive, PD responsive, root specific).

Testing and Validation of Transformation System

Due to the time period covered by this proposal and the recalcitrant nature of *Vitis vinifera* transformation, PIPRA and The Ralph M. Parsons Foundation Plant Transformation Facility will use the Thompson Seedless grape variety. Once we generate a sufficient number of independent transgenic events to obtain statistically meaningful data, we can apply the findings to a more targeted effort on select wine grape cultivars and root stocks. Additionally, system components will be tested separately before integrating all pieces into a single system. Experimental characterization of these vectors will rely on a gene of interest cassette comprised of a marker gene. The final system will offer an alternative to *Agrobacterium*-mediated transformation, P-DNA borders, alternative plant selection markers, recombinase-mediated marker gene excision and a variety of promoter options.

Explore collaborative opportunities with transgenic PD Control Strategies

The outcome of this research lends itself for collaborative projects. An important aspect of this project is adoption and improvement of the transformation system by researchers utilizing transgenic approaches for PD management. PIPRA is actively exploring collaborations within the PD consortia.

IP Strategy

Effectively accomplishing the goals of this project will require parallel approaches addressing an IP strategy as well as a technology development strategy. The IP strategy will evaluate patent landscapes related to each element in the grape transformation system. It will also refine and implement a plan to access IP rights to selected technologies that are necessary to develop and utilize the transformation technologies embodied in a series of grape-specific transformation vectors. This will require substantial bilateral and multi-lateral negotiations and the development of agreements that can be implemented across many technology users and projects. This system is envisioned to be made available under a packaged licensing agreement that will encompass all system components and be pre-negotiated on a non-profit research royalty-free and a commercial fee-per-use basis.

CONCLUSIONS

Several promising transgenic approaches have addressed the PD threat to California's wine grape industry (Aguero et al. 2005, Reisch and Kikkert 2005). Of the projects that tested transgenic strategies for PD resistance, each used proprietary technologies that could not be deployed commercially due to IP issues and would likely not survive regulatory scrutiny. Moving forward, it is important to develop a transgenic technology platform in grape with accompanying IP analysis that will allow transfer of control strategies from the laboratory to commercial fields. Anticipating potential IP roadblocks is particularly important in *Vitis* research because it has a high market value, is recalcitrant to routine transformation protocols and has a long tissue regeneration timeframe. Grapes may take 2-3 years per generation and decades to breed industry-acceptable cultivars and it is impractical to employ research strategies that ultimately need to be repeated for commercial deployment due to IP issues that were not addressed at the start of the project. PIPRA, as a clearinghouse of patented technologies, represents 41 non-profit universities and research institutions in 12 countries which account for at least 45% of the proprietary agricultural innovations developed in the public sector. Thus, PIPRA is well positioned to develop technology packages that provide a clear legal pathway for research that is targeted towards practical Pierce's disease and Glassy-winged Sharpshooter applications.

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SUPPORT FOR THE MANAGEMENT OF INTELLECTUAL PROPERTY WITHIN THE PIERCE'S DISEASE RESEARCH INITIATIVE AND RESEARCH COMMUNITY

Project Leader:
Alan B. Bennett
PIPRA
University of California
Davis, CA 95616
abbennett@ucdavis.edu

Cooperators:
Cecilia Chi-Ham
PIPRA
University of California
Davis, CA 95616
(clchiham@ucdavis.edu)

Sara Boettiger
PIPRA
University of California
Davis, CA 95616
(sboettiger@ucdavis.edu)

Josef N. Geoola
PIPRA
University of California
Davis, CA 95616
((jngeoola@ucdavis.edu))

Reporting Period:

ABSTRACT
The Public Intellectual Property Resource for Agriculture (PIPRA) and the California Department of Food and Agriculture Pierce's Disease/Sharpshooter Board (Board) began collaborations in 2005 with the goal of instituting an intellectual property (IP) management strategy inline with the Pierce's disease (PD) research consortium's mission. Within the last year, a number of information resources have been made available by PIPRA specifically tailored for the PD research community. These resources include a publicly accessible, live and comprehensive database of all PD related IP and scientific literature, an analysis of the IP and scientific literature surrounding PD research, and an IP landscape surrounding a promising PD specific technology. Collectively, these resources allow scientists to have an integrated view of the technical and legal aspects involved in their projects.

INTRODUCTION
The Public Intellectual Property Resource for Agriculture (PIPRA) is a not-for-profit research organization hosted by the University of California, Davis. PIPRA currently represents 41 public sector organizations from twelve different countries and its mission is to enable access to agricultural intellectual property (IP). PIPRA offers a range of services to address legal issues that arise during research and deployment of bio-technologies. PIPRA and the California Department of Food and Agriculture Pierce's Disease/Glassy-winged Sharpshooter Board (Board) began collaboration in 2005 to address IP issues surrounding Pierce's disease (PD) research and development. In particular, the threat PD poses to California's $16.5 billion wine industry requires foresight to seek and secure commercial deployment of feasible technologies resulting from funded research. In terms of IP, the Board would like to ensure that technologies with the potential to control PD could be promptly deployed without becoming tangled in a legal web of licenses, rights, and lawsuits.

Technologies resulting from research funded by issue-focused consortia and conducted at multiple institutions, as in the case of the PD consortium, can face three basic IP problems during research and development. First, the researchers themselves may not be aware of their obligations or opportunities with regard to patenting research discoveries. Second, once patented, new discoveries are rightfully the property of the funded research institution or university, which may have internal policies regarding licensing that may be inconsistent with the objectives of the consortia. And third, the new technologies may be blocked by already existing patented technologies. These kinds of IP issues are not uncommon in industry consortia. They are, however, often resolved up front by contractual relationships or formal joint ventures that take into account the participants' IP management strategies. Consortia of universities and other public research entities, however, typically do not have developed IP management strategies in place, in part due to the fact that public sector researchers often pay little heed to the proprietary nature of their research inputs and outputs.

PIPRA recognizes that an IP management strategy for the PD consortium needs to take a multilateral approach toward maximizing the effectiveness of the consortium's intellectual assets. Rather than focusing solely on IP protection, IP management for the PD consortia should also set milestones for technology development, assess marketing opportunities, and seek a better negotiating position during IP exchange. In essence, PIPRA seeks to aid the Board in coordinating IP to allow for access and protection, both of which are essential to the productivity of research across multiple institutions, while creating opportunities and incentives for further commercial development.

The first step toward effective IP management is the availability of information resources specifically tailored to Board funded PD researchers. Such resources provide scientists with technical and legal information critical for the deployment of marketable products with maximum security over IP rights. This report discusses the information resources specific to the PD research consortium developed by PIPRA. Included will be detailed descriptions of the IP and scientific literature database

geared towards PD specific research, an analysis of the information therein, and a biotechnology case study illustrating the types of IP issues intrinsic in emerging PD control biotechnologies.

OBJECTIVES

1. Development and maintenance of an IP and scientific literature database dedicated to PD.
2. Broad analysis of the current trend in the IP and scientific literature surrounding research in PD.
3. Analysis of the IP landscape surrounding a target technology directly related to Pierce's disease in grapes.

RESULTS

Objective 1

The IP and scientific literature database (e.g. the PD/GWSS-PIPRA database) was designed with a vision to provide state-of-the-art patent and scientific literature search and analysis tools. The PD/GWSS-PIPRA database currently contains over 6,000 IP records and over 2,500 scientific publications. This library of IP and scientific literature is updated on a quarterly basis to include the most recent IP disclosures and scientific publications available to PIPRA.

PIPRA launched an *alpha*- version of the PD/GWSS-PIPRA database in February 2006. Seventeen PD-researchers were selected to test the functionality and usability of the *alpha*-version database. The greatest concerns raised by testing researchers included slow query search speeds, the inability to connect directly to a publication of interest and the lack of a help menu. PIPRA worked with database technicians at M-CAM Inc. (http://www.m-cam.com) to resolve these issues. M-CAM optimized the searching algorithm of the PD/GWSS-PIPRA database so that search times would be cut drastically. PIPRA integrated OpenURL resolvers into the PD/GWSS-PIPRA database in order to allow researchers to directly connect to scientific publications. OpenURL resolvers are applets which can detect the end-user's host institution and connect to a publication if the institution holds a subscription to the publication. PIPRA is actively working to implement OpenURL resolvers for all institutions funded by the Board. The *beta*-version of the PD/GWSS-PIPRA database was released in May 2006. This version addresses most technical issues experienced in the *alpha*-version, including the lack of a help menu. The *beta* version (Figure 1) was also aesthetically redesigned and introduced a help menu for a more user-friendly interface. Currently, the database provides two search methods, SmartText™ and Compass™. SmartText™ allows the end-user to search across all records in the PD/GWSS-PIPRA database using a keyword or a search string. Searches can be initiated either as Boolean or advanced searches. Boolean searches allow the user to choose up to four fields to across which to search; a total of 29 fields are available. The Boolean search form also allows users to limit the returned records to patents, scientific literature, or to include both. Advanced searching is designed for generating more dynamic queries and requires that the user be familiar with creating search strings. A Compass™ search allows users to retrieve bibliographic and legal information on one or two specific patents. Compass™ also has the capability to display two patents side-by-side for easy comparison.

Figure 1: PD/GWSS-PIPRA database (**http://pierces-disease.m-cam.com**)

Scientists and IP professionals alike can utilize the PD/GWSS-PIPRA database prior to intiating or during PD related research. Valuable information, such as availablity of substitute technologies, with less IP restrictions, and complementary technologies, to ehance marketing opportunities, can be identified using the PD/GWSS-PIPRA database. Furthermore, because this database is commited to the field of PD, many of the IP records listed in the database will be the result of other Board funded research projects at other public sector universities, and in certain situations, be more readily licensable. Collectively, this information can help scientists conduct research to develop promising technologies with more ease of mind over its ultimate commercial viability.

Objective 2

PIPRA conducted an analysis of the scientific literature and IP surrounding PD research. The analysis has helped the Board and PIPRA better understand the magnitude of PD related research conducted across the United States. This information can be used early on to identify potential commercial partners and/or independent researchers who may be aligned with the consortium's goals. PIPRA will be using the information gathered in this analysis to launch a thorough survey on the impact the Board's funding has had on progress towards controlling PD.

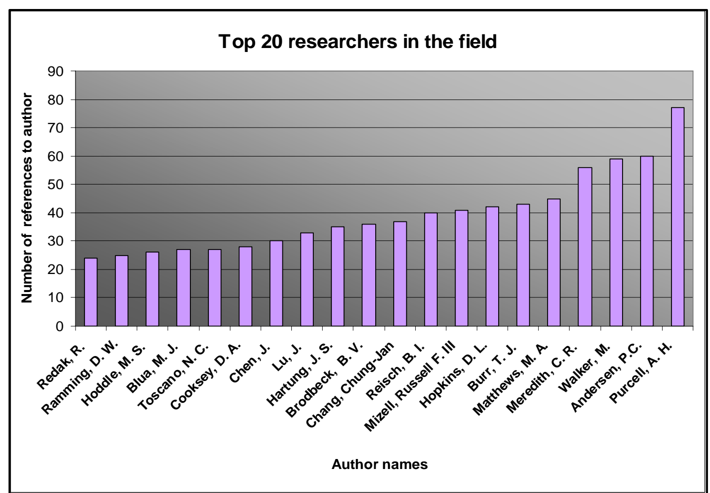

Figure 2. Top 20 Authors of PD-related publications.

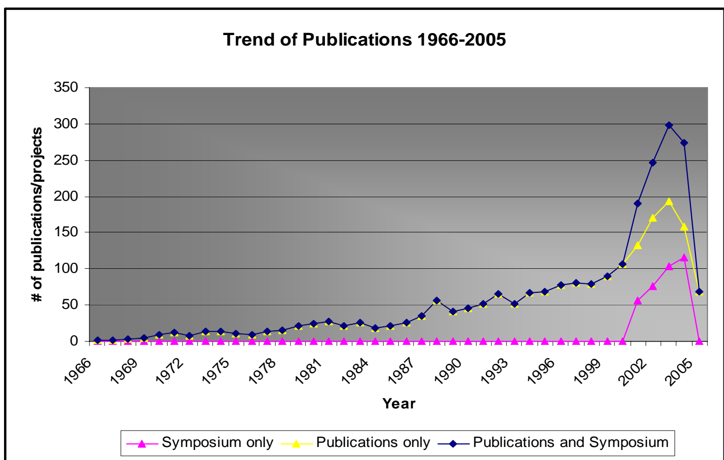

Figure 3. Number of PD-related publications per year.

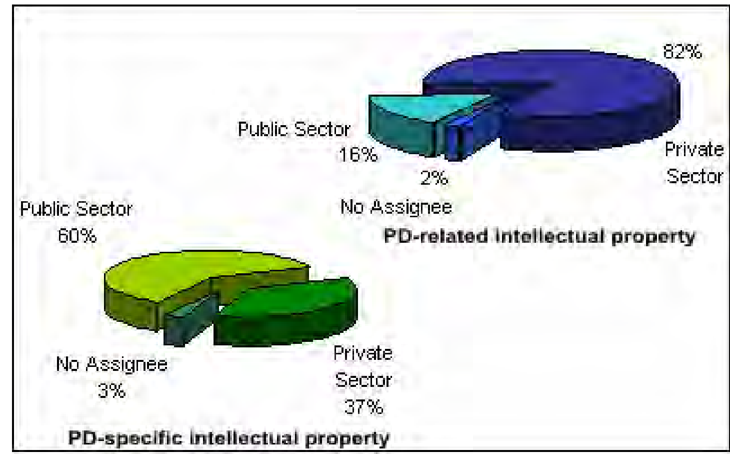

Figure 4. Distribution of IPR (patents and patent applications) in the public and private sectors.

Analysis of the scientific literature surrounding PD identified Dr. Sandy Purcell as the single most active publisher. Dr. Purcell is listed as principle investigator on over 70 publications (Figure 2). University of California, Davis and University of California, Riverside were identified as most active PD research institutions. Publishing trends in journals and symposium proceedings within the last 40 years (Figure 3) were also analyzed. A rise in the number of publications was noticeable beginning 2001. This is attributed to the increased funding PD related research received following the Board's establishment in 2001.

Analysis of the IP surrounding PD identified a total of 6,335 US patents and patent applications in the broad area of plant disease resistance but only 30 of these records described PD specific technologies. The remaining 6,305 records involved technologies applicable to *vitis*, biological control strategies and disease resistance systems. PIPRA researched the distribution of these IP rights (IPR) and found that, of the group of 30 IPR, 60 percent were owned by public sector institutions and 37 percent were owned by private industry (Figure 4). This indicated that PD-specific research was more intensive at public-sector institutions. In contrast, of the group of 6,305 broad-based IPR records, only 16 percent of the technologies were owned by public sector institutions while approximately 82 percent were owned by private industry. This distribution was not surprising since many pesticidal control systems, which were included in the 6,305 records, had been developed by chemical manufacturers in the private sector. This analysis also revealed that within the public sector, the University of Florida (Florida) had been most active in patenting PD related technologies. Collected data indicates that while only three researchers from Florida are funded by the board, the university is assignee on 42 percent of PD-related technologies within the public sector.

Objective 3

PIPRA conducted an IP analysis case study on a novel PD control technology developed by Dr. Goutam Gupta and supported by the Board. Dr. Gupta and colleagues developed an anti-microbial technology for rendering *vitis* crops resistant to PD (Dandekar 2005). A patent application (US serial no. 10/846,172) for the technology had already been filed on behalf of the inventors. For this case study, PIPRA illustrated how IP considerations, in addition to patenting, could help develop a research plan that supports commercial deployment. PIPRA researched the prior art, scientific literature, and IP landscape pertaining to a case study technology and aimed to capture IPR related to the major components and processes used by the technology.

Analysis of the IP surrounding Gupta's technology revealed a complex landscape containing many legally protected biological components. PIPRA was able to show how many of the components used by Dr. Gupta's biological construct and which required multiple licenses, could be replaced by functionally equivalent components with greater freedom-to-operate.

Moreover, the analysis also touched on the regulatory and social issues which could potentially rise during commercialization; Dr. Gupta's anti-microbial technology utilizes a human protein, a construct which is acceptable for research purposes but not desirable for a marketable ag-product. PIPRA is currently developing plant transformation enabling technologies designed to offer the possibility of incorporating, where possible, plant-derived components.

CONCLUSION

The development of a successful IP management strategy is essential to creating a strong IP portfolio. With the advent of the information resources made available by PIPRA, scientists within the PD research community are now better capable to plan research projects with proprietary values in mind. PIPRA also recognizes that these resources are only a part of a successful IP management strategy. Within the next year PIPRA will continue to build on these tools by exploring the impact Board funding has had on PD research and by conducting a thorough IP audit of a target technology in order to identify embedded IPR that could affect commercialization. These services will help implement an IP management strategy as the PD consortium prepares to advance the research and development of emerging industry solutions.

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

Project Leaders:

George Bruening
Dept. of Plant Pathology
University of California
Davis, CA 95616
gebruening@ucdavis.edu

Edwin Civerolo
USDA, ARS
SJVASC
Parlier, CA 93648
eciverolo@fresno.ars.usda.gov

Cooperators:

Paul Feldstein
Dept. of Plant Pathology
University of California
Davis, CA 95616
pafeldstein@ucdavis.edu

Marta Francis
Dept. of Plant Pathology
University of California
Davis, CA 95616
mfrancis@fresno.ars.usda.gov

Abhaya M. Dandekar
Dept. of Pomology
University of California
Davis, CA 95616
amdandekar@ucdavis.edu

Goutam Gupta
MS M888, Biol. Division
Los Alamos Natl. Lab.
Los Alamos, NM 87544
gxg@lanl.gov)

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ABSTRACT

The aim of this project is to construct and express in test plants, and then in grapevine, a protein or protein chimera ("anti-*Xf* protein") capable of inactivating or otherwise interfering with the infectivity of *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease of grapevine. Several *Xf*-cell-surface-binding peptides were selected from a random peptide library. For some of these peptides, the *Xf* cell target of binding and the stoichiometry of binding have been tentative identified. Evidence was obtained for a biologically relevant interaction between the selected peptides and *Xf* cells.

INTRODUCTION

It is likely that the development of grapevine cultivars resistant to *Xylella fastidiosa (Xf)* presents the best approach to long term, effective, economical and sustainable control of Pierce's disease (PD). Our strategy is to create transgenic rootstock(s) that will secrete a protein or proteins into the xylem for transport to scion xylem, where it will provide protection against insect vector-delivered *Xf*. An effective protein may kill *Xf* cells or merely interfere with the ability of *Xf* cells to colonize or spread in the scion xylem. Regardless of the mode of action, such proteins are here referred to here as anti-*Xf* proteins. No protein of the desired activity exists, and it is the immediate aim of this project to create anti-*Xf* protein(s). Several approaches have been taken. The approach that has been most productive is the selection of *Xf* cell-surface-binding peptides, as we describe in this report. Such peptides may be incorporated into a protein scaffold so as to generate a *Xf*-cell-surface binding protein. We have identified as a promising scaffold a protein of a T2-like bacteriophage: the tail fiber adhesion gp38 (Riede et al. 1987).

OBJECTIVES

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

RESULTS

Objective 1 (Discover peptides and proteins with high affinity for macromolecules on the *Xf* cell).

Selecting peptides that bind to Xf cells

Xf cell-binding peptides were obtained by a combinatorial biology approach: selection from a random peptide library. The source of the random peptide library was a commercial kit (New England Biolabs "Ph.D.-12 Phage Display Peptide Library," designated here RP-M13) incorporating 12 amino acid residue random peptides at the amino end of the bacteriophage M13 adhesin protein pIII (Figure 1) (Anonymous 2004). The RP-M13 (~2.7 x 10^9 peptide sequences, with ~55 particles displaying any single peptide in a 10µL aliquot) was applied using "panning," a procedures involving multiple rounds (typically four or more) of selection in which the filamentous M13 particles bearing random peptides were exposed to the target (*Xf* cells). The target was washed, typically 8 times, and any remaining bound M13 was eluted and recovered, typically at pH 2.0-2.2. The eluted M13 was titered and amplified by inoculation of male *E. coli*. M13 progeny were partially purified before initiating the next round of selection (Smith and Scott 1993, Barbas et al. 2001).

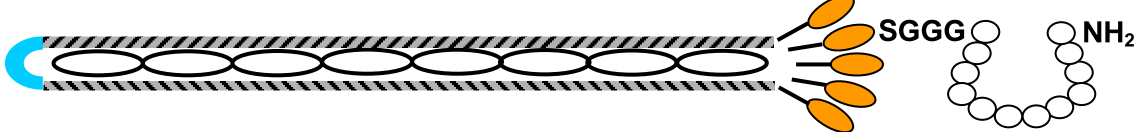


Figure 1. Diagram of a bacteriophage M13 random peptide library member. The random peptide 12-mer (open ovals) is incorporated at the amino end of the absorption protein (designated PIII or P3) of the M13 particle by a flexible tri-glycine-serine sequence and is encoded in the bacteriophage DNA. For simplicity only one of the 5 PIII proteins, orange oval, is shown with the random peptide amino end, although all five actually incorporate it.

Panning on the agar plate-derived and liquid culture (planktonic) cells proceeded differently. For the plate-derived cells, the titer increased about 10^2 at each cycle, and 14 of 20 clones obtained after 4 cycles were positive for binding to plate-derived *Xf* cells. For the planktonic cells, the titer increased only about 10^1 at each cycle, and only 1 of the first 20 M13 clones obtained after 5 cycles was found to be positive. Higher throughput methods for assaying M13 clones are in progress. Figure 2 presents an assay for *Xf*-cell-binding by selected M13 clones, and Table 1 presents results for several M13 clones.

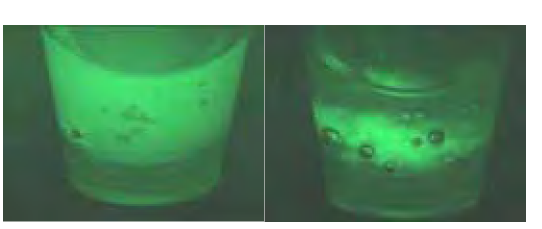


Figure 2. *Xf* cells binding of 12-mer peptide-bearing M13. Each M13 clone preparation (~10^{11} particles, 2.7μg) was incubated with *Xf* cells, and the cells were washed three times to remove unbound M13 particles. Fluorescently labeled anti-M13 IgG was added. Left panel: no reaction detected; fluorescent antibody remains in solution (M13 clone 4N2). Right panel: binding detected by agglutination (M13 clone 4N1).

Of the nine M13 clones selected with plate-derived *Xf* cells, none were found to bind to planktonic cells. In contrast, the single M13 clone obtained with planktonic *Xf* cells as the target reacted with both plate-derived and planktonic cells. Thus we have isolated reagents that are plate-derived cell-specific (9 M13 clones of the 4N and 4T series), that are planktonic-cell-specific (antibody to MopB protein, an abundant outer membrane protein, data presented in our 2004 report), or that are able to recognize *Xf* cells of both origins (M13 clone 5-19). These results suggest that plate-derived and planktonic *Xf* cells have surface compositions that are largely, but not entirely, distinct.

Table 1. Binding of M13 peptide-bearing clones to *Xf* cells

Clone identifier (a)	Target	Amino acid sequence (b)	Wt *Xf* plate cells?	Wt *Xf* planktonic cells?	H*Xf*A-minus *Xf* plate cells? (c)	H*Xf*B-minus *Xf* plate cells? (d)
4N1, 4N5	A[d]	STLHRHTPDLRLGGGS	yes	no	no	yes
4N2	A	TLPPWITTMRYQGGGS	very weak	no	no	no
4N3	A	YDLWTMSPDFKLGGGS	yes	no	no	yes
4N4, 4T1, 4T7, 4T8	A	QIVTQNVPFILRGGGS	yes	no	ND	ND
4N6	A	IISHTPVIQLGRGGGS	yes	no	ND	ND
4T2, 4T6	A	NLVYTMSSDIPLGRGS	yes	no	no	yes
4T3, 4T9	A	WTLDLWAKPIDLGGGS	yes	no	no	yes
4T4a	A	TQMNLYTPALLLGRGS	yes	no	ND	ND
4T5	A	EAGNIVIRPFYAGGGS	yes	no	ND	ND
5-19	P[d]	ATSPTRLAALAQGGGS	weak	weak	no	no
FR	A	not a clone	no	ND	no	no

(a) Bold font designates the clone selected among duplicates for subsequent experiments; FR = first round selection, which presumably is only very poorly enriched in *Xf*-cell-binding proteins
(b) GGGS is the linker sequence between the 12-mer random peptide and the amino end of the M13 adhesin P3 (Fig. 1), although in two instances the sequence was found to be altered to GRGS
(c) H*Xf*A, H*Xf*B, products of *Xf* genes PD2118 and PD1792, respectively; *Xf* cell strains with inserts in these strains were provided by Tanja Voegel and Bruce Kirkpatrick (Guilhabert and Kirkpatrick 2005)
(d) A = cells cultured on and recovered from agar plates ("plate cells"; PD3 medium); P = planktonic cells from liquid culture (PD3)
ND: not determined

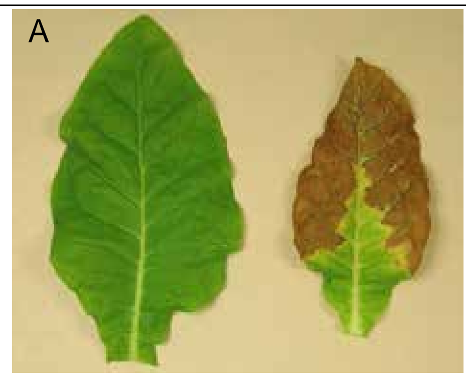
***Investigations of the possible Xf cell surface target and binding extent for M13 clone-Xf cell interactions*.**
Plate-derived *Xf* cells may be "exopolysaccharide coated." Therefore, we tested for the ability of "fastidian gum" (gift from L. Ielpi via C. Roper and B.C. Kirkpatrick), the postulated exopolysaccharide material of *Xf* (da Silva et al. 2001), to interfere with the agglutination assay for M13 binding (Figure 2). M13 bacteriophage (10^{11} particles, about 2.7µg) was exposed to 25µg of fastidian gum in 100µL of buffer for 45 minutes before addition of *Xf* cells. No interference in the agglutination reaction was observed, suggesting that fastidian gum is not involved in the *Xf* cell interactions with the selected M13 clones. Results from other experiments suggest that MopB, likely the major outer membrane protein of *Xf*, also is not the target on the *Xf* cell surface to which any of our M13 clones bind. Other potential targets for the M13 clones are two hemagglutinin-like proteins of *Xf*, H*Xf*A and H*Xf*B (Guilhabert and Kirkpatrick 2005). We did not detect binding of any of our M13 clones to mutant *Xf* cells with an inactivated *HXfA* gene, whereas *Xf* cells with an inactivated *HXfB* gene bound to all of the tested M13 clones that exhibited strong binding to wildtype *Xf* cells (Table 1).

In order to obtain a lower bound estimate of the number of M13 particles bound to an *Xf* cell, a suspension was prepared of 2 x 10^8 *Xf* cells from agar plates and 2 x 10^{12} pfu/mL of either M13 clone 4N1 or M13 clone 4N2. The suspension was incubated for 1 hr at room temperature, and the cells were recovered and washed three times with the buffer that was used in the panning experiments. Plaque assays were performed on the last wash and after a pH 2.2 elution. For 4N1, about 1000 pfu of M13 was recovered per *Xf* cell after pH 2.2 elution, whereas the value for 4N2 (known to bind to *Xf* cells poorly in the Figure 2 assay) was under 100.

Objective 2 (test proteins for interference with disease initiation)
The usual approach for the application of selected, target-binding random peptides is to incorporate them into a scaffold protein for testing against the target. We expect to have a large number of selected peptides and therefore need a less elaborate approach to evaluating their potential efficacy under biologically relevant conditions. Others have selected RP-M13 clones that bind to bacterial cells by panning and have observed the binding of the M13 bacteriophage particles to the target cells by electron microscopy (Petrenko and Sorokulova 2004). *Xf* cells were mixed with each of the six M13 clones (4N1, 4N2, 4N3, 4T2, 4T3, 5-19) as described above, incubated, and then inoculated to SR-1 tobacco plants. No M13 clone was able to prevent infection of the tobacco plants. However, interference with *Xf* infection, i.e., a potentially relevant biological activity, was observed for the set of M13 clones taken as a group (Figure 3). At the greater of two concentrations of M13 tested (3 x 10^{12} pfu/mL), the disease rating was significantly reduced (p=0.0003 assuming null hypothesis) compared to the average disease rating for plants receiving *Xf* alone. The M13 molar excess over *Xf* cells, about 10^4-fold, corresponds in magnitude to the number of copies of some abundant bacterial cell-surface proteins that could be sites for binding and is only 10-fold greater than the estimate for 4N1 M13 clone binding to *Xf* cells as observed above.

The observed interference with *Xf* infectivity supports the feasibility of the overall approach being taken in this project. However, it is important to note that Figure 3 reports results from a single experiment and, though the results are statistically significant, the experiment must be repeated to be convincing. The observed effect is small and presumably will require more effective peptides and/or incorporation of binding peptides into bactericidal constructions to create a more powerful anti-*Xf* technology.

CONCLUSIONS
The approach to solving the PD problem that is taken in this project is to create transgenic grape rootstock that will confer, on the scion, protection against infection by *Xf*. Otherwise, it is expected, the new rootstock will cause no alteration in the agronomic or quality traits of the scion compared to the situation of scion propagation on conventional rootstock. Rootstock-conferred protection is to be accomplished by expression of a xylem-targeted anti-*Xf* protein, the creation of which is the current focus of the project. Results reported here suggest that we have selected *Xf*-cell-surface-binding peptides, that these peptides have a perceptible capability for interfering with *Xf* infection of SR-1 tobacco plants (Figure 3), and therefore that a biologically relevant and at least transient interaction can occur between the selected peptides and *Xf* cells *in planta*. Our results also (i) suggest that a single *Xf* cell can bind at least 1000 peptide molecules, (ii) tentatively identify the target molecule for several of our selected peptides as the hemagglutinin-like *Xf* surface protein H*Xf*A, (iii) reveal peptide consensus sequences possibly involved in the interaction (blue font and blue underline in Table 1), and (iv) suggest that planktonic cells cultured under at least one condition do not have a significant population of exposed H*Xf*a molecules on their surface (compare columns 5 and 6 of Table 1). It is likely that the form of *Xf* cell that is released by the sharpshooter as it inoculates the plant resembles planktonic *Xf* cells more than it resembles plate-derived *Xf* cells. Therefore, we plan to discover additional peptides with affinity for planktonic cells, to identify those showing activity *in planta*, and to incorporate these peptides into suitable scaffold/fusion proteins for generation of more effective anti-*Xf* activity.



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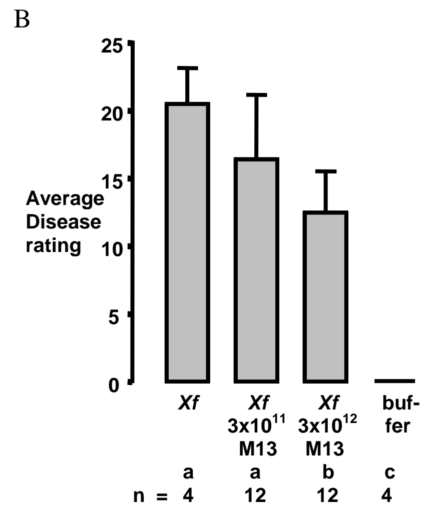

Figure 3. Biological activity of M13 clones selected for their affinity to the surface of *Xf* cells. Six M13 clones were tested for their ability to interfere with *Xf* infection of SR-1 tobacco, using six M13 clones and two plants per clone. The SR-1 plants were cut back to three leaves per plant. Temecula 1 *Xf* cell suspension from agar plates at ca. 2.4×10^8 cells/mL was mixed in 0.5X TE buffer (5 mM Tris-HCl pH7.5, 0.5 mM sodium EDTA) with M13 at 3×10^{11} or 3×10^{12} pfu/mL. After one hour, 20 µL of cell suspension was inoculated to the base of the petiole of each of the three leaves. The course of disease development was observed. Chlorosis on some leaves was observed at 3-4 weeks after inoculation, and symptoms typical of *Xf* diseases developed: scorching expanding from the leaf tip or other localized areas and separated from green tissue by a bright yellow halo. Symptoms were demonstrated to be correlated with the presence of *Xf* by quantitative PCR. At three months after inoculation, each leaf was assigned a disease rating as follows: scores of 1, 2 (leaf on right, panel A) or 3 for scorching symptoms accounting for <50%, 50-75%, and >75% of the leaf lamina area, respectively; no symptoms characteristic of *Xf* infection (leaf on the left, panel A), score of zero. The scores of all of the leaves of each plant were summed to give the disease rating of the plant. The average and standard deviation for four groups of plants are presented in panel B, with the number of plants per group at the bottom of the panel. Plants inoculated with buffer only or untreated showed no symptoms characteristic of *Xf* infection. As would be expected, the disease ratings for two plants receiving the same inoculum (root-mean-square difference 3.2) were more similar than they were for a pair of plants selected at random (standard deviation 4.7). The disease ratings were significantly different ($p<0.05$, Student's t-test) for plants receiving *Xf* exposed to 3×10^{12} Pfu/mL M13 as compared to the other three groups (lower case letters below bars).

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HOST PLANT PREFERENCE AND NATURAL INFECTIVITY OF INSECT VECTORS ON COMMON WEEDS KNOWN TO HOST X*YLELLA FASTIDIOSA*

Project Leader:
Kent M. Daane
Dept. of Environmental Science
Policy & Management
University of California
Berkeley, CA 94720

Cooperators:
Rodrigo Almeida
Dept. of Environmental Science
Policy & Management
University of California
Berkeley, CA 94720

Jennifer Hashim-Buckey
UC Cooperative Extension
Kern County, Bakersfield, CA

Researchers:
Christina Wistrom
Murray Pryor
Glenn Yokota
Dept. of Environmental Science
Policy & Management
University of California
Berkeley, CA 94720

Reporting Period: The results reported here are from work conducted in September 2006 (this project is newly funded).

ABSTRACT

Common weed species can harbor *Xylella fastidiosa* (*Xf*) and its insect vectors. Should weed control be part of a Pierce's disease control program? To address this question, we will survey weed species in agricultural areas known to host *Xf,* and determine the level of insect vector activity and the proportion of potential vectors that carry *Xf*. In the laboratory, we will compare three techniques to detect *Xf* in insects. Current methods produce mixed results; assessment of each method's accuracy will improve comparison of research projects and field survey results. This project will provide information for control decisions by investigating the importance of vegetation management in reduction of insect populations and inoculum potential.

INTRODUCTION

The emergence of Pierce's disease (PD) of grape in the General Beale Road area in Bakersfield in 2001 and 2002 exemplifies the threat posed by the glassy-winged sharpshooter (GWSS). While GWSS populations and PD are currently managed by an area-wide insecticide spray program and diseased vine removal, endemic GWSS populations are still present. This may be especially true in weedy fields, abandoned vineyards, and along roadsides and windbreaks where PD and GWSS are not managed (J. Hashim - personal communication). Numerous common weeds and windbreak species are hosts of *Xylella fastidiosa* (*Xf*) in greenhouse studies (Purcell and Saunders 1999, Costa et al. 2004, Wistrom and Purcell 2005). More importantly, nine weed species common in the Central Valley were found to be naturally infected with *Xf* (Shapland et al. 2006). Since so many different plants can harbor *Xf* to some extent, more information on sharpshooter host plant use in the field is required before those studies can be translated into concrete recommendations to growers. While GWSS have been observed feeding on a wide range of ornamental and weedy species (CDFA host list at www.cdfa.ca.gov/phpps/pdcp/index), the quantitative data on the numbers and host plant preference of GWSS in agricultural settings focused mainly on presence on citrus, grapes and urban areas, with insect choice determined on plants provided in pots (Naranjo and Toscano 2003; Perring and Gispert 2004; Daane and Johnson 2004; Phillips et al. 2004).

The first objective of this study is the identification of preferred feeding and oviposition hosts by GWSS, among plants already identified as hosts of *Xf* in agricultural areas. Year-round information on sharpshooter presence on host plants would provide information about the need for vegetation removal or modification in and around vineyards. For example, the identification of major breeding hosts of blue-green sharpshooters (BGSS) in northern California enabled the development of a riparian management plan. When the major breeding and feeding hosts of BGSS were removed and replanted with other plants less attractive to BGSS, large reductions in sharpshooter populations and Pierce's disease in adjacent vineyards resulted (Purcell et al. 1999). Similarly, in Central Valley almond orchards affected by almond leaf scorch, the identification of common sharpshooter, treehopper, and spittlebug insect vectors has just been completed (Daane et al. – unpublished data), as well as concurrent assessment of *Xf* presence in sampled weeds and sharpshooters (Shapland et al. 2006).

Field-based data is critical for practical application of treatment thresholds in development (Perring 2004), for GWSS control in areas with endemic sharpshooter populations. One important variable in the infectivity model is the proportion of vectors carrying the pathogen. This second objective of this study will determine the proportion of field-collected sharpshooters, in the San Joaquin Valley, that carry *Xf* in agricultural areas. With a functional treatment threshold, growers can predict the relationship between GWSS population and Pierce's disease potential, and better plan insecticide applications for GWSS control. The natural infectivity of BGSS captured in riparian area was highly variable, ranging from 5 to >40% (A. Purcell -

unpublished data), while in greenhouse studies, between 10 and 20 % of glassy-winged sharpshooters transmitted *Xf* (Almeida and Purcell 2003).

The third objective of this study is to compare the sensitivity and convenience of four techniques to detect *Xf* in insects in a side-by-side comparison. *Xf* transmission to grapes has only been correlated reliably with and bacterial presence in the precibarial region of sharpshooter mouthparts (Almeida and Purcell 2006). PCR-based vacuum-extraction (Bextine 2004a,b) of *Xf* in sharpshooter heads enabled more rapid, efficient, and convenient bacterial detection, in comparison to transmission tests with live plants and vectors (Purcell and Finlay 1980), or insect head culture (Newman et al. 2004). However, lyophilization and maceration, followed by chloroform/phenol extraction, also sensitively detects *Xf* in sharpshooter heads (R. Groves.- personal communication). *Xf* transmission to grapevines is highly sensitive, so sharpshooter infectivity can be assessed when endogenous bacterial populations are below the detection thresholds of culture or PCR (Hill and Purcell 1995). Culture determines the population of living bacteria, whereas PCR is a rapid technique that allows detection of bacterial DNA after the insect has died. Objective three will compare accuracy of detection, cost, turnaround time, and ease of processing between live insect transmission to plants, sharpshooter head culture, vacuum-extraction PCR, and lyophilization-maceration PCR.

OBJECTIVES

1. Determine preference of insect vectors for common weeds known to host of *Xf* in the southern San Joaquin Valley.
2. Determine the proportion of collected insect vectors that carry *Xf*.
3. Compare the efficacy of *Xf* detection methods in insect vectors.

RESULTS

Funding for this project began last month, September 206. Here, we present our planned procedures for comments and discussion to improve future work.

Agricultural sites (e.g., citrus, olives) in the southern San Joaquin Valley (Tulare to Bakersfield, California) will be selected based on the presence of high populations of weeds known to be hosts of *Xf* and moderate populations of GWSS. Each site will be divided into 4 sections or grids, which will be sampled monthly for 1 year.

The percent cover for weeds that are major host species for *Xf* will be determined throughout the year, to account for seasonal changes in vegetation. The weed species sampled will be based on previously reported records of *X. fastidisoa* host status in weeds (Costa et al. 2004; Wistrom and Purcell 2005; Shapland et al. 2006). Sharpshooter abundance will be measured with sweep netting , sticky traps, and visual counts of insects and egg masses. Although we expect sharpshooter collections to be greatest with sweep netting, sticky traps will help assess sharpshooter movement within and adjacent to the site, and visual counts of egg masses and insects, on individually sampled weed species, will determine if hosts are preferred for feeding or breeding. After timed visual inspection of each site for GWSS presence, sharpshooter frequency will be measured with GWSS collections (sweep net samples) on common weed species, similar to surveys of BGSS in riparian habitats.

Sharpshooters captured by sweep netting will be assessed for *Xf*. Live sharpshooters will be placed individually on seedling grapevines for a 4-day inoculation access period (Purcell and Finaly 1980). After removal from test grapes, the sharpshooter cohort will be divided in two. Heads of one third of the insects will be analyzed for *Xf* by culture on PWG media (Davis et al. 1983), which detects multiple strains of *Xf* (Newman et al. 2004; Hill and Purcell 1995). One-third of heads will be analyzed for *Xf* by vacuum-extraction. *Xf* DNA is purified from the extraction buffer using the DNeasy kit (Qiagen Inc.- Valenica, CA.; Bextine et al. 2004b). The remaining one-third of heads will be lyophilized and macerated in liquid nitrogen with a small hand-held pellet pestle. *Xf* DNA will be purified with a modified phenol/chloroform/alcohol extraction (R. Groves 2006 – unpublished data). After vacuum or chemical extraction, the extracted DNA will be multiplied and detected using polymerase chain reaction (PCR), allowing selective detection of *Xf* (Minsavage et al. 1994). Multiple strains of *Xf* (including grape, almond and oleander) can be detected and identified with mulitplex primers (Minsavage et al. 1994). Sharpshooter-inoculated grapevines will be assessed for *Xf* presence by culture and symptoms after approximately 12 weeks.

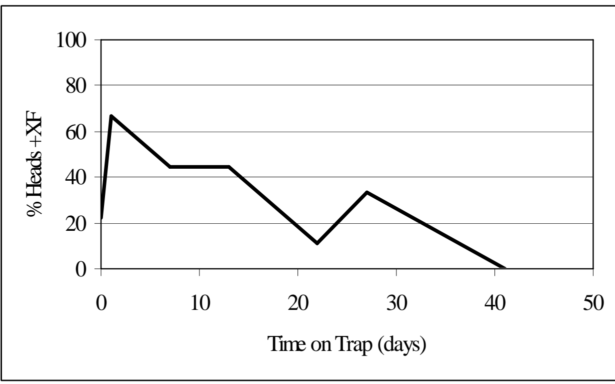

Figure 1: Recovery of *Xf* from blue-green sharpshooters with vacuum-extraction PCR, from sticky traps aged in the greenhouse (Daane and Shapland- unpublished).

Glassy-winged sharpshooters captured in sticky traps will also be analyzed for *Xf* presence using vacuum-extraction and PCR. Brief aging on sticky traps did not appear to affect the recovery of *Xf* from GWSS, (Figure 1; also Bextine et al. 2004a). If

infective sharpshooters are recovered, the weed hosts will be sampled for *Xf* presence with immunocapture PCR, which allows sensitive detection of *Xf* from plants without interference from plant-based PCR inhibitors PCR or contamination from other bacteria.

The third objective will also require greenhouse-based controls, where field-collected GWSS and BGSS will be allowed to acquire *Xf* from Pierce's diseased plants, and then allowed a four-day inoculation access period to feed on a seedling test grapevine. After the inoculation period, heads of one-third of the cohort will be cultured, one-third will be analyzed with vacuum-extraction and PCR, and one-third will be analyzed with lyophilization and PCR. Because BGSS and GWSS differ in their transmission rates of *Xf*, it will be necessary to compare detection techniques with both sharpshooter species to fulfill this objective. In preliminary comparisons, *Xf* was recovered from 73% of heads with vacuum-extraction, while 43% of the same sharpshooters transmitted *Xf* to grape (K. Daane and E. Shapland – unpublished data). To detect a 40 % difference (at 95% confidence) with these 4 techniques (transmission to plants, culture from heads, head vacuum-extraction, and head lyophilization), we will need to sample 800 sharpshooters total.

CONCLUSIONS

As this is the first month of the study there are no conclusions to report at this time.

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FUNDING AGENCIES

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EVALUATION OF SIGNAL SEQUENCES FOR THE DELIVERY OF TRANSGENE PRODUCTS INTO THE XYLEM

Principal Investigator:
Abhaya M. Dandekar
Dept. of Plant Sciences
University of California
Davis, CA 95616
amdandekar@ucdavis.edu

Cooperators:
David Gilchrist
Dept. of Plant Pathology
University of California
Davis, CA 95616

John Labavitch
Dept. of Plant Sciences
University of California
Davis, CA 95616

Ana Maria Ibanez
Dept. of Plant Sciences
University of California
Davis, CA 95616

Cecilia Aguero
Dept. of Plant Sciences
University of California
Davis, CA 95616

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ABSTRACT
Xylella fastidiosa (Xf), a gram-negative bacterium, is the causative agent of Pierce's disease (PD) in grapevines. Because *Xf* is xylem-limited, it will be essential that any anti-*Xylella* gene product be present in the xylem in an effective concentration. Work on understanding the mechanism of how proteins are targeted to this plant compartment will be relevant for the delivery of therapeutic proteins into the xylem. In addition, it will be a useful tool for *Xylella* and glassy-wing sharpshooter (GWSS) gene function studies.

We collected xylem exudate from grapevines and analyzed its protein composition by two-dimensional gel electrophoresis. Peptide spectrum and Blast analysis showed that the proteins found in the exudates are secreted proteins that share function similarities with proteins found in xylem exudates of other species. The corresponding cDNA sequences of 5 of them were found in the TIGR *Vitis vinifera* gene index. The signal sequences of xylem proteins Chi1b and similar to NtPRp27 were fused to the mature pear polygalacturonase inhibiting protein (pPGIP)-encoding gene. The expression of these chimeric genes will be evaluated in transient and permanent transformations in order to evaluate their ability to target pPGIP to the xylem. The results of this research will not only be applied in projects that test anti-*Xylella* gene products that should be delivered into the xylem but also in functional studies that are intended to target the products of *Xf* and GWSS genes to the xylem.

INTRODUCTION
Signal peptides control the entry of virtually all proteins to the secretory pathway, both in eukaryotes and prokaryotes. They comprise the N-terminal part of the amino acid chain and are cleaved off while the protein is translocated through the membrane of the endoplasmatic recticulum (Nielsen et al., 1997). Generally, signal peptides are interchangeable and secretion of non-secreted proteins becomes possible by the fusion of a signal peptide at the N-terminus of the mature protein; however, changing the signal sequence of recombinant proteins can affect the degree of protein production (Yoshida et al., 2004).

In previous research, we fused the sequence coding for the signal peptide of XSP30, a xylem-specific protein from cucumber (Masuda et al., 1999), to the green fluorescent protein (GFP) reporter gene. Contrary to what we expected, fluorescence was only detected inside the cells. Our results suggested that either the XSP30 signal peptide is not recognized by the grape secretory machinery or GFP is not secretion competent. If the first hypothesis is correct, signal sequences obtained from proteins present in grape xylem sap would constitute better candidates for delivery of transgene products to the xylem.

Interestingly, we have also found that the product of the pPGIP encoding gene from pear fruit, heterologously expressed in transgenic grapevines, is present in xylem exudates and moves through the graft union (Aguero et al., 2005). These results show that pPGIP is secretion competent in grapes and constitutes a good alternative to GFP. We intend to use the sequence encoding the mature pPGIP fused to the signal peptides for analysis.

We have collected xylem exudate from plants of *Vitis vinifera* 'Chardonnay' and analyzed its protein composition by two-dimensional gel electrophoresis. The purpose of this project is to fuse the signal sequences of these grape xylem sap proteins to the mature pPGIP-encoding gene in order to evaluate their ability to target pPGIP to the xylem.

OBJECTIVES
1. Obtain partial sequences of proteins found in grape xylem exudates and search cDNA databases for signal sequence identification and selection.
2. Design and construct chimeric genes by fusing the selected signal sequences to a sequence coding for a mature secreted protein (pPGIP).

3. Transform grapevines with the chimeric genes via *Agrobacterium tumefaciens* and *A. rhizogenes*.
4. Evaluate the efficiency of the different signal sequences in targeting protein products to the xylem tissue of grapevine through the:
   a. analysis of the expression and secretion of pPGIP in transiently transformed grapevines.
   b. analysis of the expression and secretion of pPGIP in grapevines bearing roots transformed via *A. rhizogenes.*

RESULTS

Peptide spectrum and Blast analysis showed that the proteins found in grape xylem exudates are secreted and share function similarities with proteins found in xylem exudates of other species (Buhz et al., 2004). cDNA sequences of 5 of them were found in the TIGR *Vitis vinifera* gene index. However, it was possible to predict the signal peptide in 2 contigs only (TC 39929 and TC 45857, annotated as Chi1b and similar to NtPRp27 respectively; see Figure 1). Based on their sequences, we designed primers that were used to amplify the predicted fragments from genomic DNA of 'Chardonnay' and 'Cabernet Sauvignon'. These fragments were then fused to DNA sequences that contained the mature pPGIP gene through gene splicing using a PCR-based overlap extension method (SOE) (Horton et al., 1990) and cloned into the pCR2.1-TOPO vector. These two chimeric genes were then ligated into a plant expression vector containing the 35S cauliflower mosaic virus promoter and the octopine synthase terminator and the resultant expression cassettes were then ligated into the binary vector pDU99.2215, which contains an *nptII*-selectable marker gene and a *uidA* (β-glucuronidase, GUS) scorable marker gene. The mature PGIP sequences without any signal peptide sequences was also incorporated into pDU99.2215 to serve as a control and this vector is designated pDU05.1002 (Table 1). We also incorporated signal peptides from the xylem sap protein XSP30 and the rice amylase protein Ramy3D that we have described in earlier reports. These binary vectors are designated XSP and pDU05.0401 respectively (Table 1).

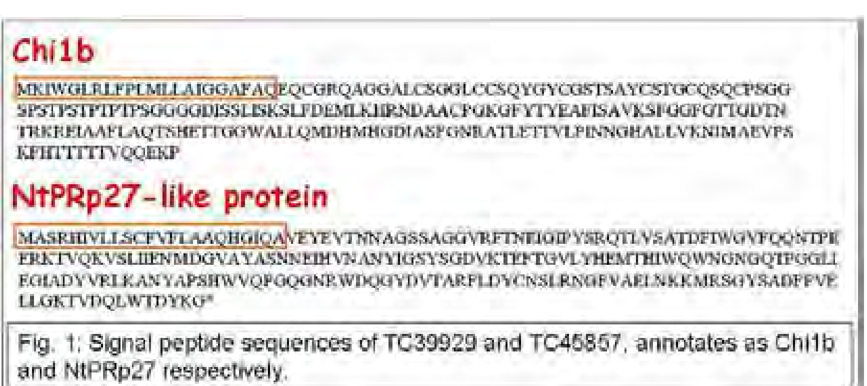

Fig. 1: Signal peptide sequences of TC39929 and TC45857, annotates as Chi1b and NtPRp27 respectively.

Table 1. Construction of vectors for the expression of mature PGIP with various signal peptide sequences.

No	Signal Peptide	Reporter Gene	Promoter	Marker Genes	Vector
1	None	Mature PGIP	CaMV35S	GUS and Kan	pDU05.1002
2	Rice amylase-Ramy3Dsp	Mature PGIP	CaMV35S	GUS and Kan	pDU05.0401
3	Xylem sap protein 30-XSP30sp	Mature PGIP	CaMV35S	GUS and Kan	XSP
4	Chi1b signal peptide	Mature PGIP	CaMV35S	GUS and Kan	pDU06.0201
5	NtPRp27 signal peptide	Mature PGIP	CaMV35S	GUS and Kan	pDU05.1910

Binary vector # 1 is the control and should be immobile although PGIP with its endogenous signal peptide is secretion competent in grape. In binary vector #2, mature PGIP has been fused to the signal sequence of rice amylase 3 (Ramy3D), which has been very effective in secretion of human α1-antitrypsin in rice cell cultures (Trexler et al., 2002). In binary vector # 3 mature PGIP has been fused to the signal sequence of cucumber XSP30, which is a xylem-specific protein. Constructs 4 and 5 have been described above. All five binary vectors have been transformed into the disarmed *A. tumefaciens* strain EHA 105 by electroporation. The next step, the permanent transformation of *Vitis vinifera* 'Thompson Seedless' has need initiated for all 5 vectors and this step takes some time. Once we obtain plants, leaf tissue will be examined for PGIP expression and the positive plants will be subjected to the analysis of the expression and secretion of PGIP.

CONCLUSIONS

Through the study of the proteins present in xylem exudates of 'Chardonnay', we have found 2 good candidates to investigate the effect of using grape signal sequences on xylem targeting. In addition we have produced 2 other chimeric genes containing the signal peptide of a xylem-specific protein in cucumber and the signal sequence of rice amylase. The results obtained with permanent transformations with these genes will provide, in the short term, valuable information for the

identification of signal peptides that will deliver proteins to grapevine xylem with high efficiency. In the long term, the development of an efficient secretory system will be essential to target therapeutic proteins to the xylem of grapevine.

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FUNDING AGENCIES

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

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

Project Leaders:

Abhaya M. Dandekar
Department of Pomology
University of California
Davis, CA 95616
amdandekar@ucdavis.edu

Goutam Gupta
B-1, MS M888, LANL
Los Alamos, NM 87545
gxg@lanl.gov

Karen McDonald
Chem Engr and Material Sci
UC Davis, Davis, CA 95616
kamcdonald@usdavis.edu

Meghan Norvell
B-1, MS M888, LANL
Los Alamos, NM 87545
mek@lanl.gov

Collaborators:

George Bruening
Dept of Plant Pathology
University of California
Davis, CA 95616
gebruening@usdavis.edu

Edwin L. Civerolo
SJVASC
Parlier, CA 93468
eciverolo@fresno.ars.usda.gov

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT

Xylella fastidiosa (*Xf*), is a gram-negative xylem-limited bacterium and causative agent of Pierce's disease (PD) in California grapevines. During very early stages of *Xf* infection, specific carbohydrates/lipids/proteins on the outer membrane of *Xf* interact with plant cells and are important for virulence (Pieters, 2001). Design of a protein inhibitor that interrupts this step of the plant-*Xf* interaction will be useful in anti-microbial therapy and controlling PD. In this UC/LANL project, we have developed a novel protein-based therapy that circumvents the shortcomings of traditional antibiotics. We have designed a chimeric anti-microbial protein with two functional domains (Figure 1). One domain (called the surface recognition domain or SRD) will specifically target the bacterium outer-membrane whereas the other will lyse the membrane and kill *Xf*. In this chimera, human neutrophil elastase (HNE; 5-10) is the SRD that recognizes MopB, the major outer membrane protein of *Xf* (Bruening et al., 2002). The second domain is cecropin B, a lytic peptide that targets and lyses gram-negative bacteria. We have combined HNE and cecropinB using a flexible linker such that both components can simultaneously bind to their respective targets. This chimeric gene was synthesized and cloned into different vectors for insect and plant transformation. Five transformed insect cell lines are being evaluated and production and processing of the protein is being optimized in liter size preps. Plant transformation experiments have been completed and we have obtained plants of *Nicotiana* tabacum var benthamiana and plants of *Vitis* vinifera 'Thompson Seedless' transformed with this gene that are being generated for the analysis of gene expression and protein production. The proteins obtained from the transgenic insect and plant cell lines will be used to test for antimicrobial activity against *Xf*.

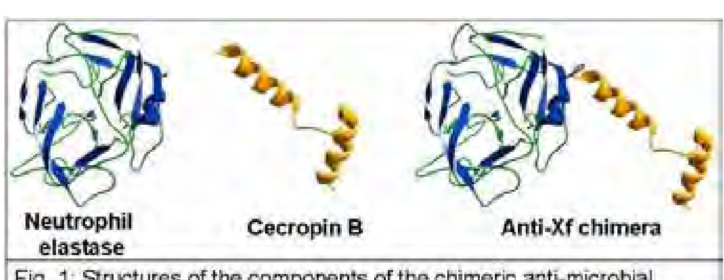


Fig. 1: Structures of the components of the chimeric anti-microbial proteins for the rapid clearance of *Xylella*.

INTRODUCTION

Globally, one-fifth of potential crop yields are lost due to plant diseases primarily of bacterial origin. *Xylella fastidiosa* (*Xf)* is a devastating bacterial pathogen that causes Pierce's disease (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, are developing 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 (Pieters, 2001; Baquero and Blazquez, 1997). Pathogen clearance by innate immunity occurs in three sequential steps: pathogen recognition, activation of anti-microbial processes, and finally pathogen destruction by anti-microbial processes. Different sets of plant factors are involved in different steps of innate immunity. Our strategy of combining a pathogen recognition element and a pathogen killing element in the chimeric molecule is a novel concept and has several immediate and long term impacts.

OBJECTIVES

Objective 1

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

Objective 2:

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

Objective 3

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

RESULTS

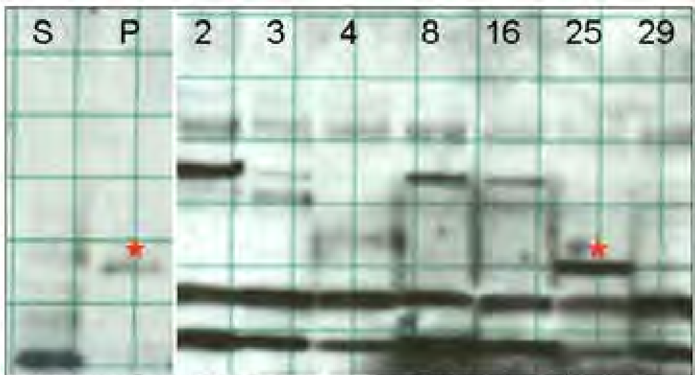


Fig. 2. Detection of anti-Xf chimeric protein expression by Western Blot. In the blot on the left, the first lane contains protein extracted from High Five cell pellet, 72 hours after infection with recombinant anti-Xf baculovirus. The second lane contains protein from the supernatant of that infection. In the blot on the right, 7 samples of callus transformed with anti-Xf chimera were probed for recombinant protein expression. Lanes containing recombinant protein of expected size (~28 kDa) are marked with a red star. The primary antibody used for detection is rabbit derived anti-cecropin B, and the secondary antibody is a peroxidase conjugated goat derived anti-rabbit IgG.

Following our successful accomplishment of Objectives 1a, b and c in the first year of our project, where functional activity of HNE (SRD for MopB) and Cecropin B (defensin) components were tested individually, we designed a chimeric protein of Cecropin B and HNE (Objective 2a). The covalent attachment of Cecropin B to HNE is proposed to increase the stability of the peptide by lowering the conformational entropy of its unfolded state and to increase the overall affinity for the bacterial surface by minimizing the degrees of motion at the binding site, thereby increasing binding between the ligands and the surface.

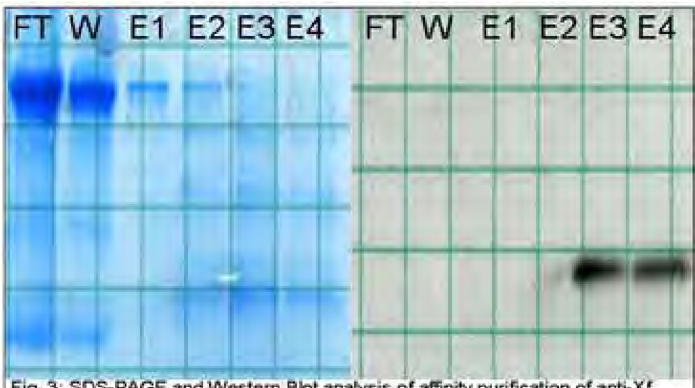


Fig. 3: SDS-PAGE and Western Blot analysis of affinity purification of anti-Xf chimeric protein. Fractions from batch chromatography on elastin-agarose were run on a denaturing gel (on left). FT = flow through, W = wash, E = elution. Recombinant anti-Xf protein is detected in elution fractions 3 and 4 only, by Western Blot (on right).

The HNE-Cecropin B chimera gene was synthesized and cloned into pBacPAK8 baculovirus vector. The chimeric gene inserted into pBacPAK8 was co-transfected with BacPAK6 viral DNA into Sf21 cells. Recombinant viruses formed by homologous recombination were amplified, and the protein expression was optimized in High Five cells (Invitrogen, Carlsbad, CA), derived from *Trichoplusia ni* egg cell homogenates. High Five cells have been shown to be capable of expressing significantly higher levels of secreted recombinant proteins compared to Sf9 and Sf21 insect cells. Optimal conditions for the expression have been worked out in High Five cells; suspension cells in logarithmic growth are infected with recombinant *Xf* chimera baculovirus, with a multiplicity of infection of 10, and grown for 72 hours. About 25-50% of the expressed chimeric protein is secreted into the supernatant and is detected on a Western Blot as a single band (Figure 2). The supernatant is collected, concentrated and dialyzed. Concentrated supernatant is then run on an elastin affinity column, chimeric protein containing fractions are pooled and dialyzed, and the dialyzed fractions are run on a weak cation exchange column (Figure 3). All chromatography steps are carried out by gravity flow. Chimeric protein containing fractions are pooled and dialyzed and tested for elastase activity. By these methods, we are able to purify ~250 µg active protein from 50mL supernatant. These conditions are being scaled up to produce the amounts required for testing against *Xf* (currently purifying liter size preps).

Currently recombinant anti-*Xf* protein is being purified and then it will be quantitated by UV spectroscopy, flash frozen in 50 mM Na-Acetate pH 5, 0.1M NaCl, 50% glycerol and shipped from LANL to UC Davis and the ARS for testing in *Xylella* cultures. We have also cloned the chimera into a plant vector (Fig. 4) that was electroporated into disarmed *Agrobacterium tumefaciens* strain EHA 105 creating a functional plant transformation system that has been used to transform pre-embryogenic callus of *Vitis vinifera* 'Thompson Seedless' successfully.

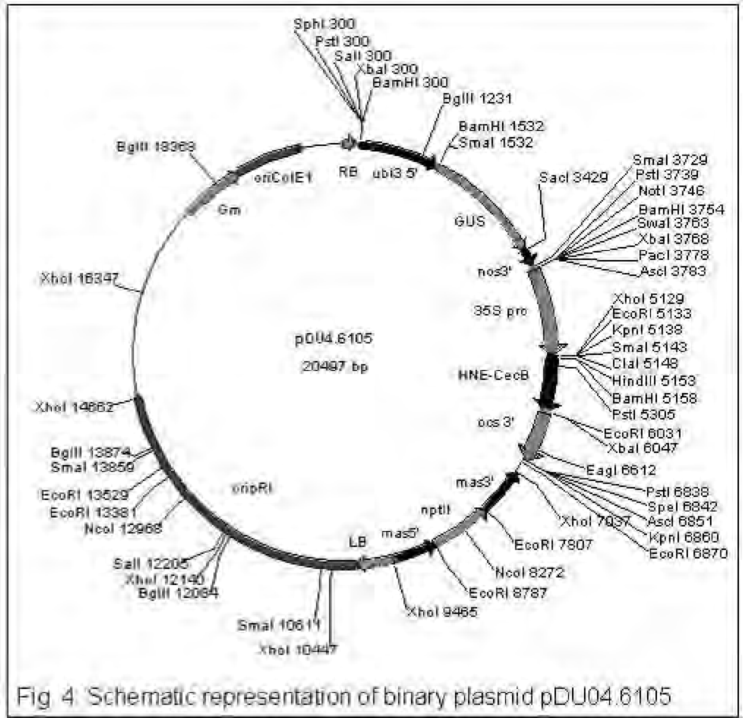
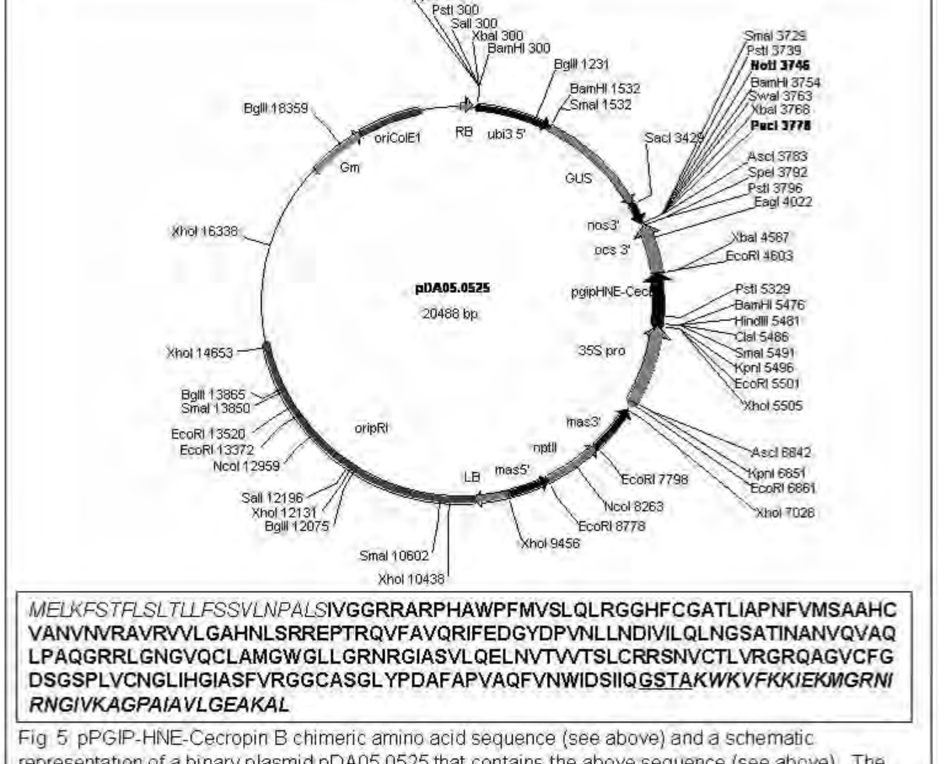


Fig. 4. Schematic representation of binary plasmid pDU04.6105

We have obtained transformed callus of 'Thompson Seedless (TS)' and have regenerated ~49 independent lines of TS. These are being propagated and transferred to the greenhouse. Transformation of the rootstocks 'St George' and '110' will be initiated. The binary vector has also been used to transform *Nicotiana tabaccum* 'benthamiana' and 13 independent transformed lines were selected, these were placed on callus inducing media to induce callus formation. These callai will be evaluated for HNE-CecropinB production. This construct will also be transformed into *Nicotiana tabaccum* 'SR1' to obtain plants. At the moment we have selected 18 shootlines and these will be rooted and then transferred to the greenhouse for seed production. Seedlings will then be tested for sensitivity to *Xf* inoculation and disease resistance.

In addition, the same experiments have been performed using a second construct in which the coding sequence of the signal peptide of HNE was replaced with that of the pear polygalacturonase inhibiting protein (pPGIP). The amino acid sequence of this chimeric gene product is shown in Figure 5. Our hypothesis is that the pPGIP signal peptide will direct/improve the secretion of the chimeric protein and, as a consequence, increase its concentration in the xylem. This hypothesis in based in previous results that have shown that the product of the pPGIP encoding gene, heterologously expressed in transgenic grapevines, is present in xylem exudates and moves through the graft union (Aguero et al., 2005).



*MELKFSTFLSLTLLFSSVLNPALS***IVGGRRARPHAWPFMVSLQLRGGHFCGATLIAPNFVMSAAHC VANVNVRAVRVVLGAHNLSRREPTRQVFAVQRIFEDGYDPVNLLNDIVILQLNGSATINANVQVAQ LPAQGRRLGNGVQCLAMGWGLLGRNRGIASVLQELNVTVVTSLCRRSNVCTLVRGRQAGVCFG DSGSPLVCNGLIHGIASFVRGGCASGLYPDAFAPVAQFVNWIDSIIQGSTA*****AKWKVFKKIEKMGRNI RNGIVKAGPAIAVLGEAKAL***

Fig 5. pPGIP-HNE-Cecropin B chimeric amino acid sequence (see above) and a schematic representation of a binary plasmid pDA05.0525 that contains the above sequence (see above). The signal peptide of HNE (MTLGRRLACLFLACVLPALLLGGTALASE) has been replaced with the predicted signal peptide of pPGIP (italics) which has been fused to the N-terminal of the mature HNE (bold). HNE is attached to Cecropin B (bold italics) by the GSTA linker, which is underlined.

We have obtained transformed callus of 'Thompson Seedless (TS)' with the pPGIP-HNE-Cecropin B gene and have regenerated ~27 independent shoot lines of TS. These are being propagated and then will be transferred to the greenhouse. The pPGIP-HNE-Cecropin B binary vector has also been used to transform *Nicotiana tabaccum* 'benthamiana' and this transformation that was recently carried out is still in progress. This construct was also transformed into *Nicotiana tabaccum* 'SR1' to obtain plants. At the moment we have selected 29 shoot lines and these will be rooted and then transferred to the greenhouse for seed production. Seedlings will then be tested for sensitivity to *Xf* inoculation and disease resistance.

CONCLUSIONS

The main objective of this project is to develop a potent therapy against *Xf* by utilizing the principles of innate immunity by which plants recognize pathogens using their surface characteristics and then rapidly clear them by cell lysis. We have developed a chimeric anti-microbial protein containing two functional domains. One domain (called the surface recognition

domain or SRD) will specifically target the *Xylella* outer-membrane whereas the other will lyse the membrane and kill *Xylella*. In this chimera, elastase is the SRD that recognizes mopB, the major outer membrane protein of *Xf*. The second domain is cecropin B, a lytic peptide that targets and lyses gram-negative bacteria. We have successfully tested each of these components individually and demonstrated that they each (elastase and cecropin B) display activity against *Xf*, which is synergistic when both proteins are combined. We have tested the protease activity of elastase against the purified mopB and intact *Xf* cells to demonstrate that the *Xylella* protein is degraded and therefore, a target for elastase. We have successfully combined the elastase and cecropinB using a flexible linker such that both components can simultaneously bind to their respective targets. This chimeric gene has been synthesized, cloned into a pBacPAK8 baculovirus vector, and packaged into recombinant baculovirus in Sf21 insect cells. Optimization of chimeric protein production is ongoing. We successfully made two vector constructs for expression in plants. Plant transformation experiments are ongoing in grapevine and tobacco to test chimeric protein production in plants. With the first construct transgenic *V.vinifera* L. 'Thompson Seedless' shoots have been obtained. Additional transformations of grapevine scion and rootstocks are in progress to obtain transgenic plants. Once obtained the transgenic plants will undergo acclimation in the greenhouse and then they will be inoculated with *Xf* and tested for PD tolerance/resistance.

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FUNDING AGENCIES

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

RESPONSE OF UNGRAFTED GRAPE ROOTSTOCKS TO *XYLELLA FASTIDIOSA* AT A PIERCE'S DISEASE SITE IN TEXAS.

Project Leaders:

James S. Kamas
Dept. of Horticultural Sciences
Texas A&M University
Fredericksburg, TX
m-black@tamu.edu

Mark C. Black
Dept. of Plant Pathology & Microbiol.
Texas A&M University, AREC
Uvalde, TX 78802
j-kamas@tamu.edu

Cooperators:

Penny Adams
Dept. of Horticultural Sciences
Texas A&M University, TCE
Fredericksburg, TX

James L. Davis
Dept. of Plant Pathology & Microbiol.
Texas A&M University, AREC
Uvalde, TX 78802

Alfred Sánchez
Dept. of Plant Pathology & Microbiol.
Texas A&M University, AREC
Uvalde, TX 78802

Reporting Period: The results reported here are from work conducted March 2005 to September 2006.

ABSTRACT

There were highly significant differences in year 2 among 12 commonly used grape rootstocks in TX for Pierce's disease (PD) symptoms and *Xylella fastidiosa*-serology. The rootstocks Salt Creek, Dog Ridge, and Champanel were the most PD resistant and 1616C, Freedom, and Harmony were most susceptible. Growers in areas at high risk for PD should consider PD reactions with other traits when selecting rootstocks for specific vineyard sites. There were no correlations in year two between rootstock vigor and either PD symptoms or serology results.

INTRODUCTION

Pierce's disease (PD), caused by the bacterial pathogen *Xylella fastidiosa* (*Xf*), is the most limiting factor for growing grapes in much of TX and other U.S. gulf-coast states. Multiple management strategies are needed to improve PD control, including genetic resistance of scions and rootstocks, site selection, vegetation management, and vector management.

OBJECTIVES

1. Evaluate *Xf* reactions among commonly planted grape rootstocks in TX at a vineyard with a history of PD.

RESULTS

This report summarizes results midway through year two of a planned three-year rootstock study. Planting was initiated in 2005 in Llano County, TX at a site where two previous plantings of *V. vinifera* cultivars succumbed to PD. Planting continued as plants became available and to replace plants lost to transplant shock and PD (Table 1). Entries were 5BB, 5C, 110R, 1103P, 1613C, 1616C, Champanel, Dog Ridge, Freedom, Harmony, Salt Creek and SO4. There were five plants per plot and five replications. Leaves with PD symptoms in cv. Black Spanish (Le Noir) border rows consistently tested positive for *Xf* with ELISA and *Xf* was isolated. We anticipate collecting data on symptoms and ELISA reactions through 2007.

There were significant differences among entries for PD leaf scorch, proportion of plants positive with *Xf*-serology, mean optical density from *Xf*-ELISA, and plant vigor (Table 2). Harmony and 5C were least vigorous. SO4, 1613C, 1103P, 1616C and Salt Creek were most vigorous. PD symptoms were most prominent in 1616C, Freedom, and Harmony, but lowest in Salt Creek, Dog Ridge, Champanel and 110R. The proportion of plants positive for *Xf* with ELISA and the mean ELISA optical density were greatest for 1616C, Harmony, Freedom and SO4. Proportion of plants positive for *Xf* with ELISA and the mean ELISA optical density were lowest for Champanel, Salt Creek, and Dog Ridge. There were significant correlations between PD leaf scorch symptoms and both serology parameters (Table 3).

CONCLUSIONS

The rootstocks Salt Creek, Dog Ridge, and Champanel apparently have lower risk for PD than 1616C, Freedom, and Harmony. Growers in areas at high risk for PD should consider these PD reactions when selecting rootstocks for specific vineyard sites.

No rootstock entry in this trial was completely free of PD at mid-season of the second year of this study but there were highly significant differences among entries for symptoms and *Xf* serology. Serology (ELISA) is a general indicator of the concentration of *Xf* cells in grape tissue, and acquisition of *Xf* by vector insects is more efficient during feeding on plants with high populations (Hill and Purcell, 1997). The interaction of rootstock, scion and *Xf* has not been carefully studied, but our hypothesis is that vine mortality can be delayed or reduced if both scion and rootstock are not highly susceptible.

Genetic resistance in commercially-grown *Vitis* genotypes is useful in PD management in southern and southeastern U.S. (Hopkins and Thompson, 1984). Several rootstock cultivars derived from crosses with native *Vitis* species apparently have

some resistance to *Xf* based on preliminary results from this study. Once we quantify resistance in the most commonly used rootstocks (ungrafted) in TX, we can assess the potential of certain rootstock X scion (grafted) interactions in PD management. Growers need multiple techniques, even if some have small effects, that can be combined to delay yield decline and plant death in vineyards at risk from PD. Grower strategies for PD management should include partial rootstock resistance along with site selection distant from riparian habitats, vector monitoring, timely insecticide applications, and selection of scion varieties adapted to the site.

Table 1. Planting dates[1] for rootstock plants rated and sampled on 14Aug06.

	Number of plants						
Entry	**28Mar05**	**12Apr05**	**11Aug05**	**26Sep05**	**14Mar06**	**20Apr06**	**31May06**
1103P	22					3	
110R	21						
1613C			20	4			
1616C	25						
5BB		23					2
5C	25						
Champanel			15		4	5	1
Dog Ridge			23			1	
Freedom	18						6
Harmony			23			1	
Salt Creek			25				
SO4	25						

[1]Planting date variation due to availability of planting materials and replanting due to transplant shock and PD mortality.

Table 2. Serology on samples collected 14Aug06, leaf scorch symptoms, and relative vigor of ungrafted grape rootstocks planted at a high PD-risk site in Llano County, TX.

Entry	Serology reaction[1]	O.D.[2]	Scorch[3]	Vigor[4]
1616C	1.0	1.7	2.8	1.9
Harmony	0.9	1.5	2.5	3.0
Freedom	0.9	1.7	2.7	2.4
SO4	0.8	1.4	2.0	1.8
1613C	0.7	1.1	1.9	1.8
1103P	0.6	1.0	1.6	1.9
5C	0.6	1.0	1.8	2.7
5BB	0.6	1.0	1.9	2.2
110R	0.5	0.8	1.3	2.1
Dog Ridge	0.3	0.4	1.2	2.3
Salt Creek	0.2	0.2	1.0	2.0
Champanel	0.1	0.1	1.2	2.3
Mean	**0.6**	**1.0**	**1.8**	**2.2**
LSD 0.05	**0.3**	**0.5**	**0.4**	**0.6**
CV, %	**37**	**35**	**18**	**21**

[1]Proportion of plants in a plot positive for *Xf* using DAS ELISA; OD<0.300 negative; OD≥0.300 positive.
[2]Mean optical density. Data analyzed were mean O.D. from individual plant samples in one plot.
[3]1 to 5 visual index of leaf scorch; 1=no symptoms, 4=plants with severe PD symptoms, 5=dead.
[4]1 to 5 visual index of plant vigor with 1=most vigorous, 4=very little growth, and 5=dead.

Table 3. Correlations of variables among rootstocks planted at a high risk PD site in Llano Co., TX.

	Serology reaction[1]	**O.D.**[2]	**Vigor**[3]
Leaf scorch index[4]	0.76 ***[5]	0.82 ***	0.2 N.S.[5]
Serology reaction		0.94 ***	-0.13 N.S.
O.D.			-0.06 N.S.

[1]Proportion of plants per plot positive with ELISA using O.D. threshold of ≥0.300 for positive reactions.
[2]Data analyzed were mean ELISA optical densities per plot.
[3]1 to 5 plant vigor index with 1=most vigorous, 4=very little growth, and 5=dead.
[4]1 to 5 leaf scorch index where 1=healthy, 4=plants with severe Pierce's disease symptoms, and 5=dead.
[5]*** indicates significant Pearson correlation at $P<0.001$; N.S. indicates not significant at $P=0.05$

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FUNDING AGENCIES

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

EVALUATION OF GRAPEVINE ENDOPHYTIC BACTERIA FOR CONTROL OF PIERCE'S DISEASE

Project Leader:
Bruce Kirkpatrick
Department of Plant Pathology
University of California
Davis, CA 95616
bckirkpatrick@ucdavis.edu

Cooperators:
Margot Wilhelm
Department of Plant Pathology
University of California
Davis, CA 95616
mwilhelm@ucdavis.edu

Reporting Period: The results reported here are from work conducted July 2005 to September 2006.

ABSTRACT

In this reporting period we optimized our antagonism assay using our previously known antagonists. We found that a *Xylella fastidiosa* (*Xf*) lawn with a concentration of 10^5- 10^6 cfu/ml is optimal for visualizing zones of inhibition. Ten new isolates were shown to have antagonistic capability, including *Bacillus subtilis*, *Pseudomonas* sp., and *Pantoea agglomerans*.

Fourteen isolates chosen for their antagonistic or competitive ability *in vitro* were assayed *in planta* for systemic movement. Of these isolates, six were able to move upwards 30cm from the point of inoculation.

We continued our evaluation of Dr. Darjean-Jones' initial biological control experiment started in 2003. During fall of 2005, xylem sap extracted from these vines with a pressure bomb yielded approximately 220 new isolates. Of these, 169 are from vines inoculated with isolate #169 *Bacillus subtilis* and isolate #161*Bacillus* sp. Eighty-five of our new isolates were sequenced and 44 of these are *Bacillus* sp. Nine isolates had the same sequence as the *Bacillus* species that was originally inoculated into the vines. In February, bud-wood from Dr. Darjean-Jones' biological control experiment was collected from each treatment and propagated in the greenhouse. Ten vines per original treatment along with young Cabernet Sauvignon vines purchased from a nursery were inoculated with Stagg's Leap strain of *Xf* in mid July, 2006. At 14 weeks post inoculation these vines will be assessed for *Xf* infection using Immunocapture (IC)-PCR and rated for Pierce's disease symptom severity.

A new biological control experiment was started in the greenhouse this spring. This experiment is comprised of seven endophyte treatments in 15 Thompson seedless vines per treatment. Vines were mechanically inoculated with the endophytes and 6 weeks later they were inoculated with Stagg's Leap *Xf*. *Xf* infection and symptoms will be assessed after 14 weeks. We are testing the protective capabilities of three *Bacillus* sp. strains, three *Pseudomonas* strains, and one treatment co-inoculated with two *Pseudomonas* sp.

INTRODUCTION

The environment inside grape vine xylem vessels is a distinct ecological niche that supports a sparse microbial community. *Xylella fastidiosa* (*Xf*), the causative agent of Pierce's disease (PD), is one possible inhabitant. But our research, as well as work done in Nova Scotia reveals a diversity of other bacterial species capable of surviving in grape xylem (Bell et al., 1994). Endophytes are microbial organisms that do not visibly harm the host plant but can be extracted from surface sterilized tissue (Hallman et al., 1997). Some bacterial endophytes have been proven beneficial to plant health and are used to promote growth or as biological control treatments for fungal and bacterial pathogens. Previous researchers in our lab isolated an extensive library of endophytes collected from healthy grapevines, PD-infected vines, and asymptomatic vines in areas of high PD incidence (escape vines). We hypothesize that some of these bacterial endophytes may be antagonistic or compete with *Xf* for nutrients. Our library includes endophytes, such as *Pantoea agglomerans* and *Pseudomonas* sp., already tested as biological control agents in other crop systems (Stockwell et al., 2002, Barka et al., 2002). At this time our assays have yielded three additional endophytes that are antagonistic to *Xf in vitro* and are also capable of moving systemically within the grapevine. In this reporting period we have confirmed previous results and streamlined our *in vitro* antagonism procedure. Current PD management practices primarily involve keeping vector numbers low and removing infected vines. Biological control utilizing a systemic bacterial endophyte would be an implementable and environmentally desirable solution to this problem.

OBJECTIVES

1. Finish screening our existing library and recently acquired grape endophytic bacteria to identify potential antagonists of *Xf*.
2. Determine if *Xf*-antagonistic endophytes can systemically move in grapevines.
3. Evaluate the biocontrol abilities of endophytes against *Xf* including
   i) prevention of infection
   ii) suppression of Pierce's disease symptoms in greenhouse and field studies
   iii) long term health and survival of infected vines in the field
4. Isolate additional endophytes from escape vines and characterize these for antagonistic traits.

RESULTS AND CONCLUSIONS

Optimization of Antagonism assay

During this reporting period we optimized our procedure for the *in vitro* antagonism assay. Briefly, seven day old liquid cultures of *Xf* Temecula or our *Xf* hemagglutinin mutant (HxfA) which forms a confluent "lawn" of cells on solid medium are centrifuged and cells are re-suspended in PD3 media to a spectrophotometer OD 600 reading of 0.1-0.13. The resulting suspension contains 10^5– 10^6 cfu/ml. One hundred micro liters of this suspension are spread onto PD3 solid medium and incubated for 4 days. Each endophyte isolate, stored in glycerol solution at -80C, is streaked onto the media in which it was first isolated. The next day, one loop-ful of each overnight endophyte culture is suspended in sterile water. Three, 5µl droplets of each suspension are placed onto the 4 day old *Xf* lawns and allowed to dry. Endophyte inoculated plates are incubated for 5-7 days and then evaluated for zones of inhibition surrounding the endophyte colony.

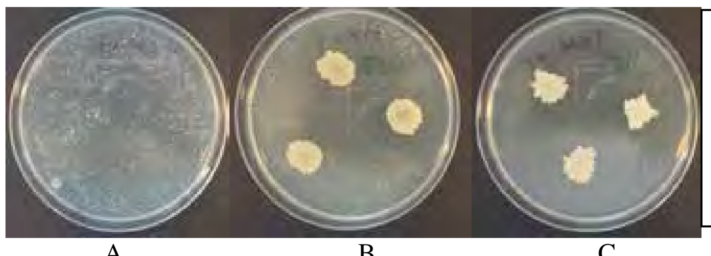
During this period we screened approximately 100 new isolates from our existing library and from our new additions collected during fall 2005. The optimized screening protocol is more efficient than our previous protocol and we expect to finish the library evaluation by December 2006. Table 1 summarizes our current results with 17 isolates that show some degree of *Xf* inhibition *in vitro*. Strains of *B. subtilis* and *Pseudomonas* have shown the greatest antagonism *in vitro*. Isolates #4, #11, and #37 have been included on the table even though their inhibition zones are smaller because *Pantoea agglomerans* strains have been successfully used in biocontrol research (Stockwell et al., 2002), and may be good candidates for further study in this project.

In addition to the antagonism assay we have started evaluating each isolate showing some degree of inhibition on crystal violet polypectate media (CVP). A positive result on this media indicates pectin degradation. It is possible that isolates capable of pectin degradation may also be able to degrade pit membranes connecting xylem vessels and be able to move systemically within the vine.

Table 1. Bacterial isolates screened in 2005-2006 showing some degree of antagonism toward *Xf*.

Endophyte	Identification	Zone of clearing (a)
169	*Bacillus subtilis*	16-20mm
69	*Bacillus subtilis*	complete
17	*Bacillus subtilis*	7-15mm
197	*Pseudomonas virdiflava*	20mm-complete
393	*Pseudomonas virdiflava*	rg over entire plate
403	*Pseudomonas syringae*	complete
205	*Pseudomonas sp.*	complete
329	*Pseudomonas sp.*	complete
100	*Bacillus subtilis*	15-20mm
147	*Bacillus subtilis*	rg over entire plate
11	*Pantoea agglomerans*	rg 3-8mm
37	*Pantoea agglomerans*	rg 1-2mm
4	*Pantoea sp. (Erwinea sp.)*	rg over entire plate
W157 (b)	*Bacillus pumilus*	rg 6-10mm
154	*Bacillus subtilis*	8-12mm
139	*Bacillus sp.*	rg 10-15mm
200	*Pseudomonas sp.*	complete

(a) zone attained on lawn plates with *Xf* concentration of 10^5-10^6 cfu/ml.
(b) "W" indicates an isolate collected October 2005 from our 2003 biocontrol experiment in the field.
rg = reduced growth in these areas, ie. *Xf* colonies aren't cleared but are much smaller compared to controls



A. Temecula *Xf* lawn control plate
B. Isolate 17 on a lawn of HxfA showing zones of inhibition
C. Isolate 169 on a Temecula plate showing almost complete inhibition

A B C

Figure 1. *In vitro Xf* Antagonism assay.

Assessment of endophytes' ability to colonize and move systemically in grape xylem
Fourteen isolates exhibiting good antagonism or competition *in vitro* were assessed for their ability to move systemically in grape xylem. Two Chardonnay vines per isolate were pinprick inoculated with an endophyte suspension of approximately 10^8 cfu/ml. Mechanical pinprick inoculation with the endophyte suspension is similar to mechanical inoculation of *Xf*: a 20 µl drop of suspension is pipetted onto the stem and the drop is punctured with a needle 1-5 times until the drop is sucked into the stem. We inoculated plants on two places on the stem near the 3rd or 4th internode from the graft junction. Plants were inoculated once and then again three days later to ensure success. After seven weeks, four, 2 cm sections including the point of inoculation (POI), first petiole after the POI, 9-11 cm, and 28-30 cm from the POI, were cut from the vine and cultured. Each 2 cm section or petiole was surface sterilized in 10% bleach, 70% ethanol, and three washes in sterile water for one minute. The section was then put into a sterile grinding bag with 2 ml phosphate buffered saline (PBS) and pulverized. One hundred micro liter aliquots of this solution were plated onto solid media of the same type on which the endophyte was first isolated. Total colonies that were morphologically similar to the endophyte were counted and recorded. Representative colonies morphologically resembling the original endophyte at the POI and 28-30 cm were sequenced to confirm their identities. Out of 14 isolates tested for systemic movement, 5 were able to move past the point of inoculation and up to the 28-30cm section. Isolates capable of moving 30cm or into the petiole, are likely capable of degrading pectins in the pit membranes connecting xylem elements, and would thus be able to colonize the entire vine over time.

Table 2. Endophytes that possessed some ability to systemically colonize Chardonnay vines. Numbers reflect cfu/ml resembling original isolate in each stem section.

Isolate	POI	Petiole (a)	9-11cm	28-30 cm
W121	1.00×10^6	3.00×10^5	5.25×10^6	3.00×10^5
69	2.60×10^5	128	270	69
169	6.37×10^5	lawn	7.90×10^4	3.50×10^3
17	3.07×10^5	0	1.20×10^4	1.09×10^3
11	5.99×10^5	9.50×10^4	3.35×10^4	2.89×10^3
Agro556 (b)	6.07×10^5	1.66×10^5	2.55×10^5	10

(a) First leaf petiole up from the POI.
(b) *Agrobacterium vitis* 556 is an avirulent strain obtained from Thomas Burr.

Continuing evaluation of biocontrol experiment initiated in 2003
During September and October 2005, xylem sap from vines in Dr. Darjean-Jones' original biocontrol experiment, was extracted with the pressure bomb technique as described in previous reports. These vines had been in the field for two years. Approximately 220 bacterial isolates were streaked to purity and then frozen at -80C for further characterization. Of these, 176 isolates were isolated from vines originally inoculated with a putative *Cellulomonas* species (#169) or *Bacillus* species (#161). In the 2003 experiment these two isolates best protected vines from developing PD symptoms in the field. Last fall we re-sequenced #169 and found that the putative *Cellulomonas* isolate was in fact a *Bacillus subtilis* strain. Because three different researchers have worked independently on this project, we cannot be sure when the misidentification occurred. It is possible that Dr. Darjean-Jones' vines were inoculated with *Cellulomonas* and the isolate has been lost, or her vines were initially inoculated with *B. subtilis*. However, our current research shows that isolate #169, *B. subtilis*, has strong antagonistic ability *in vitro* and moves well within the vine, properties that were originally reported for the *Cellulomonas* strain #169.

We also wanted to determine, if after 2 years in the field, the original endophytes used for the experiment could be re-isolated. Out of 220 newly isolated samples, 122 of these were extracted from vines originally treated with strain #169. The 16S rDNA of 45 of these was sequenced. Twenty-five were identified as *Bacillus* species and three were specifically identified as *Bacillus subtilis*. While it is possible we were able to re-isolate some of the original #169 endophyte strain, we

also isolated *Bacillus subtilis* strains from field grown vines that were not originally inoculated with #169. Out of 54 isolates from vines originally treated with *Bacillus* sp (#161), 20 were sequenced, and six were identified also as species of *Bacillus*. We will do more sensitive genetic typing of these isolates using rep-PCR to determine if the *Bacillus* strains that were isolated from endophyte inoculated vines are the same as those that were originally inoculated in 2003. We expect to finish characterizing the other isolates this winter 2006-2007.

To determine if these vines were still protected against *Xf*, bud wood cuttings from these vines were propagated in the greenhouse this spring and challenged with *Xf*. Cuttings were taken from all vines in the field testing negative for *Xf* infection by IC- PCR. Each cutting had 2-4 buds and was rooted in a plastic callus chamber for 6 weeks. Cuttings with callus formation were grown in the greenhouse and trained to a single shoot for 3 months. Ten propagated vines representing each original endophyte treatment were then mechanically inoculated with Stagg's leap strain of *Xf*. Stagg's leap strain was chosen because it is one of the more virulent *Xf* strains. Nursery grown Cabernet Sauvignon plants were used as positive and negative controls. These plants will be evaluated for *Xf* infection with IC-PCR, rated for symptoms at 14 weeks post inoculation, and planted in the field in spring, 2007. Final results from this experiment should determine if our propagated cuttings or the original vines retain their resistance to PD.

New Biological control experiment for 2006

This year we started a new biological control experiment consisting of eight different endophyte treatments. Each treatment consists of 15 Thompson seedless vines inoculated with endophyte suspensions or H_2O controls in the same manner as movement assay vines. We are testing 3 *Bacillus* isolates, 3 *Pseudomonas* isolates and one co-inoculation of 2 *Pseudomonas* species. Six weeks after endophyte inoculation, the vines were inoculated with Stagg's leap strain of *Xf*. After 14 weeks or when PD symptoms appear these vines will be evaluated for *Xf* infection with IC-PCR and rated for symptoms. During spring of 2007, surviving vines will be planted out in the Armstrong field plots at UC Davis, California.

Isolate additional endophytes from escape vines in the field

This spring we sampled xylem sap from 10 escape vines in Napa vineyards. The first sampling took place in late April 2006 when vines were "bleeding". Sap was collected in a 40 ml Falcon tube and directly plated onto 523 and PD3 media. Resulting colonies were streaked to purity and then stored in glycerol at -80C. In June we sampled the same vines and extracted xylem sap with the pressure chamber. All isolates have been cataloged and will be tested for antagonism later this winter.

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

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

Cooperator:
Melody Meyer
Department of Plant Pathology
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT

Pierce's disease does not occur in colder regions of North America. Although the "cold curing" phenomenon has been well documented, little is known about the physiological mechanisms that mediate cold therapy. To better understand the cold therapy phenomenon, we planted control and *Xylella fastidiosa (Xf)* infected *Vitis vinifera* "Pinot Noir" (PN) and "Cabernet Sauvignon" (CS) grapevines across 4 locations in Northern California and exposed control and infected grapevines across 4 temperature conditions in cold rooms. After treatment periods, xylem sap was extracted using a pressure bomb and the composition of the sap was analyzed for pH, osmolarity, glucose, sucrose, fructose, Ca^{2+}, and Mg^{2+}. Differences were found across the different field locations and cold rooms. Similar results for CS inoculated vines were found for the cold room experiments. Results for the PN field plots revealed differences in pH for both inoculated and control vines across the field plot locations. Osmolarity values of PN control vines were different across the field plot locations and in the cold room treatments.

Effects of buffer and xylem sap on the survival of *Xf* and various cold temperatures were reported in the 2004 PD/GWSS Proceedings. Abscisic acid (ABA) levels are elevated in many cold-treated plants and ABA has been shown to induce the synthesis of certain pathogenesis related (PR) proteins that in some case possess anti-fungal properties. However, we are proceeding with experiments to determine if exogenous applications of ABA on non-chilled grapevines can elicit PR proteins.

INTRODUCTION

Xylella fastidiosa (*Xf*) is a xylem-limited, gram-negative bacterium that causes Pierce's disease (PD) in grapevines. One factor that has been shown to be associated with the observed limited geographical distribution of PD in North America is the severity of winter temperatures in those regions. Purcell (1977, 1980) demonstrated that relatively brief exposures to sub-freezing temperatures eliminated *Xf* in cold treated *Vitis vinifera* grapevines. Purcell also found that a higher percentage of grapevines that were moderately susceptible to PD such as 'Cabernet Sauvignon', were cured by cold therapy treatments compared to susceptible varieties such as 'Pinot Noir.' More recently, Purcell's group also showed that whole, *Xf* infected potted vines exposed to low temperatures had a higher rate of recovery than PD-affected detached bud sticks exposed to the same cold temperatures (Feil, 2002). This implies that some factor(s) expressed in the intact plant, but not in detached bud sticks, helped eliminate *Xf* from the plants. Despite documentation of the cold curing phenomenon, little is known about the physiological/biochemical basis that mediates cold therapy. Our objective is to elucidate the physiological/biochemical basis that mediates cold therapy and to identify the physiological/biochemical factor(s) that occur or are expressed in cold treated vines that eliminate Xf. If such a factor(s) is/are found, it may be possible to induce their expression under non-freezing temperatures and potentially provide a novel approach for managing PD.

OBJECTIVES

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

RESULTS

Objective 1

Using the same varieties used in our 2004-2005 field and cold room studies (PD vs. temperature treatment results were reported in the 2005 Pierce's Disease Symposium Proceedings), Pinot Noir (PD-susceptible) and Cabernet Sauvignon (moderately resistant to PD) grapevines were prepared as described in the 2005 Pierce's Disease Symposium Proceedings. In October/November 2005, prepared grapevines were transported to 4 sites that were selected because of their varying winter temperatures. Sites included: UC Hopland Research Station (Mendocino County), McLaughlin Reserve (Lake County), Foresthill (Placer County), and UC Davis (Yolo County).

Due to the unacceptably high mortality rate at the Fall River plot in 2004, we established the 2005 plot in Lake County at the Donald and Sylvia McLaughlin Reserve. The plants for the Blodgett plot (El Dorado County) were initially planted in the beginning of November 2005. However, due to an unforeseen problem, the plot was moved to Foresthill (Placer County) in December of 2005. The Placer County site is at an elevation similar to Blodgett and we monitored the temperature data at both locations using HOBO dataloggers. Plants were picked up from the four locations in April and planted in the field at UC Davis. Plants are being rated for PD symptoms using the same symptom severity index as in the 2004-2005 season and the presence of Xf is being detected with IC-PCR. In the spring of 2006, new Pinot Noir and Cabernet Sauvignon vines prepared as described above. The grapevines will be transported to the 4 field locations in October/November 2006.

Grapevines, using the same varieties and prepared as described for the field studies but grown in 6" standard pots, were exposed to different temperature regimes in cold rooms located at the Department of Pomology, UC Davis during the winter of 2005-2006. Plants were prepared as described in the 2005 Pierce's Disease Symposium Proceedings. Once in the cold rooms, plants were not moved daily, as in year 1, to attempt to see if we could achieve greater differences between treatments (i.e., all year 1 vines were exposed to the same -5 night temperature, thus differences between treatments were not that great). Plants prepared in 2005 were subjected to one of 4 temperature regimes:

Regime 1: -5°C   Regime 3: +2.2°C
Regime 2: +0°C   Regime 4: +5°C day

After 3 months of treatment, xylem sap was extracted, and the vines were planted in the Plant Pathology field area. The grapevines for the 2005-2006 cold room experiments are currently being evaluated with ICPCR and being rated for PD symptoms to determine the most effective temperature regime for curing without causing unacceptable plant mortality.

Objective 2

2a) El Dorado vs. Yolo County (2004)

Preliminary work using 'Pinot Noir' and 'Cabernet Sauvignon' field materials collected from El Dorado and Yolo counties showed some differences in xylem sap pH and osmolarity (Table 1). These results were obtained from Pinot Noir and Cabernet Sauvignon vines growing in El Dorado County and at the Foundation Plant Services vineyard at UC Davis. Both varieties were grown in the same manner at each site; however, management practices at the two sites were not identical and the clones were not the same. In 2004, dormant cuttings were collected in late February and xylem sap was extracted using a custom-made pressure bomb. These same parameters were examined again in 2005 from grapevines growing at the same two locations in late March, approximately one month later than in 2004. 2004 and 2005 data can be found in 2005 Pierce's Disease Symposium Proceedings. Differences in pH and osmolarity could possibly be explained by differences in the sampling date, management differences, or weather differences at the time of sampling. To try to elucidate these differences, xylem sap samples were collected in late February and again in late March/ early April of 2006 (Tables 1and 2).

Table 1: Osmolarity and pH of xylem sap collected from grapevines from El Dorado County and Yolo County on February 26, 2006.

		El Dorado	Yolo
pH	Pinot Noir	5.74	5.70
	Cabernet Sauvignon	6.24	5.83
Osmolarity mmol/kg	Pinot Noir	60.4	39.5
	Cabernet Sauvignon	52.0	77.0

Table 2: Osmolarity and pH of xylem sap collected from grapevines from El Dorado County and Yolo County on March 25, 2006 and April 7, 2006.

		El Dorado	Yolo
pH	Pinot Noir	5.53	5.57
	Cabernet Sauvignon	5.39	5.50
Osmolarity mmol/kg	Pinot Noir	34.3	16.6
	Cabernet Sauvignon	26.4	23.5

2b) Xylem sap analysis of field and growth chamber plants

In 2004-2005 and 2005-2006 seasons, field grown and growth chamber plants prepared as described in Objective 1, were sampled for potential changes in pH, osmolarity, protein profiles, total sugars, and calcium and magnesium concentrations in xylem sap (Table 3). In both the field experiments and the cold chamber experiments, pH and osmolarity of xylem sap from cold treated vines was lower than found in PD3 media used to grow *Xf*. Sugar and select ion concentration analysis of Cabernet Sauvignon grapevines show greater amounts of glucose and fructose in the –5ºC cold room treatment; where as Ca^{2+} levels are greater in the warmest treatments. Very little sucrose was found in the samples (data not shown). Osmolarity of collected xylem sap is greatest in the coldest treatments and decreases with increasing temperature and shows an interesting relationship with total sugars, calcium, and magnesium concentrations. Conversely, in Pinot Noir grapevines glucose and fructose levels are the lowest in the coldest treatments. Ca^{2+} levels show a similar trend with Cabernet Sauvignon vines, with increased Ca^{2+} levels with the warmer temperature treatments. Temperature appears to have a less direct effect on osmolarity in Pinot Noir grapevines.

Our hypothesis is that changes in xylem sap components in vines that undergo cold treatment may have significant effects on *Xf* viability. Previous research on several plant species has shown that a number of plant genes are expressed in response to freezing temperatures (reviewed by Thomashow, 1998). In some plants, these freeze-induced proteins are structurally related to proteins that plants produce in response to pathogens, i.e. pathogenesis-related proteins (Hon, et al. 1995; Kuwabara, et al, 2002). Thus it may be possible that cold-stressed grapevines could produce proteins that are deleterious to *Xf*.

We are continuing to concentrate proteins in xylem sap by acetone precipitation and electrophoresing the proteins on 1-dimensional polyacrylamide gels (PAGE). Unique protein bands that were found in the cold stressed plants were cut from the gel, and end terminally sequenced. Initial sequencing results of xylem proteins from cold-treated vines showed proteins that are similar to stress proteins that are produced by Cabernet Sauvignon berries and Pinot Noir roots. Understanding the role of these proteins is limited by the *Vitis vinifera* genome not being fully sequenced to date. The appropriate databases will be checked periodically to compare our sample protein sequences against new sequencing information. Due to constraints in xylem sap sample volume, the total organic acids and peroxidase analyses were delayed until the 2005-2006 samplings and will be tested soon.

Table 3: 2005-2006 Mean osmolarity and pH of xylem sap from grapevines growing at 4 field locations around California. 1st sampling = early February 2006; 2nd sampling = late March 2006.

			Davis		Hopland		Foresthill		McLaughlin	
			1st	2nd	1st	2nd	1st	2nd	1st	2nd
pH	Pinot Noir	Control	6.14	5.56	6.17	5.74	6.17	6.10	6.06	5.96
		Inoculated	6.08	6.03	6.34	5.99	5.92	6.08	6.09	6.22
	Cabernet Sauvignon	Control	5.92	5.84	6.63	6.02	6.21	6.27	6.41	5.92
		Inoculated	6.07	5.96	6.32	5.96	6.24	6.23	6.19	6.06
Osmolarity	Pinot Noir	Control	91.1	42.6	72.7	44.2	179.5	67.5	118.7	37.9
		Inoculated	51.4	31.0	41.5	58.0	67.2	57.8	89.5	56.8
mmol/kg	Cabernet Sauvignon	Control	189.8	26.8	64.7	38.2	134.7	61.1	78.9	44.4
		Inoculated	213.1	30.7	67.2	22.0	86.6	53.1	84.3	28.1

Objective 3

We have assessed the effect of pH and osmolarity on the viability of *Xf* cells *in vitro* using various buffers and media and then exposed to various temperatures (28ºC, 5ºC, 2.2ºC, 0, -5ºC, and -20ºC). Everyday, samples were collected and diluted and plated out onto PD3 and allowed to grow for seven days. After seven days, colonies were counted to determine the potential effect each treatment had on the viability of *Xf* cells

Results reported in the 2004 Pierce's Disease Research Symposium Proceedings demonstrated that *Xf* can survive at 28ºC in most media except water and at lower temperatures, our results were similar to those found by Feil (2002). The 2004 proceedings demonstrated that *Xf* survived best in potassium phosphate buffer at 6.8. Survival at pH 6.6 quickly tapered off after a few days. With all other pH buffers (5.0-5.8) *Xf* rapidly died after 1 day. Interestingly, this data supports what has previously reported by Davis (1978), but the pH at which *Xf* survives in culture is higher than what was found in xylem sap samples from our field and cold chamber experiments. We used the same temperatures that were used in the cold room experiments for comparison. *Xf* grew best in PD3 at all temperatures.

Objective 4

Kuwabara et al. (2002) elicited cold-shock proteins at 23°C in winter wheat with an ABA concentration of 100ppm. ABA treated plants elicited proteins that were able to inhibit fungal growth when exposed to exogenous applications of ABA. Though the mechanism is not thoroughly understood, endogenous ABA has shown to induce pathways that are involved in induced resistance to plant pathogens (Ton & Mauch-Mani, 2004; reviewed in Bostock, 2005).

4a) ABA concentration in xylem sap

ABA concentrations in xylem sap have been measured for the four field sites and the cold chamber experiments. The concentration of ABA in the sap was determined using a commercially available immunoassay that has a sensitivity of 0.0064-0.16 picomoles ABA/ml (Phytodetek ABA Test Kit, Agdia) and only requires a small volume of xylem sap. ABA concentrations of xylem sap are being determined for the vines in the cold chambers and in the field. Experiment will be replicated in the 2006-2007season.

4b) ABA applications to grapevines

In November 2005, healthy and *Xf*-inoculated Cabernet and Pinot vines prepared as stated in Objective 1 were sprayed with solutions of ABA. Valent Biosciences representatives provided us with 2 types of ABA. There were 5 treatments with *Xf*-infected vines and healthy controls:

Control: 8 Pinot/ 8 Cabernet plants sprayed with water
1000ppm spray: 8 Pinot/ 8 Cabernet plants sprayed with VBC-30054
100 ppm drench: 8 Pinot/ 8 Cabernet plants sprayed with VBC-30054
100 ppm spray: 8 Pinot/ 8 Cabernet plants sprayed with VBC-30030
10 ppm drench: 8 Pinot/ 8 Cabernet plants sprayed with VBC-30030

To determine effectiveness of ABA and synthetic ABA treatments, vines are currently being evaluated for PD symptoms and being tested with ICPCR. Experiments will be replicated in the 2006-2007 season.

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FUNDING AGENCIES

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USE OF THE *E. COLI* α-HEMOLYSIN SECRETION SYSTEM IN BACTERIA DESIGNED FOR SYMBIOTIC CONTROL OF PIERCE'S DISEASE IN GRAPEVINES AND SHARPSHOOTERS

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

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

Thomas A. Miller
Department of Entomology
University of California
Riverside, CA 92521

Reporting Period: The results reported are from work conducted from April 2006 to October 2006.

ABSTRACT
Strains of *Alcaligenes xylosoxidans denitrificans* (*Axd*) that secrete anti-*Xylella* factors are being developed for use in a strategy to prevent the spread of *Xf* or possibly to cure infected grapevines. We built constructs that fused the last 60 amino acids of the autotransporter α-hemolysin from *E. coli* to test proteins. These were two different forms of anti-BSA single chain antibodies (scFvs) which are surrogates for anti-*Xylella* effector proteins. These proteins were efficiently secreted from *E. coli* when co-expressed with the proteins HlyB and HlyD. HlyB, HlyD, and TolC together form the membrane structure used by α-hemolysin to cross both the inner and outer membrane of the cell. We report here on efforts to move this system into *Axd*, a species not closely-related to *E. coli*, but that can survive in both grapevine and sharpshooters.

INTRODUCTION
The glassy-winged sharpshooter (GWSS) is the principal vector of the xylem-limited bacterium *Xylella fastidiosa* (*Xf*), which causes Pierce's disease (PD) in grapes. Limiting the spread of this pathogen by rendering GWSS incapable of pathogen transmission or by interfereing with the replication of *Xf* in the plant may stop the spread of PD. These endpoints can be accomplished by genetically modifying bacteria that live in the sharpshooter, the plant, or both in a method called symbiotic control. Symbiotic control seeks to modify the phenotype of an organism indirectly by modifying its symbiotic bacteria.

Symbiotic control 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 et al., 2001), interference with HIV attachment to its target cells in the reproductive tracts of humans (Chang et al., 2003; Rao et al., 2005), and the elimination of persistent *Candida* infections from biofilms in chronically infected human patients (Beninati et al., 2000). Symbiotic control has also been applied to deliver cytokines mammalian guts to relieve colitis (Steidler et al., 2000; Steidler, 2001). Thus, the method has wide applicability.

Alcaligenes xylosoxidans denitrificans (Axd) is Gram negative pseudomonad-like species that can colonize the GWSS foregut and cibarium, as well as various plant tissues, including grape xylem. It is non-pathogenic in insects, plants and healthy humans. Given these characteristics, *Axd* has become the focus of our symbiotic control efforts to control PD in grapes. Over the past several years we developed the technology to stably modify *Axd* by inserting genes into its chromosome via transposition, have developed methods to suppress horizontal gene transfer, and have isolated a single chain antibody that recognizes an epitope on the surface of the PD strain of *Xf*. (Bextine et al., 2004). We are currently engaged in combining these systems in order to produce strains of *Axd* that are suitable for environmental release in a practical strategy symbiotic control strategy for PD.

One way to deliver anti-*Xylella* protein factors from *Axd* in grapevine is by secretion. Secreted anti-*Xylella* factors might circulate throughout the plant, reaching foci of infection across physical xylem boundaries. Secretion from Gram-negative bacteria, however, is complicated by the fact that these species have two membranes that a protein must cross before appearing outside the cell. Gram negatives contain at least 6 identified types of secretion systems. Unfortunately, many of these systems are unpredictable when expressed heterologously. One system that seems to have wide applicability is the a-hemolysin autotransporter from *E. coli* (Fernandez et al., 2000). This protein is secreted in a single energy-dependent step across both membranes of Gram negative bacteria when the other components of the system are also present (the proteins HlyB, HlyD, and TolC). Fusion of the last 60 amino acids of the protein is sufficient to target any N-terminal passenger protein for secretion.

We report here the evaluation of the *E. coli* α-hemolysin system for use in *Axd* to secrete soluble anti-*Xylella* protein effectors in grapevine and insects.

OBJECTIVES
1. Test the *E. coli* α-hemolysin secretion system in *E. coli* and *Axd*.

RESULTS

Secretion of scFv-hemolysin fusions from *E. coli*. We used a two plasmid system to secrete two test constructs from *E. coli*. One plasmid carried the *hlyB* and *hlyD* genes and were encoded on a low copy number plasmid based on pSC101. The others carried either an antiBSA scFv gene fused to a sequence encoding the last 60 amino acids of HlyA or an antiBSA scFv capable of dimerizing to form a diabody that was similarly fused to HlyA (Fernandez et al., 2000; Fraile et al., 2004). These plasmids were grown in the same strain overnight, the supernatant harvested, and small samples of the supernatant loaded on SDS-PAGE gels. A subsequent western blot used an antibody directed at an epitope on the scFv constructs. These results are shown in Figure 1. Neither scFv constructs alone (scFv-HlyA) nor the HlyB-D plasmids produced any detectable secreted protein. When both plasmids were grown together, single chain antibody proteins could be detected easily in the supernatants of their respective cultures.

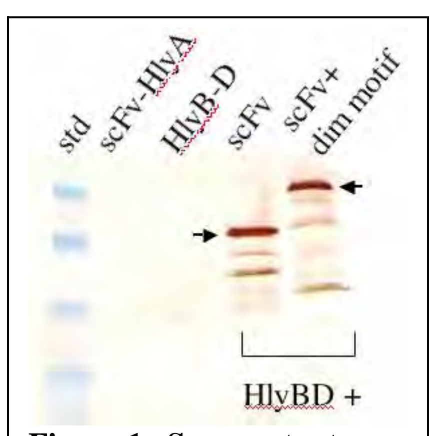

Figure 1. Supernatants from *E. coli* cultures expressing secretable forms

Progress toward secretion of a single chain antibody constructs from *Axd*. We added origins of transfer (*oriT*) to each of the plasmids in the *a*-hemolysin secretion system described above and attempted to move these plasmids into *Axd* via mating from *E. coli*. We recovered no exconjugants whatsoever. Control matings from one *E. coli* strain to another yielded abundant exconjugants. From these data we conclude that the origins of replication of the secreting plasmids do not function in *Axd*. Efforts at constructing and testing the secretion system on broad host range plasmids like pRO1600 are underway (Shanks et al., 2006).

CONCLUSIONS

The *E. coli* hemolysin secretion system is a robust and powerful way to secrete various proteins in a single step across both membranes of Gram negative bacterial cells. In the presence of HlyB and HlyD proteins, fusions of two different scFv to the last 60 amino acids of HlyA were secreted into the growth medium of the E. coli cells carrying them. Attempts to use these fusions in *Axd* failed because the origins of replication (ColE3 and pSC101) were not functional in this species. Moving the secretion system onto plasmids that do replicate in *Axd* (like pRO1600 and its derivatives) should result in secretion from this species as well. It should then be possible to secrete anti-*Xyllela* effector proteins within grape plants that can circulate within the xylem of the plant.

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FUNDING AGENCIES

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GENOTYPIC CHARACTERIZATION OF *ALCALIGENES XYLOSOXIDANS* SUBSP. *DENITRIFICANS* (*AXD* HC01) AND FOUR RELATED STRAINS

Project Leader:
Jennifer Parker
Department of Entomology
University of California
Riverside, CA 92521
Jpark001@student.ucr.edu

Project Director:
Thomas A. Miller
Department of Entomology
University of California
Riverside, CA 92521
Thomas.Miller@ucr.edu

Cooperators:
Carol R. Lauzon
Department of Biological Sciences
California State University
Hayward, CA 95452
clauzon@bay.csuhayward.edu

David J. Lampe
Department of Biological Sciences
Duquesne University
Pittsburgh, PA 15282
lampe@duq.edu

Reporting period: The results reported here are from work conducted September 2005 to September 2006.

ABSTRACT
In symbiont therapy, an insect's natural symbionts are genetically modified to prevent the transmission of a pathogen. This strategy is currently under investigation as a way to control the spread of Pierce's disease (PD) of grapevine. PD is caused by the bacterium *Xylella fastidiosa* (*Xf*), which is transmitted by the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*). The five GWSS symbionts used in this research were identified through biochemical testing as *Alcaligenes xylosoxidans denitrificans* (*Axd*) Hc01, *Axd*1, *Axd*2, *Axd*3, and *Axd*4. The genetic relatedness of these bacteria, as well as their relationships to other bacterial species was analyzed using two highly conserved prokaryotic genes, the 16S rDNA sequence and the gyrase B sequence. These sequences were used to construct phylogenetic trees using the neighbor-joining method. Analysis of the 16S tree indicated that all of these bacteria were closely related to members of the genus *Pseudomonas*. The phylogenetic trees that were constructed using the gyrase B gene also supported the conclusion that these bacteria are closely related to members of the genus *Pseudomonas*. Further testing using the 16S-23S intergenic spacer region one is currently underway.

INTRODUCTION
One new potential management strategy for Pierce's disease (PD) of grapevine is the use of symbiont therapy. Symbiont therapy exploits the interactions among a pathogen-transmitting organism, its bacterial symbionts, and the pathogenic organism itself (Beard 2002). First, a bacterial symbiont that occupies the same niche as the pathogen must be identified. These symbionts are genetically modified to produce a molecule that hinders the spread of the pathogen in question. The genetically modified bacteria are re-introduced into the vector so that they can reduce its ability to transmit the pathogen in question. For this approach to be successful, the bacterial symbiont must be easily cultured and manipulated *in vitro*, and the genetic modification cannot alter their value to the host organism or their ability to occupy their niche. In addition, the bacterial symbionts cannot be pathogenic to either their host or to non-target organisms before or after the genetic modification (Durvasula 2003). Symbiont therapy has been investigated as a way to control the spread of Chagas disease (Beard 2002; Durvasula 2003), murine colitis (Steidler 2000), and HIV (Chang 2003).

For symbiont therapy to be effective in limiting the spread of PD, a culturable symbiont that inhabits the pre-cibarium and cibarium of the glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis)* is required, since these areas are colonized by *Xf*. Three bacterial species that meet these requirements are *Chryseomonas* spp, *Ralstonia* spp, and *Alcaligenes* spp (Bextine 2004). The *Alcaligenes* species were of particular interest because they were frequently isolated from wild GWSS (Kuzina 2004) and because they could also successfully colonize the xylem of various plants, including citrus (Araujo 2002; Bextine 2005). Five *Alcaligenes* species were isolated from the mouthparts of GWSS and identified as *Alcaligenes xylosoxidans* subspecies *denitrificans* (*Axd*) using standard morphological and biochemical tests. Four of these species were designated as *Axd*1, *Axd*2, *Axd*3, and *Axd*4. The other *Alcaligenes* species that was found in GWSS was designated as *Axd* Hc01 and selected for further study (Bextine 2004). However, the classification of *Axd* Hc01 remains unsettled.

OBJECTIVE
1. If *Axd* Hc01 is to be used as part of a symbiont therapy program, the issues surrounding its identity must be resolved. One way to help clarify its identity and relationship to other identified *Axd* strains is to construct phylogenetic trees based on the sequences of universally present, highly conserved prokaryotic genes (Laguerre 1994). The goal of this research is to help identify *Axd* Hc01 by placing it in phylogenetic trees based on 16S, gyrase B, and 16S-23S intergenic spacer region sequences.

RESULTS

The phylogenetic trees based on 16S sequences indicate that *Axd* Hc01 is most closely related to members of the genus *Pseudomonas* (Figure 1). The second 16S phylogenetic tree shown also indicates that *Axd*1 and *Axd*2 are more closely related to *Axd* Hc01 than *Axd*3 and *Axd*4 (Figure 2). In the two gyrase B trees (Figures 3 and 4), *Axd* Hc01 does not group with the other *Alcaligenes* species used in the study, but some of its relatives do. In agreement with the 16S studies, *Axd* Hc01 groups most closely with a member of the genus *Pseudomonas* in the gyrase B trees (Figure 3). However, in the second gyrase B tree (Figure 4), *Axd* Hc01 does not group with members of this genus. Instead, *Axd*4 does. In the most current 16S-23S ITS 1 trees, *Axd* Hc01 does not group with any of the organisms included in the study (Figure 5). Its relatives *Axd*2 and *Axd*4 do cluster together in the bottom 16S-23S ITS 1 tree, and *Axd*1 groups with *Alcaligenes faecalis* 16.7 (Figure 6). The 16S-23S ITS 1 trees do not yet include all of the organisms that the first two trees do, and this could be one reason for the noted discrepancies. Once the 16S-23S ITS 1 trees are completed, the relationships among these five species will be further investigated and clarified using DNA-DNA hybridization techniques.

CONCLUSIONS

From a preliminary analysis of these results, it can be concluded that *Axd* Hc01 and its relatives are related to members of the genus *Pseudomonas*. However, more work using the 16S-23S ITS region will be necessary to provide more information concerning the identity of *Axd* Hc01 at the species and subspecies level and to clarify its relationship to *Axd*1, *Axd*2, *Axd*3, and *Axd*4. The successful identification of the *Axd* Hc01 bacterium and its relatives will help contribute to a strategy based on symbiont therapy to control PD.

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FUNDING AGENCIES

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Figure 1. 16S phylogenetic tree inculding r*Axd*.

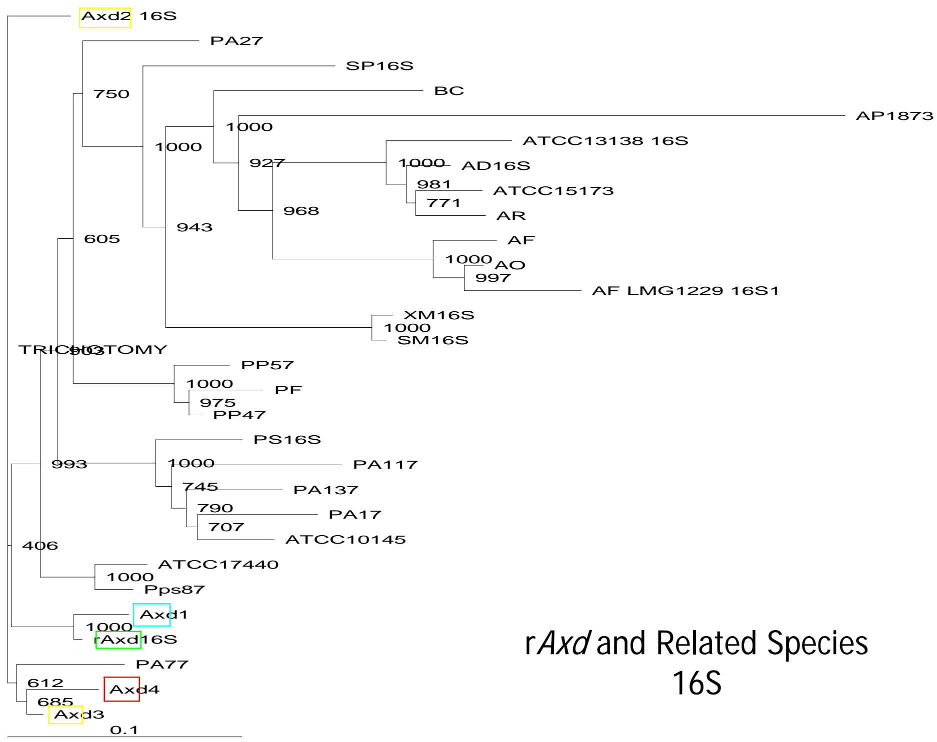


Figure 2. 16S phylogenetic tree including r*Axd* and its relatives.

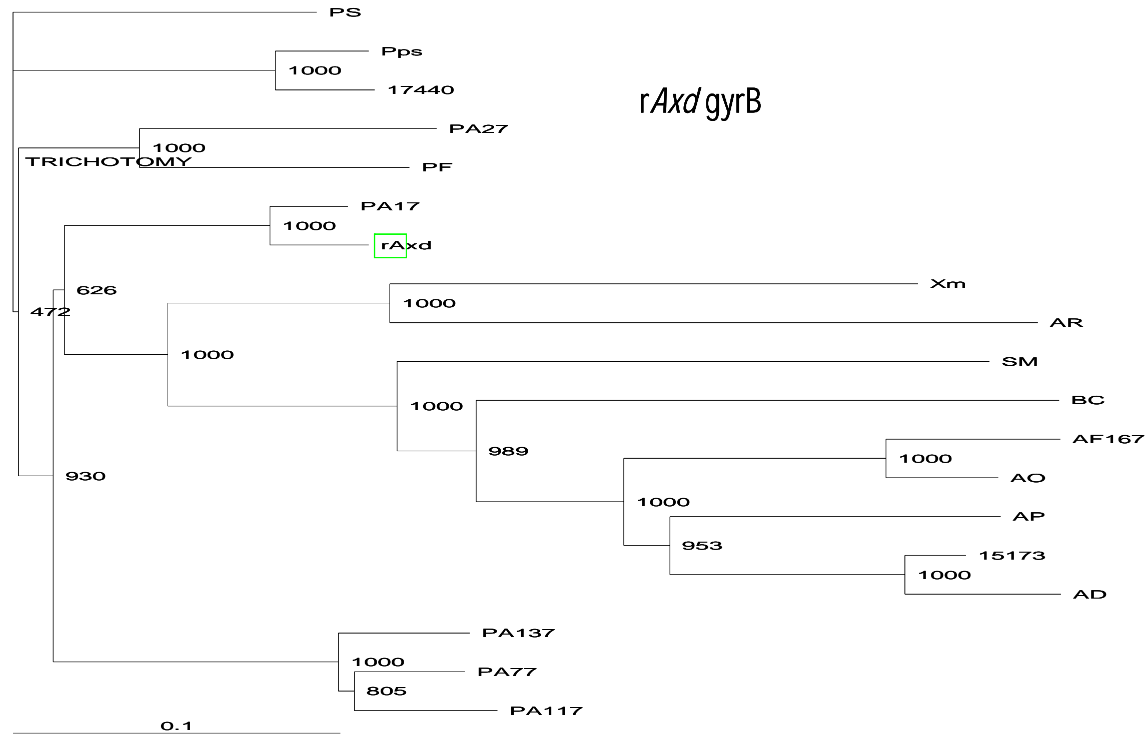


Figure 3. gyrB phylogenetic tree for r*Axd*.

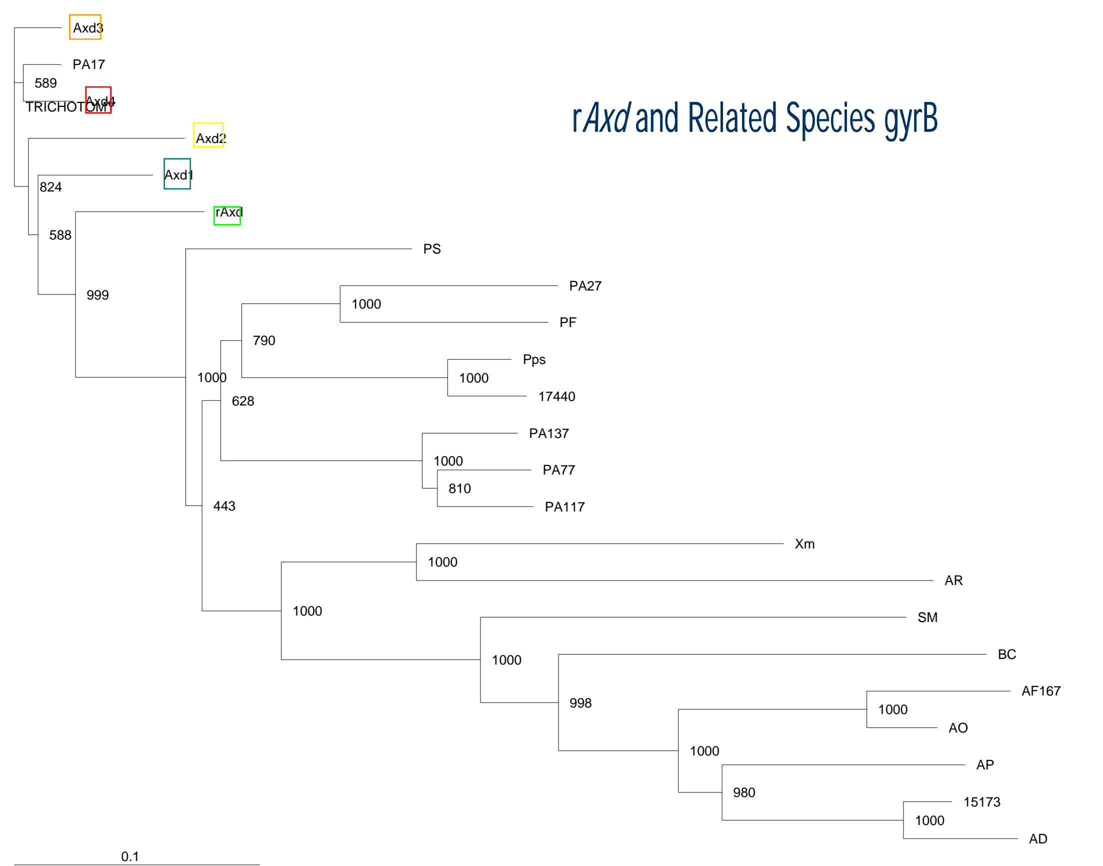


Figure 4. gyrB phylogenetic tree including r*Axd* and its relatives.

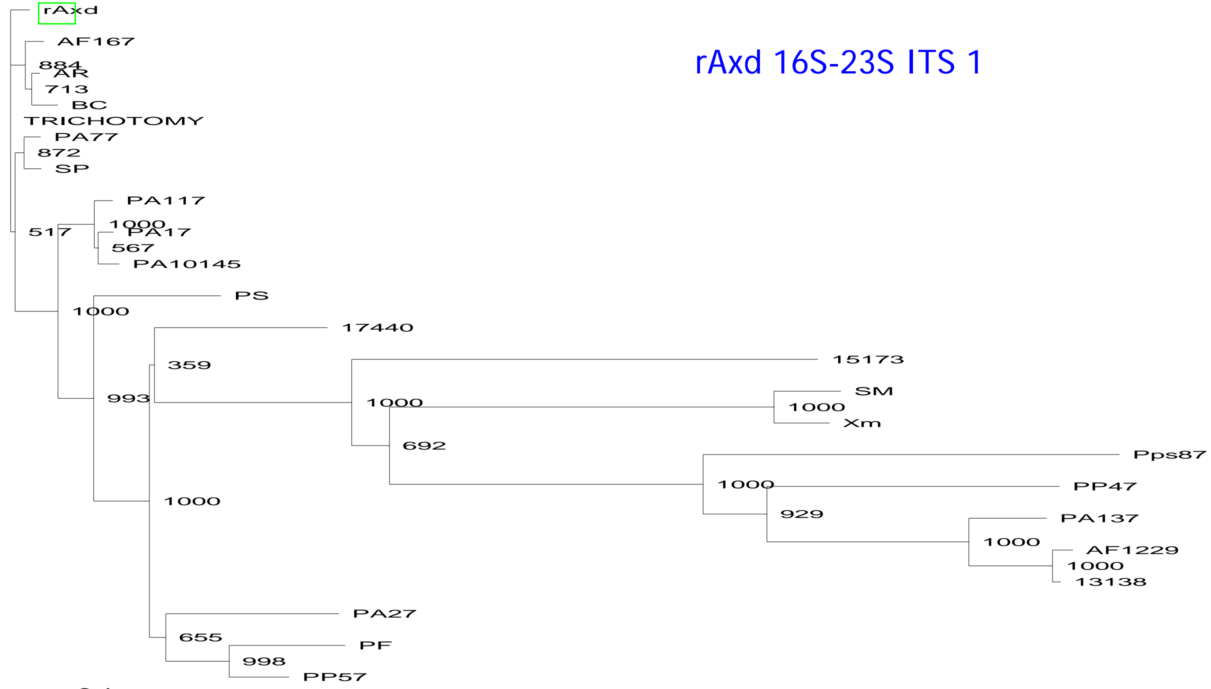


Figure 5. 16S-23S ITS 1 phylogenetic tree for r*Axd*.

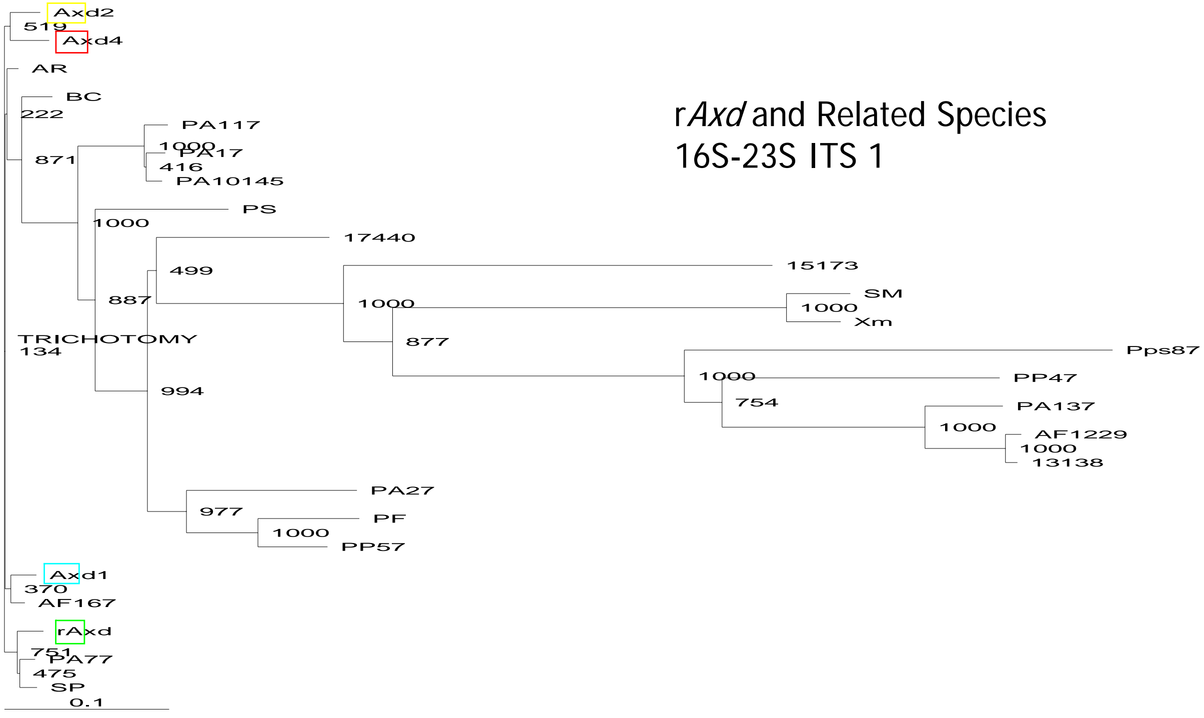


Figure 6. 16S-23S ITS 1 phyogenetic tree including r*Axd* and its relatives.

REGULATION OF BIOTECHNOLOGY APPLIED TO AGRICULTURE

Project Leader:
Thomas Miller
Department of Entomology
University of California
Riverside, CA 92521
Thomas.Miller@ucr.edu

ABSTRACT
Regulatory agencies are faced with an increasing number of permit applications using biotechnology to solve agricultural problems. Newer innovations include engineered biopesticides, transgenic and paratransgenic insects such as the symbiotic control project aimed at controlling Pierce's disease. Review panels point to the regulatory burden as one reason not to fund new technology. The results of a Workshop held 7-9 November 2006 in Washington DC to address these and other regulatory issues will be described.

Section 5:
Crop Biology and Disease Epidemiology

ROLE OF ALFALFA IN THE EPIDEMIOLOGY OF *XYLELLA FASTIDIOSA* IN CALIFORNIA

Project Leader:
Rodrigo Almeida
Dept. of Env. Science, Policy & Mgmt.
University of California
Berkeley, CA 94720
rodrigo@nature.berkeley.edu

Cooperators:
Kent Daane
Dept. of Env. Science, Policy & Mgmt.
University of California
Berkeley, CA 94720

Russell Groves
Department of Entomology
University of Wisconsin
Madison, WI

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

ABSTRACT
Alfalfa occurs widely throughout the Central Valley, often adjacent to grape and other plants susceptible to *Xylella fastidiosa (Xf)*. A previous epidemic of Pierce's disease in the Central Valley was associated with migration of infective insects from alfalfa fields to vineyards. We will determine the importance of alfalfa as a host of different strains of *Xf* and its role as a source of the pathogen for vector transmission. These studies provide basic information on these interactions, which would be incorporated in disease management strategies that include alfalfa as a host of *Xf* and sharpshooter vectors.

INTRODUCTION
The role of alfalfa in the maintenance and spread of *Xylella fastidiosa (Xf)* in California is poorly understood, despite the fact that the epidemic of Pierce's disease (PD) in the Central Valley during the 1940s was linked to alfalfa fields. We will conduct studies aimed to understand the importance of alfalfa in the epidemiology of *Xf* in California in relation to grape and almond hosts, and native and invasive insect vectors. We will conduct transmission experiments with different *Xf* strains and different vector species to determine what factors are of important in the spread of this bacterium among different host plants. Recent research on *Xf* has also examined its biology within alfalfa plants. Our work will also study the movement and multiplication of *Xf* in alfalfa and the potential role of this crop as a reservoir of *Xf* for vector acquisition. Information gathered in this project will lead to determination of the importance of alfalfa in the epidemiology of *Xf*.

OBJECTIVES
1. Determine the fate and role of *Xf* in alfalfa.
2. Determine the transmission efficiency of *Xf* strains to/from alfalfa to grape and almond by three sharpshooter vector species.

RESULTS
This project is being initiated. We have no results to report at this point.

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

WHERE, WHEN AND HOW DO INGESTION AND OTHER FEEDING BEHAVIORS OF THE GLASSY-WINGED SHARPSHOOTER ALLOW INOCULATION OF *XYLELLA FASTIDIOSA*?

Project Leader:
Elaine Backus
USDA, ARS, PWA
Crop Dis., Pests & Gen.
Parlier, CA 93648
ebackus@fresno.ars.usda.gov

Researchers:
Sebastien Dugravot
Dept. of Entomology
University of California
Riverside, CA 92521

Brendon Reardon
Dept. of Entomology
University of California
Riverside, CA 92521

Holly Shugart
USDA, ARS, PWA
Crop Dis., Pests, Gen.
Parlier, CA 93648

Cooperators:
Jianchi (JC) Chen
USDA, ARS, PWA
Crop Dis., Pests & Gen.
Parlier, CA 93648
jichen@fresno.ars.usda.gov

Thomas Miller
Dept. of Entomology
University of California
Riverside, CA 92521
Thomas.miller@ucr.edu

Steven Ruzin
Biological Imaging Facil.
University of California
Berkeley, CA 94720
ruzin@nature.berkeley.edu

David Morgan
Calif. Dept. Food & Agric.
Mt. Rubidoux Field Stn.
Riverside, CA 92501
dmorgan@jps.net

Reporting Period: The results reported here are from work conducted November 1, 2005 to September 30, 2006.

ABSTRACT

This year, we completed the laboratory portion of work that identified an aspect of electrical penetration graph (EPG) ingestion waveform (C) of glassy-winged sharpshooter (GWSS) that definitively represents xylem ingestion. We also demonstrated that extravasation is correlated with the B1 waveform. Both B1 and C waveforms may play a role in the behavior that facilitates inoculation of *Xylella fastidiosa* (*Xf*). Results this year support that both amount and location of *Xf* binding in the foregut of GWSS ("vector load") are critical for success of subsequent inoculation. The precibarium is the first area colonized during acquisition, and also may be the most important location for subsequent inoculation. If insects become "maximally loaded" with *Xf*, preliminary results suggest that a single probe by a single vector can cause a lethal infection. These results will help solve the PD/GWSS problem by providing powerful new tools for future studies, answers to numerous questions about how vector transmission works, and new potential targets for host plant resistance.

INTRODUCTION

The behaviors comprising within-plant feeding (a.k.a. stylet penetration) of hemipteran vectors are intricate and complex, and vary enormously among species. Yet, a deep understanding of stylet penetration is particularly important for sharpshooter vectors because behavior plays a crucial role in transmission of non-circulatively transmitted pathogens like *Xylella fastidiosa (Xf)*. Thanks to EPG monitoring, sharpshooter stylet penetration can now be observed in detail, in real-time. Two stylet penetration behaviors emphasized in this project likely control *Xf* inoculation. They are uptake of plant fluids into the gut (ingestion) and expulsion of bacteria-laden fluids (egestion or extravasation).

OBJECTIVES

1. Characterize ingestion behavior, especially to: (a) identify in which cell types various durations of ingestion (C) are occurring, and (b) how to recognize that by EPG alone.
2. Characterize extravasation behavior, especially to: (a) correlate the B1 waveform with fluid flow in and out of the stylets, and (b) determine in which plant cells this behavior occurs.
3. Characterize behavior-*Xf* interactions that permit inoculation, especially to (a) identify the behaviors (i.e. ingestion, extravasation or both) during which bacteria are expelled, and (b) whether bacterial expulsion is into xylem, or any plant cell type penetrated, or both.

RESULTS

Insect Availability

Last year's progress report announced that we had solved all problems with availability of experimental plants, insects and bacteria. Unfortunately, one month after writing that report, our source for clean glassy-winged sharpshooters (GWSS), D. Morgan of CDFA, told us he could no longer provide insects due to unforeseen colony issues. For most of this year, we have attempted to use GWSS lab-reared from egg masses laid by adults that we field-collected from ornamental plants in Bakersfield (for *Xf* inoculation studies) or we have substituted smoketree sharpshooter (STSS) (for waveform correlation studies without *Xf*). These attempts were partially successful, but consequences were severe for one experiment. As of this writing (late September 2006), we have been promised a few clean insects from Morgan, to re-do key experiments.

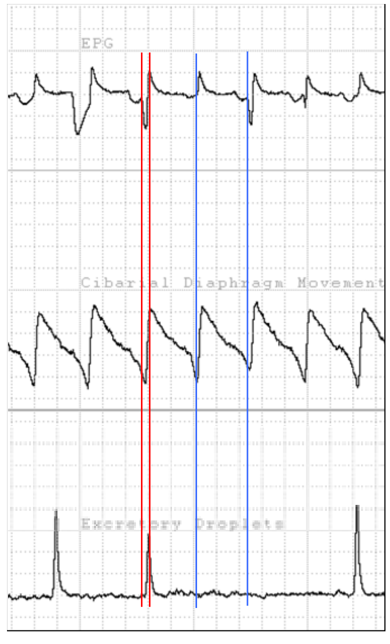


Figure 1. Three simultaneously recorded voltage signals from EPG (top), cibarial diaphragm movements (middle) and production of excretory droplets (bottom). Light-detecting diodes were placed on the video screen to generate voltage signals for diaphragm movements (increased voltage = more uplifted diaphragm) and excretory droplet expulsion (peaks).

Objective 1. Correlation of ingestion with EPG waveforms

Study a: Ingestion-activity correlations and cell types in which it occurs

An electromyographic (EMG) study of cibarial dilator muscle activities was completed by post-doc S. Dugravot (Dugravot et al., ms. in prep.). The project temporally correlated EMG signals of muscle potentials with video images of cibarial diaphragm movements (visible when backlit) and production of excretory droplets (Figure 1). The C waveform was 100% correlated with cibarial dilator activity. The valley portion of C represented the rapid, muscular uplift of the diaphragm (i.e. sucking; Figure 1, red lines) and the plateau represented the slower, non-musclular drop of the diaphragm (i.e. swallowing; Figure 1, blue lines). In addition, once sustained ingestion was underway, excretory droplets were also correlated with cibarial pumping; 2 – 4 pumps produced a single droplet (Figure 1). Droplets ceased when cibarial pumping ceased, especially during interruption (N) waveforms corresponding to salivation into xylem (Backus and Labavitch 2006) (data not shown). However, the synchrony of droplets and cibarial pumping did not hold for the beginning and end of ingestion. Each insect varied greatly in the onset of droplet production in relation to onset of pumping, and likewise, the end of pumping did not immediately signal the end of droplet production. Thus, we confirm that production of excretory droplets is an excellent correlate of sustained ingestion. However, we caution that droplet production cannot be used to time onset or cessation of ingestion, which differed by up to 40 min.

Data from this EMG study were complemented by a second study performed by Backus at the Argonne National Lab. EPG waveforms were recorded from wired GWSS that were subjected to high-energy X-ray imaging during feeding, which allowed the cibarial muscle movements to be directly viewed. Video images and waveforms are being analyzed, but preliminary results support the above findings. Significantly, it was also observed that atypical C waveform shapes, hypothesized to correlate with ingestion from non-xylem cells, were actually caused by unusual cibarial muscle contractions. In fact, all aspects of C waveform fine structure were correlatable with cibarial muscle movements alone. Other correlations will be possible as data are analyzed

A third project completed this year was a histological study to determine which ingestion events were performed in xylem, and whether that ingestion tissue can be identified by waveform appearance alone. We examined salivary sheath branches of artificially terminated probes made by STSS feeding on cowpea stem (see sample waveform in Backus and Labavitch 2006). Preliminary results strongly support that waveform C is virtually always correlated with xylem, but can occur in any of several xylem cell types. Very early, especially short-duration, C events usually occurred in pri-mary protoxylem cells or small, unlignified secondary xylem cells. In contrast, somewhat later, especially longer-duration, C events occurred in large, lignified secondary xylem cells.

Study b: Recognizing ingestion from waveforms alone

Results from Study a (to date) support that waveform C represents ingestion, but its fine structure is not correlated with ingestion tissue type. Yet, C predominantly occurs in xylem, and the type of xylem cell (and perhaps function-ality) appears to be correlated with the C event order and/or the event duration.

Objective 2. Correlation of extravasation with EPG waveforms

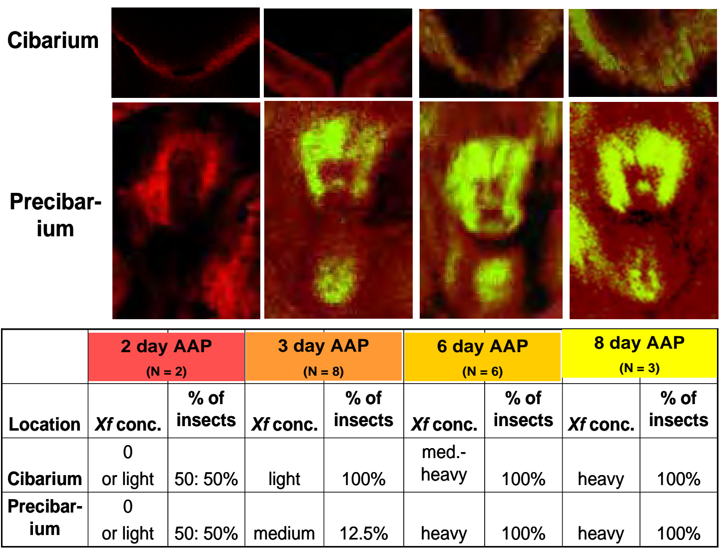
Study a: Correlate B1 waveform with muscle movements and fluid flow in and out of stylets

The EMG study described under Objective 1 above also examined muscle potentials from the precibarial valve muscles, which are hypothesized to control extravasation. Results conclusively showed that the precibarial valve is voluntarily moved

only during pathway waveforms, not during C. The valve muscle potentials occurred throughout pathway, and strongly resembled waveform B1 spikelet bursts (B1s) (data not shown). Because B1 is the only waveform that is ubiquitous throughout pathway, this supports that B1s represents valve fluttering. The directionality of streaming potentials implies that fluid moves in and out of the precibarium during this behavior. This work supports earlier findings correlating B1 with *Xf* inoculation. B1 apparently represents a combination of fluid uptake and expulsion (extravasation) (via precibarial valve fluttering) for tasting, plus small amounts of salivation.

Study b: Determine in which plant cells B1 occurs
A combination of the GWSS EMG study and STSS sheath histology studies above, plus results from earlier studies, continues to support that B1 occurs throughout pathway, in all cell types penetrated by the stylets. Work detailed in Backus and Labavitch 2006 shows that B1 can occur in xylem cells, just prior to or interrupting ingestion events.

Objective 3. Characterize behavior-*Xf* interactions that permit inoculation
Study a: Identify the behaviors (ingestion, extravasation or both) during which bacteria are expelled
Last year's progress report detailed findings by the previous post-doc, P.H. Joost, that green fluorescent protein (GFP)-expressing *Xf* were seen embedded in salivary sheaths and floating freely in artificial diet on which putatively inoculative GWSS fed. Yet, only 1 out of 40 EPG-recorded probes revealed GFP. In the year since that work, we have demonstrated that the low success rate of Joost's experiment was due to not enough GFP-*Xf* available in infected plants, so that his ultra-clean GWSS from Morgan could not acquire enough *Xf*. We perfected our acquisition protocol, and produced confocal laser scanning microscope (CLSM) images that demonstrated that GFP-*Xf* are acquired first into the precibarium and then into the cibarium over an 8 day acquisition access period (AAP) (Figure 2). These images were showcased in our poster at the 2005 PD Symposium (Backus et al. 2005). We term these insects "clean, maximally loaded." This year, now more confident of our acquisition protocols, a second post-doc (S. Dugravot) attempted anew to perform this diet-inoculation experiment.



	2 day AAP (N = 2)		3 day AAP (N = 8)		6 day AAP (N = 6)		8 day AAP (N = 3)	
Location	*Xf* conc.	% of insects	*Xf* conc.	% of insects	*Xf* conc.	% of insects	*Xf* conc.	% of insects
Cibarium	0 or light	50: 50%	light	100%	med.-heavy	100%	heavy	100%
Precibar-ium	0 or light	50: 50%	medium	12.5%	heavy	100%	heavy	100%

FIG 2. CLS micrographs of representative epipharyngeal halves of cibaria (top) and precibaria (bottom; more magnified) at 2, 3, 6 or 8 days AAP.

After four months of efforts this year, we concluded that the attempt was again unsuccessful. Like last year, however, we learned much from the problems encountered. Our efforts to rear clean (non-inoculative) GWSS from egg masses in the absence of Morgan's insects were apparently unsuccessful. None of the nearly 60 probes that were observed in artificial diets showed signs of GFP-*Xf*, even though the insects were given 8 – 13 d AAP's on infected acquisition grape plants. Indeed, when we dissected the GWSS heads and examined their foreguts via CLSM, we invariably found much less GFP-*Xf* than last year's maximally loaded insects (data not shown). Large, flocculent aggregations of autofluorescing (non-GFP) microbes were visible in both areas, but primarily in the cibarium; GFP-*Xf* were merely tucked in amongst these, in small to medium aggregations. We concluded that the insects had already acquired competing microbes into the prime acquisition (and inoculation) sites, and again there was too little GFP-*Xf* present to inoculate into the diet. We term these insects "dirty, topped-off." These findings support the hypothesis that competition for binding sites occurs in the insect's foregut. A third post-doc, B. Reardon, will attempt this diet-inoculation project again, after we have received (hopefully) ultra-clean GWSS from Morgan.

Study b: Determine into which plant cells bacteria are expelled
In November 2005 we performed a plant inoculation experiment to complement the diet inoculation study described above. For this test, we were able to use the last of the ultra-clean insects provided to us last year by Morgan. Recent CLSM of their heads has shown that the precibaria of these insects acquired GFP-*Xf* (Figure 3, next page) during their 5 – 8 day AAP's, prior to use in the inoculation test. GFP-*Xf*-inoculative insects were each EPG-recorded for a single, standardized probe composed of pathway plus 3-6 min of ingestion on a healthy 'Cabernet Sauvignon' grapevine petiole. Each plant was then held in an insect-exclusion cage in the greenhouse for one of 4 time periods: 0, 10, 20 or 40 d, whereupon the petiole tissue in the immediate vicinity of the marked probe site was prepared, sectioned, and examined using epifluroescence light microscopy (ELM) with a GFP filter cube. The 0 d tissue actually was excised 2 – 4 min after the end of the probe. In addition, when plants became obviously symptomatic for PD (at 3 months), 8 – 10 leaves above the fed-upon petiole were assayed for *Xf* using PCR.

Results are still being fully analyzed, but preliminary findings are startling. First, 100% of the 36 plants (9 reps per treatment) became unambiguously symptomatic for PD and died within 6 months after the inoculation probe. Control,

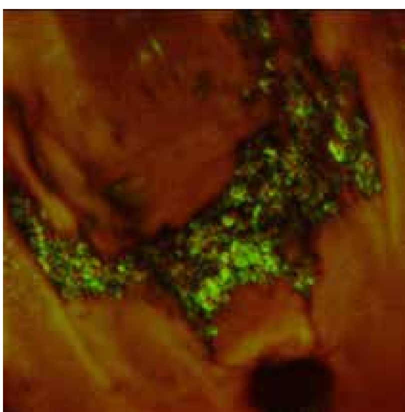


Figure 3. Close-up view of the precibarial valve and the distal half of the epipharyngeal basin in a GWSS allowed a 5 day AAP, showing green GFP-*Xf*. The plant was PCR-positive for *Xf* . Note better resolution than for Fig. 2, due to improved CLSM protocols. 320x

healthy plants subjected to all the same treatments (except inoculation) thrived, and none died. All plants were kept on an automatic irrigation regime in the greenhouse, and none became dessicated. PCR tests were encouraging, but require further optimization in a November repeat of the experiment. A majority of plants in each treatment were positive for *Xf*: 100% of the 0 d plants, and 56% each for the 10, 20 and 40 d plants. We suspect that the 44% negative were false negatives, due to either concentration of template below the detection limit, or problems with inhibitors. The strong symptom results and adequate PCR results suggest that 100% of these grape plants were inoculated with *Xf* by a single GWSS probe, which led to a lethal, systemic PD infection. If an upcoming repetition of this experiment verifies our findings, it will be the first time that GWSS has been experimentally shown to exhibit 100% vector efficiency per individual insect, let alone from a single probe. Future refinement of the procedure could provide a bioassay for reliable, natural insect-inoculation that could be useful for comparative host plant resistance studies.

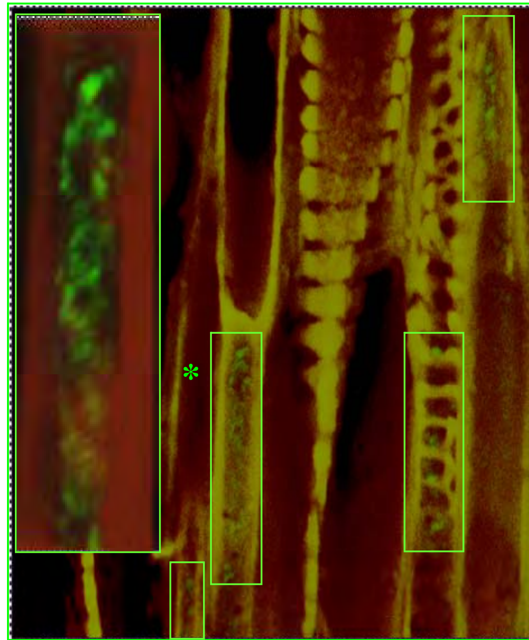


Figure 4. GFP-*Xf* revealed (small green boxes) in paraffin-embedded and longitudinally-sectioned stems from mechanically inoculated grape. Inset: a section adjoining the box with the *, magnified. This represents ~70% of the green signal that will be visible using the final protocol. 300x.

Second, unfortunately, our histological preparations were not successful, but we have determined the cause and are developing new protocols to solve the problem. In order to locate salivary sheaths in plant tissue, we must be able to prepare, embed and section the tissue. However, standard, alcohol-based histological preparations quench the fluorescence of GFP; hence, all researchers using confocal visualization of GFP-*Xf* use unsectioned tissues. Our GFP-*Xf* samples fluoresced slightly, but at the same (yellow) wavelength as lignified xylem cell walls (as in Figure. 4). After much effort and with the help of S. Ruzin, we have devised the first-ever protocols for classical histological preparation of GFP in plant tissues. Although improvements still will be made in the coming weeks, we now can definitively identify GFP-*Xf* in longitudinally-sectioned xylem cells and spectrally separate bacterial cells from cell walls (Figure 4).

CONCLUSIONS

Our findings will help solve the PD/GWSS problem by providing: 1) A powerful tool in EPG for studies of host plant resistance, including a natural, insect-inoculation bioassay and eventual development of a resistance index for genotype screening (the Stylet Pentration Index); 2) Insights into the mechanism of *Xf* transmission (acquisition and inoculation); 3) Numerous spin-offs from such basic findings, such as information for risk assessment models, with implications for all levels of the *Xylella*-sharpshooter-grape pathosystem, including ecological, epidemiological and management; and 4) Knowledge of new potential targets for grape breeding and transgenic resistance.

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THE ROLE OF GLASSY-WINGED SHARPSHOOTER SALIVARY ENZYMES IN INFECTION AND MOVEMENT OF *XYLELLA FASTIDIOSA*

Project Leaders:
Elaine A. Backus
USDA, ARS, PWA
Crop Dis., Pests & Gen.
Parlier, CA 93648
ebackus@fresno.ars.usda.gov

John M. Labavitch
Dept. of Plant Sciences
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

Researchers:
Holly Shugart
USDA, ARS, PWA
Crop Dis., Pests & Gen.
Parlier, CA 93648

L. Carl Greve
Dept. of Plant Sciences
University of California
Davis, CA 95616

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ABSTRACT
The purpose of this project is to determine whether a vector's enzymatic saliva aids the establishment of the few 'pioneer' *Xylella fastidiosa* (*Xf*) cells that are inoculated into a plant; thus the bacteria would co-localize with the saliva. Previous work showed that watery saliva of glassy-winged sharpshooter (GWSS) degrades cell walls, and is injected into xylem during feeding. Saliva contains cell wall-degrading enzymes, notably β-1,4-glucanase (EGase), which can degrade pit membranes that impede cell-to-cell movement of bacteria. We plan to immunoprobe for salivary EGase, in grape stems fed-upon by GWSS that have acquired green fluorescent protein (GFP)-transformed *Xf*, to co-localize *Xf* with both sheath and watery saliva. This year, we: 1) improved protocols for histology of salivary sheaths using epifluroescence light microscopy, 2) dissected 500 pairs of salivary glands to extract EGase and raise antibodies, 3) showed histologically that the salivary sheath dissolves over time, resulting in cellular abnormalities typical of cell-wall loosening, and 4) showed that sheath and watery saliva are directly injected into and travel through xylem cells.

INTRODUCTION
Many researchers, including ourselves, are investigating how *Xf* moves from cell to cell**,** because it is a crucial mechanism for the earliest stages of infection. *Xf* can cause PD only if bacteria can 'break out' of the initial, imprisoning inoculation xylem vessel(s) to produce an increasing population that becomes systemic. Thus, initial infection success is dependent, in part, on *Xf* lateral movement through adjacent vessels. Recent evidence by Labavitch and colleagues studying *Xf* movement through stems supports the idea that pit membranes limit bacterial movement. Sufficient quantity of cell wall-degrading enzymes can digest parts of the primary cell wall network of the pit membrane, allowing bacteria to pass. The enzymes polygalacturonase (PG) and β-1,4-glucanase (EGase, often identified as cellulase in the literature) are produced by mature *Xf* populations, typical of the biofilms in xylem that are seen in later stages of infection, and may function in this way. It seems to us, however, that the few pioneer bacteria inoculated by a vector are unlikely to produce a sufficient titer of enzymes to digest through the pit membrane. Fewer than 200 cells are typically inoculated by sharpshooters (Hill and Purcell 1995).

Although it is routine to histologically image sheath saliva in fed-upon plants (e.g. Leopold et al, 2003, Backus et al 2005b), no researcher has ever directly visualized watery saliva in plants, for any hemipteran. Due to its fluid and dispersive nature, it is unstainable by conventional means. However, Backus and colleagues have defined electrical penetration graph (EPG) waveforms that represent salivation and ingestion (Joost et al. 2006). Correlating EPG with histology of probed plant tissues revealed the cell types into which saliva is injected (Backus et al. 2005). Watery saliva becomes mixed with, and spreads out from, the salivary sheath and enters the xylem. Labavitch and colleagues recently have found very high activity of EGase and other cell wall polymer-degrading enzymes in GWSS salivary gland fractions. The evidence that copious quantities of cell wall-degrading saliva are injected along with very few bacterial cells led Backus to hypothesize that the small number of pioneer bacteria initially inoculated are aided in their cell-to-cell movement (therefore their ultimate infection) by the enzymatic salivary secretions of their vector.

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

RESULTS
Award notification for this new grant was received in June 2006. However, as usual, the official start of the project will be delayed until spring 2007, due to budgeting and paperwork circumstances beyond our control. Nevertheless, we made progress in these four months analyzing pertinent previous research, using Backus's in-house ARS funds.

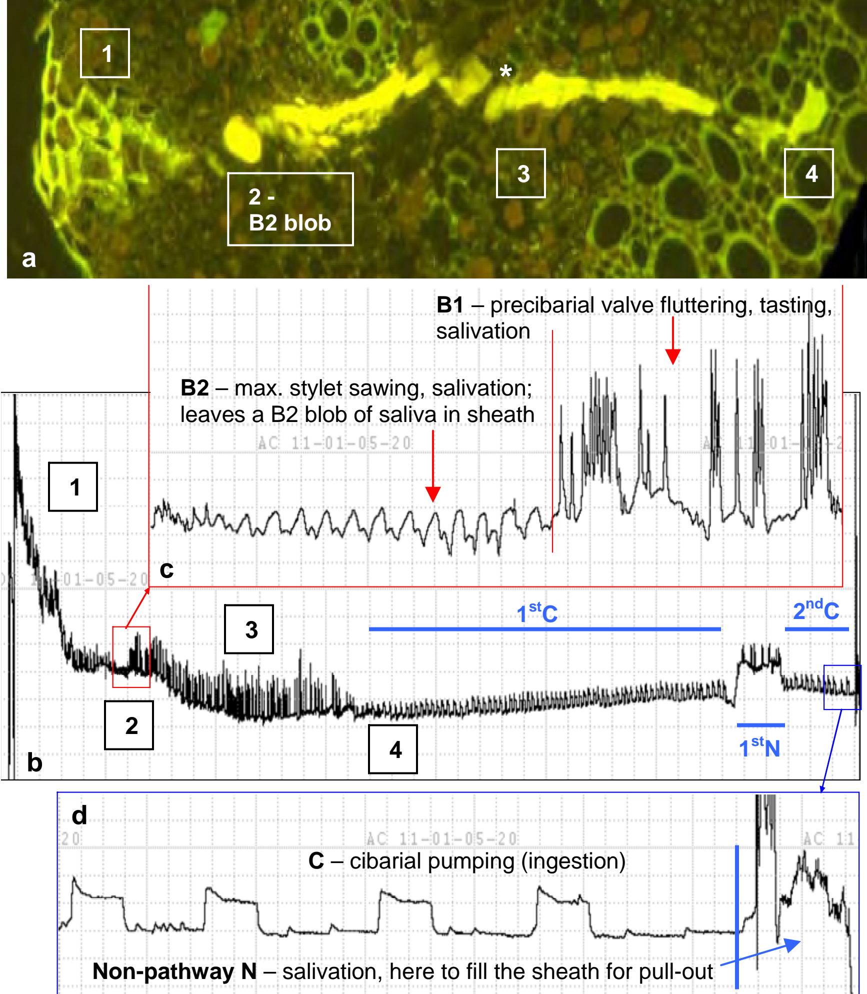


Figure 1. a. Montage of images (merged at *) from two adjoining sections of a long GWSS salivary sheath in healthy 'Cabernet sauvignon' grape petiole, excised 20 d after the probe. The sheath bypassed a small vascular bundle and penetrated straight to a secondary xylem cell in an interior vascular bundle. The cell is filled with sheath saliva because the probe was naturally terminated by the insect. Numbered labels identify spatial sheath correlations with temporal waveform correlations in part b. 200x. **b.** Entire EPG waveform trace recorded from this probe. **c, d.** Enlarged views of the red-boxed section of pathway waveform (**c**), and blue-boxed section of the last ingestion waveform (**d**), respectively, in part b, showing waveform details.

General Methodologies
Strong progress was made this year in developing new methods that will be applicable to this grant's research, as well as to other projects in the Backus lab. Among them are further fine-tuning of the histological methods used to visualize GWSS salivary sheaths in fed-upon grape tissues using confocal laser scanning microscopy (CLSM) and epiflurescence light microscopy (ELM), especially in relation to correlated EPG recordings of stylet penetration. We found that the salivary sheath autofluoresces very brightly at almost all wavelengths of light, and thus it can be imaged using both CLSM and ELM without time-consuming staining and counter-staining of tissues (Fig. 1a). The sheath is bright yellow under a GFP filter, and can be spectrally separated from the surrounding plant tissues (**Fig. 1a**) and GFP. We also further perfected the method for simultan-eous EPG recording, marking the probing site, histological preparation, then correlating waveforms (Figure 1b-d) with salivary sheaths. Landmarks in the sheath that correspond with known waveform types (e.g. the B2 blob; Figure 1 a-c) allow spatial and temporal correlations of behaviors (compare numbers on sheath and waveform pictures).

Objective 1. Purify and characterize β-1,4-glucanase and develop antibodies.
Study a: Enzyme purification
Salivary glands were dissected by Backus and her colleagues from nearly 500 GWSS, field-collected from ornamental shrubs in Bakersfield, CA, during the summer of 2006. Glands were frozen in extraction buffer at -20 C. Plans are to dissect another 300-500 pairs of glands before January 2007, when protein extraction, purification, and assaying of EGase will begin in the lab of Labavitch and his colleagues. Antibodies to purified EGase will be raised by a core facility at UC Davis, for later Objective 2 work.

Objective 2. Determine whether GWSS salivary proteins affect the presence/distribution of inoculated *Xf*.
Studies a and b: Immunocytochemistry of probes by clean vs. GFP-*Xf *inoculative GWSS
Our ultimate goal for this objective is to combine five challenging procedures into one experiment: 1) using ultra-clean GWSS, allow one group of insects to maximally acquire GFP-*Xf* and the other (control) group to remain clean, then 2) EPG-record a single, standardized probe as described in Backus (2006), then 3) excise, histologically prepare, and section the fed-upon grape tissue, then 4) probe the sectioned tissue with Cy5-fluorescently conjugated antibody to EGase (from Objective 1), and finally 5) use CLSM to simultaneously locate and image autofluorescent salivary sheaths and cell walls, GFP-*Xf* and Cy5-stained EGase/watery saliva. In addition, this study will include a time-course, in which fed-upon plants are held for varying time periods before excision and preparation for microscopy. In this way, we hope to visualize the location of both watery saliva (i.e. EGase) and sheath saliva in relation to presence, location and movement of *Xf* bacterial cells, during certain EPG waveforms. This year, we made substantial progress developing each of the individual protocols to be combined in the larger test, with the following findings.

The salivary sheath dissolves, resulting in cellular abnormalities typical of cell-wall loosening

In the plant inoculation experiment described more fully in Backus 2006, we developed the EPG and time course methods. We also used ELM to examine the appearances of the salivary sheaths after 0, 10, 20 and 40 days following an EPG-identified, standardized probe. We found that the outermost region of the salivary sheath (the "trunk" area, where the mandibular stylets are braced) shows signs of cell enlargement over time (Figure. 2a-d), similar to that caused by hemipterans that cause saliva-mediated, direct feeding damage via cell wall-degrading enzymes, such as *Empoasca fabae* and *Lygus hesperus* (Backus et al. 2005a, Shackel et al. 2005, respectively). This is strong, albeit indirect, evidence that such enzymes are active in GWSS saliva, ultimately leading to a cellular response typical of such agents.

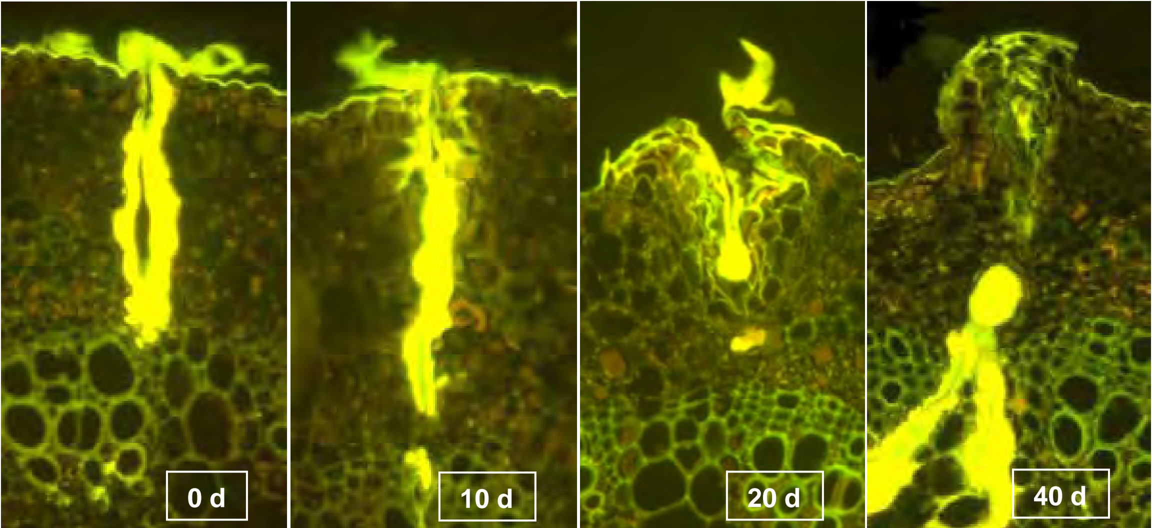


Figure 2. Naturally terminated, GWSS salivary sheaths in healthy 'Cabernet Sauvignon' grape. Although all sheaths terminated in xylem, not all chosen sections show that. **a.** Sheath excised at 0 d time period (actually, 2 – 3 min after end of probe). Note the distinctness of the sheath edges. **b.** Sheath excised 10 d after the probe. Note slight spread of saliva into adjoining cells, especially cell walls, and less distinct edges of the sheath, which is beginning to dissolve. **c.** Sheath excised 20 d after probe. Note strong dissolution and wide area of saliva into adjoining cells and cell walls, enlargement of some cells, and start of swelling of tissue. **d.** Sheath excised 40 d after probe. Note less sheath saliva (now absorbed) and strong cellular enlargement, swelling of adjacent tissue. 200x.

Sheath and enzymatic watery saliva are directly injected into xylem cells, and they can travel

ELM images of salivary sheaths from the plant inoculation-time course study (described in Backus 2006) usually showed sheath saliva within an ingestion xylem cell at the point of stylet entry, as in Figure 1. This is because the wired insects were allowed to naturally terminate their probes, and sharpshooters typically fill their sheaths as they back their stylets out of the plant (Backus et al. 2005). In addition, sheath saliva also was commonly seen in sections adjacent to the sheath's entry point. In the most extreme example to date, sheath saliva was found in each of 22 contiguous sections, i.e. up to 220 µm away from the salivary sheath (Figure 3). While a sheath branchet to a xylem cell was typically 20 – 40 µm wide, the stylet entry point was usually within one 10 µm-wide section. It is reasonable to hypothesize that less viscous, more dispersive watery saliva could travel even further within xylem vessels.

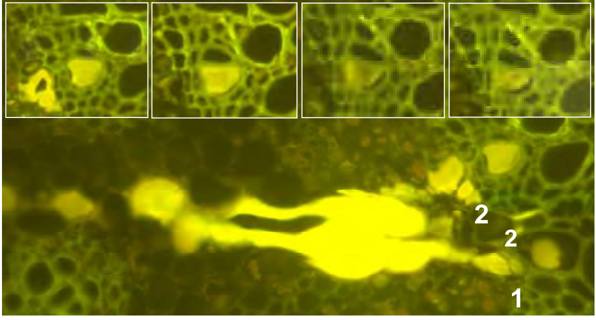
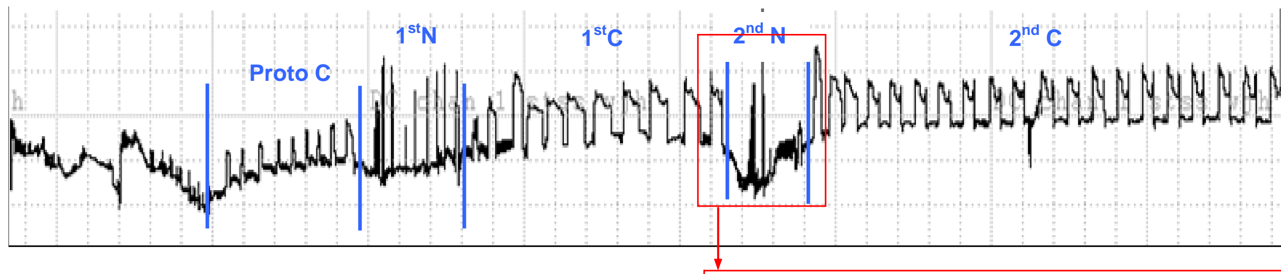


Figure 3. Naturally terminated, GWSS salivary sheath into healthy 'Cabernet Sauvignon' grape, excised 2-3 min after the probe. The sheath had four branches, two shown here, into secondary xylem cells filled with sheath saliva (1 and 2). Saliva in branch 2 moved 22 sections away from this entry point. Representative appearances of the sheath saliva in sections 3, 8, 18 and 19 (from left to right) are shown in insets. 200x.

We also have EPG evidence that salivation occurs in xylem ingestion cells. This year we definitively identified the sharpshooter waveforms representing watery salivation into a xylem cell (Figure 4, below). We performed an experiment to determine when smoke tree sharpshooter (STSS) ingestion waveforms are definitely from xylem. Preliminary data analysis is described in Backus 2006. But the following finding is pertinent to this project. Several correlated salivary sheaths had single branches leading directly to single xylem cells. In one example, the sheath (not shown) led to a large, lignified secondary xylem cell that appeared empty, i.e. it lacked occluding sheath saliva (because the insect's stylets were abruptly pulled out before it could fill the cell). This was the first xylem cell contacted; there was no evidence of branching into or extension from a different cell. Therefore, the two correlated ingestion

(C) waveform events (1st C and 2nd C) must have both occurred in the same cell. It follows then that the interruption waveform (N) between them (2nd N) must represent in part (non-stainable) watery salivation into that xylem cell (Figure 4).



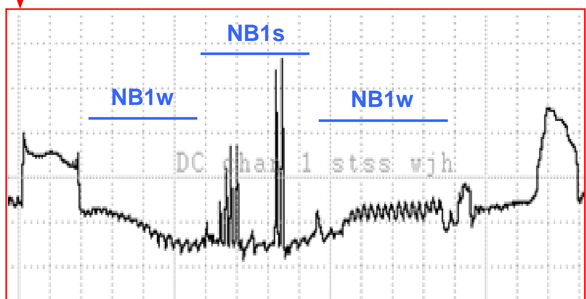


Figure 4. a. Last part of the waveform trace from an artificially terminated probe by a STSS into healthy cowpea. Waveforms from STSS and GWSS are identical. The waveform spike at the far right is the artificial pull-out spike generated when the insect was plucked off the stem. As a result, the salivary sheath was hollow, providing definitive correlation of its termination cell with the final ingestion event, 2nd C. **b,** Enlarged views of the red-boxed section, showing details of the salivation wave, N. NB1w represents salivation; NB1s represents precibarial valve fluttering.

CONCLUSIONS

The described findings support the following hypotheses, which will be further tested this year: 1) watery saliva is injected during the earliest stages of stylet penetration, as well as further along the pathway and into a xylem ingestion cell, 2) GFP-*Xf* exit the stylets during all parts of the probe, and become embedded in the salivary sheath, as well as injected directly into xylem cells, and 3) the bacteria move into areas first traversed by the watery saliva. Findings from this study will help solve the PD/GWSS problem by opening up all-new avenues for transgenic host plant resistance. Novel transgenes could be developed by engineering an inhibitor of the salivary components that aid inoculation.

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FUNDING AGENCIES

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SIGNIFICANCE OF RIPARIAN PLANTS IN THE EPIDEMIOLOGY OF PIERCE'S DISEASE

Project Leader:
Kendra Baumgartner
USDA-ARS
Department of Plant Pathology
University of California
Davis, CA 95616
kbaumgartner@ucdavis.edu

Cooperators:
Sarah Greenleaf
Department of Plant Pathology
University of California
Davis, CA 95616

James Quinn
Dept. of Environ. Science and Policy
University of California
Davis, CA 95616

Joshua Viers
Dept. of Environ. Science and Policy
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted July 1, 2005 to June 30, 2006.

ABSTRACT
We examined the relationship between the occurrence of Pierce's disease (PD) in Napa Valley vineyards and both adjacent and distant vegetation types. Because the vector, *Graphocephala atropunctata* (blue-green sharpshooter, BGSS), is mobile and has a broad host range, disease risk is influenced by vector migration among vegetation types. Therefore, certain combinations of vegetation types surrounding vineyards are more likely to be associated with PD. To test this hypothesis, we surveyed for PD in a total of 41 vineyards located adjacent to either riparian woodland (vector habitat), urban land (vector habitat), other vineyards (vector habitat), or oak woodland (habitat status unknown). The proportions of the four vegetation types distant from the sites (within 0.5, 1, 1.5, and 2 km) were quantified with a geographical information system. Vineyards were surveyed for PD in 11/05. Pathogen presence was confirmed by ELISA. Multiple binary logistic regression showed that both adjacent and distant vegetation type significantly predicted PD presence. Vineyards were more likely to have PD if they were adjacent to riparian woodland and surrounded by more vineyards or urban land. These results suggest that vineyards and urban lands may be important in PD epidemiology. Given that uninfected vineyards adjacent to riparian woodland were also surrounded by large amounts of riparian and upland woodland, it is also possible that riparian woodland in more forested landscapes hosts lower vector densities or a lower proportion of infective vectors. Alternatively, more expansive woodland may be associated with lower PD risk because it decreases the spread of infective BGSSs.

INTRODUCTION
Riparian areas contribute to Pierce's disease (PD) in North Coast vineyards, as evidenced by a correlation between disease incidence and proximity of vines to riparian woodland (Purcell 1974). Purcell (1975) concluded that the bluegreen sharpshooter (BGSS) acquires *Xylella fastidiosa* (*Xf*) mainly from riparian hosts in spring, as the pathogen is not detectable in vines early in the growing season (Hopkins 1981). Our findings of few infected riparian hosts in spring suggest that feeding on such hosts is unlikely to result in *Xf* acquisition by the BGSS at this time (Baumgartner and Warren 2005). Therefore, either BGSSs acquire *Xf* from riparian hosts in summer or autumn, when *Xf* populations are sufficient, or they acquire *Xf* from other hosts.

The generalist feeding habit of the BGSS (Hewitt et al. 1949) makes it difficult to predict which hosts are important inoculum sources (competent reservoirs). *Xf* has a broad host range that includes all winegrape varieties and some riparian plants (Hewitt et al. 1949, Severin 1949, Freitag 1951), but its limited persistence and low titers in most species means that not all hosts are competent reservoirs (Purcell and Saunders 1999, Baumgartner and Warren 2005). *Xf* hosts have been identified mainly from greenhouse studies (e.g. Hill and Purcell 1995). In the field, such hosts are situated within plant communities (vegetation types), where their relative abundance can vary. In addition, the *Xf*-conducive environment in a greenhouse likely over estimates the host range. Therefore, field-based investigations are needed to identify vegetation types that contribute most to the spread of PD.

Our aim was to determine the relationship between PD and the spatial arrangement of vineyards among other vegetation types (landscape structure). Landscape structure is a key factor in the spread of vector-borne mammalian diseases, such as Lyme disease (Allan et al. 2003), bubonic plague (Collinge et al. 2005), and malaria (Guerra et al. 2006), and the invasive forest pathogen, *Phytophthora ramorum* (Meentemeyer et al. 2004). Our approach was to randomly select 41 Napa Valley vineyards adjacent to riparian woodland (vector habitat), urban land (vector habitat), other vineyards (vector habitat), or oak woodlands (habitat status unknown). The proportions of the four vegetation types distant from the sites (within 0.5, 1, 1.5, and 2 km) were quantified with a geographical information system (GIS; ArcGIS v9.1, ESRI, Inc., Redlands, CA). Each site (standardized to a 500-vine block) was sampled for PD in October 2005. *Xf* presence was confirmed by Dr. Barry Hill, using ELISA (Hill and Purcell 1995). Multiple binary logistic regression was used to identify combinations of adjacent and distant

vegetation types that were significantly correlated with PD. We also examined the relationship between winter temperatures, adjacent and distant vegetation types, and PD.

OBJECTIVES

1. Determine if vegetation types distant from vineyards are correlated with PD occurrence in North Coast vineyards.
2. Identify combinations of adjacent and distant vegetation types that are correlated with PD occurrence in North Coast vineyards.
3. Examine the relationship between winter temperatures, adjacent and distant vegetation types, and PD.

RESULTS

Multiple binary logistic regression showed that both adjacent and distant vegetation type significantly predicted PD presence. PD was significantly more likely to be present in vineyards adjacent to riparian woodland. Conversely, PD was less likely to be present in vineyards surrounded by a higher proportion of riparian woodland. This pattern was significant at the buffer radius of 1.0 km, based on both the likelihood ratio and Wald significance tests, and it was significant at the radius of 1.5 km, based on the likelihood ratio test (Table 1). A similar, but nonsignificant pattern existed for the 0.5 km (Figure 1) and 2.0 km radii. Similarly, we found that PD was significantly likely to be absent from vineyards surrounded by a higher proportion of upland woodland at all spatial scales, based on the likelihood ratio test.

The opposite pattern existed for vineyards and urban land. Vineyards were significantly more likely to have PD if they were adjacent to riparian woodland and if more vineyards were present within 0.5, 1.0, 1.5, or 2.0 km (Table 1; Figure 2). Similarly, vineyards surrounded by more urban land were significantly more likely to have PD at all spatial scales.

There were correlations between some vegetation types at some spatial scales (*data not shown*). Proportions of upland woodland and riparian woodland were positively correlated ($P<0.05$) at 1.0, 1.5, and 2.0 km. Proportions of urban land and vineyard were also positively ($P<0.05$) correlated at 1.0, 1.5, and 2.0 km. All other pairs of independent variables (riparian woodland vs. vineyard, upland woodland vs. vineyard, upland woodland vs. urban, riparian woodland vs. urban) were negatively correlated ($P<0.05$) at all four spatial scales.

PD was more likely to be present in vineyards with colder minimum January temperatures. Multiple binary logistic regression with minimum January temperature and type of adjacent vegetation as independent variables showed that the overall model was significant ($\chi^2=14$, $P=0.001$; Nagelkerke $R^2=0.43$). Both minimum January temperature (Wald=6.9, $P=0.008$) and type of adjacent vegetation (Wald=4.6, $P=0.03$) contributed significantly to the model. Minimum January temperature was positively correlated with the proportion of total woodland within 0.5, 1.0, 1.5, and 2.0 km of the vineyards ($P<0.05$).

CONCLUSIONS

Our results show a clear pattern - vineyards are more likely to have PD if they are adjacent to riparian woodland and surrounded by more vineyards or urban land. Several mechanisms, not mutually exclusive, may result in this pattern. For example, because all winegrapes and certain ornamental plants can host both the vector and the pathogen, vineyards and urban lands may be important in PD epidemiology. Northern California vineyards and gardens, which are irrigated in the summer, may provide relatively attractive forage for the BGSS. In addition, reports of pathogen titers show that grapevines are among the highest (Hill and Purcell 1995). Therefore, the focal host species in this disease system, the grapevine, may itself be a competent reservoir. However, given that the proportions of vineyards and urban lands were significantly positively correlated, and that both were negatively correlated with the proportion of woodland, it is not possible to determine if spatial patterns of PD result from the presence of vineyards and urban lands and/or the absence of woodland.

Although PD was more common in vineyards adjacent to riparian woodland, the concomitant absence of PD from the majority of such vineyards (63%) suggests that either not all riparian woodland contributes to PD risk or that factors other than proximity to riparian woodland are more important. Given that uninfected vineyards adjacent to riparian woodland were also surrounded by large amounts of riparian and upland woodland, it is possible that riparian woodland in more forested landscapes hosts lower vector densities. Similar results were found in Lyme disease studies, where both vector density and the proportion of infective vectors are higher in smaller forest patches (Allan et al. 2003). Riparian woodland is thought to be preferred BGSS habitat because of the succulent vegetation. In vineyards situated in more forested landscapes, BGSS densities may be controlled by natural enemies. Insect predators are often more vulnerable to loss of natural habitat than are their prey (Hunter 2002). This differential effect of habitat fragmentation on natural enemies and prey would be expected to result in high BGSS densities in riparian woodland adjacent to vineyard only when natural habitat has been lost in the surrounding landscape.

Smaller patches of riparian woodland may be associated with higher PD risk because they may host a higher density of competent reservoirs, compared to larger woodland patches. More expansive woodlands are typically characterized by higher plant diversity, possibly resulting in lower densities of competent reservoirs via a dilution effect (Schmidt and Ostfeld 2001). PD is characterized by several features required for such a relationship to exist between disease risk and host diversity

(Schmidt and Ostfeld 2001): the absence of transovarial transmission (Purcell and Finlay 1979), a generalist vector (Hewitt et al. 1949), and high variation in reservoir competence (Purcell and Saunders 1999). Furthermore, reservoir hosts that harbor some of the highest reported pathogen titers include the non-native species, *Rubus discolor* and *Vinca major* (Baumgartner and Warren 2005). Given their invasive nature, it is possible that remnants of riparian woodland have a higher frequency of such hosts and, therefore, increase the risk of PD in adjacent vineyards. Alternatively, more expansive woodland may be associated with lower PD risk because it decreases the spread of infective BGSSs.

Temperature is a potentially confounding variable in our study. Grapevines are more likely to recover from infection by *Xf* in regions with colder winters (Feil et al. 2003). Thus, if colder vineyards were more likely to be situated in landscapes dominated by woodland, winter temperature might have explained the relationship between woodland and PD. We found the opposite pattern; colder vineyards were more likely to be infected with PD and were more likely to be in landscapes with low amounts of woodland. Since our results are limited to correlative relationships among these factors (not causal relationships), we can only conclude that the relationship between PD and winter temperature may be more complex than previously thought. It is possible that factors other than winter temperatures, that are also correlated with the amount of nearby woodland, are responsible for increased risk of PD.

From an epidemiological perspective, our findings suggest that both local and remote factors have interactive effects on the risk of PD. The occurrence of PD is not correlated with the presence of an adjacent riparian woodland unless the surrounding landscape is also dominated by vineyards or urban lands. This complicates management decisions, which are targeted at the vineyard scale. If vegetation types and their proportions within 2 km of a vineyard have significant effects on PD, then control practices focused on the vineyard and its immediately surrounding area have to counteract these distant factors.

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Table 1. Results from multiple binary logistic regression of the relationship between PD occurrence, adjacent vegetation type, and distant vegetation type (within 0.5, 1, 1.5, and 2 km of each vineyard site).

Continuous independent variable						Likelihood ratio test of significance of continuous independent variable		Type of adjacent vegetation (riparian vs. non-riparian)			Overall model	
Variable	Radius (km)	Nagel-kerke R^2	Parameter estimate*	Wald	***P***	χ^2	***P***	Parameter estimate	Wald	***P***	χ^2	***P***
Riparian	0.5	0.14	-8.0	1.0	0.3	3.6	0.06	2.2	3.5	0.06	4	0.1
Riparian	1.0	0.27	-37.4	3.9	0.049	5.1	0.02	3.0	5.3	0.02	8	0.02
Riparian	1.5	0.27	-42.7	3.7	0.05	5.2	0.002	2.6	5.5	0.02	12	0.003
Riparian	2.0	0.22	-36.3	2.8	0.09	1.1	0.029	2.2	5.0	0.03	7	0.04
Upland	0.5	0.29	-5.6	3.6	0.06	5.2	0.02	2.1	5.0	0.03	9	0.01
Upland	1.0	0.31	-5.3	4.1	0.04	6.5	0.01	2.3	5.5	0.02	10	0.008
Upland	1.5	0.31	-5.1	4.2	0.04	6.5	0.01	2.4	5.5	0.02	9	0.009
Upland	2.0	0.27	-4.6	3.7	0.05	5.8	0.02	2.4	4.9	0.03	8	0.02
Vineyard	0.5	0.33	5.4	5.4	0.02	9.6	0.002	2.6	5.8	0.02	10	0.006
Vineyard	1.0	0.39	6.0	6.6	0.01	10.0	0.002	2.6	5.8	0.02	12	0.002
Vineyard	1.5	0.41	6.3	7.0	0.008	9.4	0.002	2.8	5.9	0.02	13	0.001
Vineyard	2.0	0.40	6.7	6.2	0.01	7.3	0.007	3.1	5.6	0.02	13	0.002
Urban	0.5	0.19	4.9	2.2	0.14	2.4	0.12	1.9	4.4	0.05	5	0.07
Urban	1.0	0.40	20.0	4.4	0.04	9.9	0.002	3.8	5.1	0.02	13	0.002
Urban	1.5	0.51	26.8	5.6	0.02	14.2	0.0002	4.7	6.0	0.01	17	0.001
Urban	2.0	0.51	25.0	6.9	0.008	14.3	0.0002	4.0	6.3	0.01	17	0.001

*Negative parameter estimate, likelihood of PD decreases with increasing proportions of the vegetation type; positive parameter estimate, likelihood of PD increases with increasing proportions of the vegetation type.

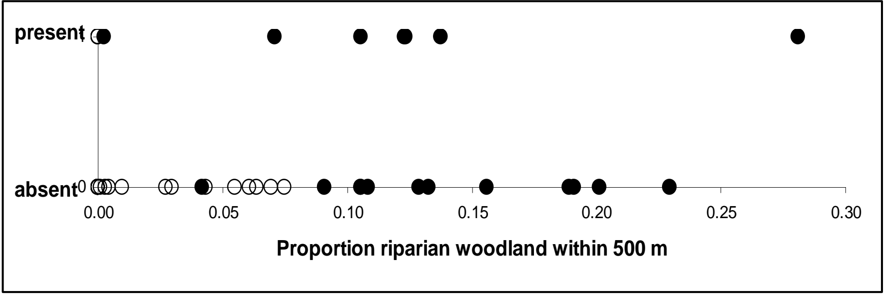


Figure 1. Relationship between PD, type of adjacent vegetation, and the proportion of riparian woodland within 500 m. Closed circles, vineyards adjacent to riparian woodland; open circles, vineyards adjacent to urban land or other vineyards; y-axis, PD presence/absence. A similar pattern was found for the proportion of riparian woodlands within 1, 1.5, and 2 km (*data not shown*).

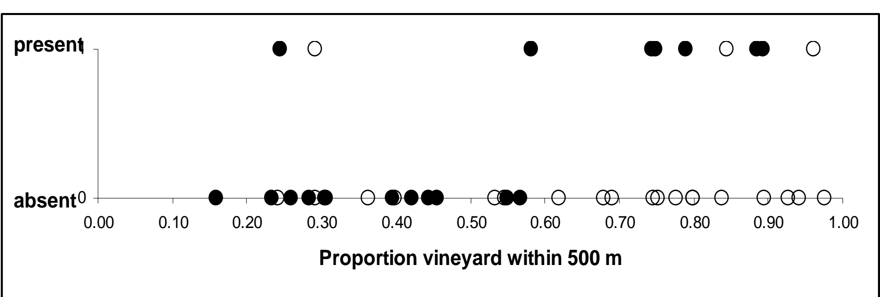


Figure 2. Relationship between PD, type of adjacent vegetation, and the proportion of vineyards within 500 m. Closed circles, vineyards adjacent to riparian woodland; open circles, vineyards adjacent to urban land or other vineyards; y-axis, PD presence/absence. A similar pattern was found for the proportion of vineyards within 1, 1.5, and 2 km (*data not shown*).

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

Project Leader:
Douglas Cook
Department of Plant Pathology
University of California
Davis, CA 95616
drcook@usdavis.edu

Collaborators:
Francisco Goes da Silva
Department of Plant Pathology
University of California
Davis, CA 95616
(fdgoesdasilva@ucdavis.edu)

Hong Kyu Choi
Department of Plant Pathology
University of California
Davis, CA 95616
(hchoi@ucdavis.edu)

Alberto Iandolino
Department of Plant Pathology
University of California
Davis, CA 95616
(Currently with Monsanto Corp.)

Reporting Period: The results reported here are from work conducted July 2005 through September 2006.

ABSTRACT

Susceptible *Vitis vinifera* responds to *Xylella* infection with a massive re-direction of gene transcription involving >800 genes with strong statistical support. This number is increased from previous estimates based on use of a more sensitive and robust statistical method known as linear models for microarray data (LIMMA). The transcriptional response to *Xylella* infection is characterized by increased transcripts for phenlypropanoid and flavonoid biosynthesis, ethylene production, adaptation to oxidative stress, and homologs of pathogenesis related (PR) proteins, and decreased transcripts for genes related to photosynthesis. A survey of 22 transcripts by means of *in situ* hybridization reveals that a majority of transcriptional activity is associated with phloem and cortical tissues, consistent with the presence of the pathogen in adjacent xylem elements. DNA sequence analysis of regions 5' to the transcription site for ~200 differentially expressed genes provides a rich source of new gene promoters and the possibility of *in silico* analysis of regulatory cis-elements.

In addition to highlighting potential metabolic and biochemical changes that are correlated with disease, the results suggest that susceptible genotypes respond to *Xylella* infection by induction of a limited, but apparently inadequate, defense response. We have also tested the hypothesis that Pierce's disease results from pathogen-induced drought stress. We compared the transcriptional and physiological response of plants treated by pathogen infection, low or moderate water deficit, or a combination of pathogen infection and water deficit. Although the transcriptional response of plants to *Xylella* infection was distinct from the response of healthy plants to moderate water stress, we observed synergy between water stress and disease. In particular, water stressed plants exhibit a stronger transcriptional response to the pathogen. This interaction was mirrored at the physiological level for aspects of water relations and photosynthesis, and in terms of the severity of disease symptoms and pathogen colonization, providing a molecular correlate of the classical concept of the disease triangle.

INTRODUCTION

All organisms adapt to external stressors by activating the expression of genes that confer adaptation to the particular stress. In the case of Pierce's disease, such genes are likely to include those coding for resistance or susceptibility to *Xylella fastidiosa (Xf)*.

Genomics technology offers an opportunity to monitor gene expression changes on a massive scale (so-called "transcriptional profiling"), with the parallel analysis of thousands of host genes conducted in a single experiment. In the case of Pierce's disease of grapes, the resulting data can reveal aspects of the host response that are inaccessible by other experimental strategies. In May of 2004, the first Affymetrix gene chip was made available for public use, with ~15,700 *Vitis* genes represented. This gene chip has been developed based primarily on collaboration between the Cook laboratory and researchers at the University of Nevada-Reno (Goes da Silva et al., 2005). With the arrival of the Affymetrix gene chip, we are poised to make a quantum leap in the identification of host gene expression in response to *Xf*.

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

Three co-lateral benefits from the identification of pathogen-induced genes are: (1) the promoters for such genes are candidates to control the expression of transgenes for resistance to Pierce's disease, (2) the protein products of induced genes may have roles in disease resistance, and (3) knowledge of host gene expression can be used to develop improved diagnostic assays for disease. In a related project, we are currently characterizing pathogen-responsive promoters, which will facilitate testing of candidate genes for resistance phenotypes.

OBJECTIVES

1. Identify genes and gene pathways in susceptible *Vitis vinifera* correlated with *Xylella infection*: (a) identify *Xylella*-responsive genes in *V. vinifera*, (b) distinguish early from late gene expression, and (c) determine the correlation between drought stress and Pierce's disease.
2. Determine host genotype affects on gene expression in response to *Xylella* infection: (a) susceptible *Vitis vinifera* compared to tolerant and/or resistant genotypes of *Vitis* species, and (b) comparison of pathogen-induced gene expression with gene expression triggered by salicylic acid and ethylene.
3. Detailed analysis of candidate genes: (a) Real Time PCR to validate candidate genes identified in objectives 1 and 2, (b) Real Time PCR to study kinetics and specificity of the host response in susceptible and resistant genotypes, and (c) in situ hybridization to establish precise location of plant gene expression relative to bacterial infection.

RESULTS

Objective 1 Activities

Microarray experiments under Objective 1 were completed in the prior year of this project. In the current project period we have reanalyzed the Affymetrix data set by a more robust and sensitive statistical approach known as LIMMA (Smyth 2005). Figures 1 and 2 present the overall data in the context of a Boolean diagram and 2-dimensional hierarchical heat map, respectively. Briefly, we have identified 883 differentially expressed genes, of which 448 are up-regulated and 435 are down-regulated. We are currently annotating this expanded gene set by means of MapMan and AraCyc tools, so that genetic and biochemical pathways are more readily inferred from the data.

Figure 1.

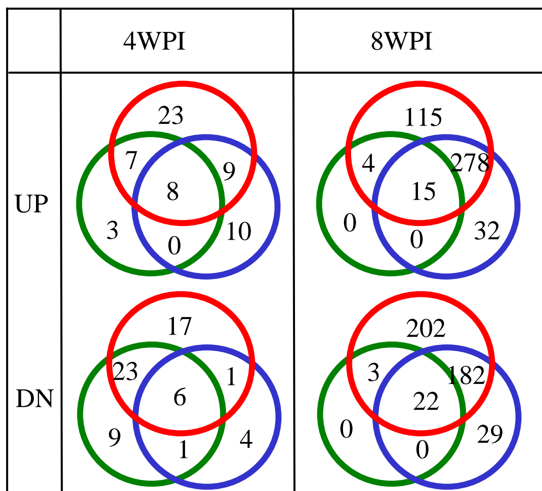


Figure 2.

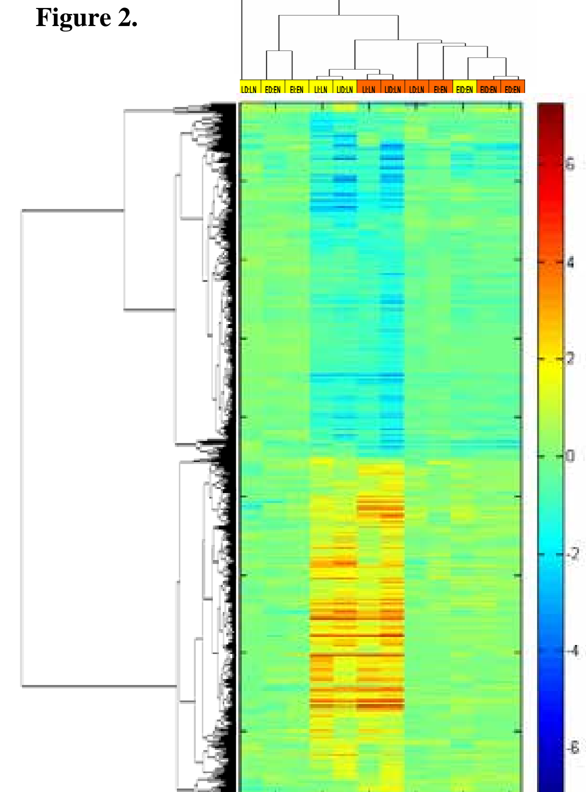


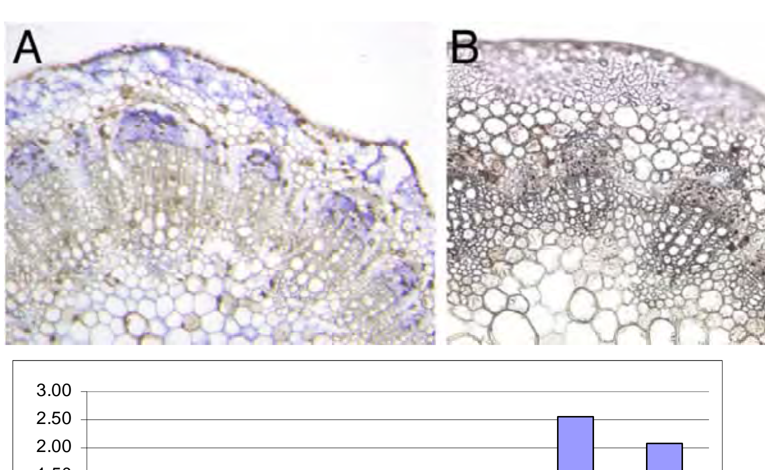
Figure 1. Boolean diagram of 883 differentially regulated genes. WPI, weeks post inoculation; UP, up regulation; DN, down regulation; O, drought; O, infection; O, drought + infection.

Figure 2. 2-D cluster and heat map of LIMMA pairwise comparisons for 883 genes. The horizontal axis presents experiments by treatments (drought, infection, infection + drought, healthy well watered). The vertical axis presents hierarchical clustering of 883 differentially expressed genes. Colors in the heat map correspond to levels of gene induction/repression, with red-yellow representing induced transcripts and blue representing repressed transcripts. Lanes 4-7 correspond to genes induced or repressed in infected symptomatic plants, with or without water deficit.

Within the context of the recent LIMMA analysis, we have determined that less than 4% of the genes influenced by *Xf* infection are also influenced by water deprivation. This limited overlap suggests that the host response to *Xylella* is substantially dissimilar to a response to water stress. Among the *Xylella*-responsive genes, 50 genes exhibited significant transcriptional induction or repression within four weeks of infection, considerably in advance of symptom development. We are particularly interested in using real time RT-PCR to determine whether any of these 50 genes may represent rapid responses to the presence of the pathogen. Major categories of early genes include cell wall modifying enzymes/proteins, proteins involved in production of the stress hormone jasmonic acid, pathogenesis-related (PR) proteins, enzymes of the flavonoid biosynthesis pathway, and regulatory proteins involved in gene transcription and protein turnover. The largest category of down-regulated early genes is implicated in photosynthesis.

Objective 2 Activities
To test the effect of host genotype on gene expression, we conducted a replicated greenhouse experiment involving susceptible plants of *Vitis vinifera* Cabernet Sauvignon and reportedly tolerant hybrids (Blanc du Bois, Champanel, and Black Spanish). Four replicates of each genotype were inoculated with the Fetzer strain of *Xf* and petiole and leaf samples were collected at 2, 4, 8 and 12 weeks post inoculation. Contrary to expectations, severe symptoms developed in Champenele and Black Spanish vines, while only limited symptoms were evident in Blanc du Bois. Petioles are currently being analyzed to determine (1) the extent of pathogen movement in each variety over time, and (2) host gene expression using real time RT-PCR. Depending on the results of these ongoing analyses, we will determine whether to use to the collected leaf samples for Affymetrix profiling experiments.

Objective 3 Activities
24 candidate genes were selected from the Affy chip analysis and used to develop a RealTime PCR assay; this significantly extends our previous RealTime data involving 4 genes. The assay was used to validate results from the Affy chip, especially with respect to quantitative estimates of gene expression. The specificity of host gene expression was addressed by RealTime PCR experiments on field grown plants. *In situ* hybridization experiments were used to evaluate the spatial distribution of transcripts in petiole and leaf samples, with the majority of 22 genes tested revealing transcript accumulation in phloem and cortical tissue, as shown by example in Figure 3.



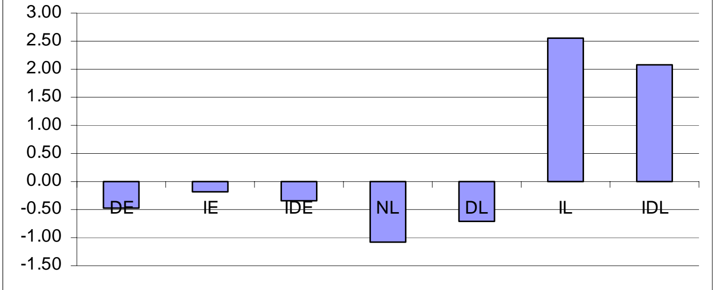


Figure 3. *In situ* hybridization analysis and Affymetrix expression data for a *Xylella*-induced thaumatin transcript.

Panel A, transcript is evident as blue coloration in phloem and cortical tissue of an infected petiole.
Panel B, limited transcript is evident in the cortical tissue of a non-infected petiole.
Panel C, transcript abundance in leaves determined by means of Affymetrix microarray analyses. Scale represents log2 values for gene expression. DE, early drought; IE, early infection; IDE, early drought + infection; NL, non-infected late; DL, late drought; IL, late infection; IDL, infection + drought late. The early time point corresponds for 4 weeks post inoculation (prior to symptom development), while the late time point corresponds to 8 weeks post inoculation (subsequent to symptom development).

Promoter analysis of *Xylella*-induced genes

Promoter sequences for 240 *Xylella*-induced transcripts are being isolated by means of BLASTN analysis against the *Vitis vinifera* whole genome shotgun dataset. In an initial analysis, transcripts for 32 genes were used used to query the dataset, yielding promoter fragments for 30 genes of which 25 promoters contained 1-15kbp of sequence 5' of the translation start site. BLAST analysis with a larger set of the 240 most induced/repressed transcripts is currently underway. Ultimately we intend to query all ~800 *Xylella*-induced transcripts for 5' promoter sequences, producing a sizeable dataset for mining putative cis-regulatory elements and promoter selection.

CONCLUSIONS

We have identified numerous genes where expression is induced strongly in diseased tissue. The largest fraction of the *Xylella*-responsive transcriptome is synergistically modified in plants that are doubly-treated by pathogen infection and moderate drought stress. One important class of synergistically up-regulated genes encode enzymes of the flavonoid biosynthesis pathway, while the predominant class of synergistically down regulated are from the photosynthesis pathway. A smaller fraction of *Xylella*-responsive transcripts are responsive to the pathogen, but apparently not sensitive to water status. Many of these later genes are annotated as PR proteins implicated in host defense. Taken together, the results are consistent with the existence of two distinct classes of transcriptional response in grapes to *Xylella*. One response is sensitive to plant water status and results in redirection of flavonoid synthesis and photosynthesis genes, and one response is independent of plant water status leading to the activation of defense-related transcripts. Although we observed limited overlap in the genes induced in response to moderate drought stress and the genes induced in diseased tissue, we cannot rule out the possibility that a more severe drought stress may lead to an increase in the coincidence of Pierce's disease and drought-associated gene expression.

Real time RT-PCR with 24 candidate genes was used to evaluate gene expression as a check of the Affymetrix microarray data. The RealTime data confirm the major conclusions drawn from the Affymetrix GeneChip, including the correlation between pathogen infection and gene expression, and the synergisitc interaction between infection and water deficit as it relates to the "strength" of gene induction or repression. Genes identified in the Affymetrix microarray analysis were also subject to *in situ* hybridization analysis of petiole tissue. The results indicate that the majority of *Xylella*-induced transcripts exhibit up-regulation specifically in phloem and cortical tissues. Current work focuses on localizing gene expression in infected and non-infected leaf tissue.

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

Project Leaders:

David Gilchrist
Department of Plant Pathology
University of California
Davis, CA 95616
dggilchrist@ucdavis.edu

Douglas Cook
Department of Plant Pathology
University of California
Davis, CA 95616
drcook@ucdavis.edu

James Lincoln
Department of Plant Pathology
University of California
Davis, CA 95616
jelincoln@ucdavis.edu

Reporting Period: The results reported here are from work conducted October 1, 2005 to September 20, 2006.

ABSTRACT

Several projects working toward understanding the genetic basis for susceptibility or resistance at the molecular level in grape to Pierce's disease need to test the ability of candidate genes to alter disease progression or activity of *Xylella fastidiosa (Xf) in planta*. A major limitation to developing assays to test candidate genes is the ability to regulate expression of these genes in time and space relative to the presence of the pathogen. Currently, we are able to express any candidate transgene constitutively and at high levels but cannot modulate gene expression in relation to pathogen presence. The goal of this research is to clone and characterize unique DNA sequences from grape that specifically regulate the expression of grape genes in tissues that are infected with *Xf*. The result will be the delivery of one or more *Xylella*-responsive promoters from grape to drive the bioassay of any candidate gene at locations where the bacteria reside or where bacterial signals extend. The promoters also would be capable of either increasing or suppressing the expression of a gene of interest. Grower acceptance as well as enabling the most subtle level of transgene expression requires that these genes be expressed only when and where they are needed.

INTRODUCTION

Among the potential solutions to Pierce's disease (PD) in grapes are approaches based on gene transfer technology. One research priority identified in 2004 by the PD/GWSS Program was the need to identify, clone and characterize unique DNA sequences that specifically regulate the expression of grape genes in tissues that are infected with *Xylella fastidiosa (Xf)*. This means the candidate genes driven by such promoters will be off (not expressed) and only on (expressed) when the bacteria or their secreted signals are present in the vascular system of the grape plant. Emphasis was placed on the urgency and practical utility of isolating promoters of PD responsive genes. Transgenic technology offers the possibility of modifying specific traits (e.g., PD susceptibility) based on the introduction of novel genes. One of the major bottlenecks in the genetic engineering of grape (or any plant) is the absence of suitable promoters - sequences that regulate gene expression in particular tissues (e.g., vascular tissue) or in response to particular situations (e.g., sharpshooter feeding or *Xylella* infection). In the absence of tissue or response-specific promoters, transgenic strategies for control of PD can use only so-called constitutive promoters. By definition, constitutive promoters are expressed in all cells all the time. By contrast, *Xylella*-inducible promoters have the potential to confer transgene expression at the time and location of bacterial infection, thus delivering therapeutic proteins more precisely to their intended site of action. In addition to increasing the specificity of transgene expression, such promoters should reduce the possibility of unintended side affects in non-target tissues.

In addition to their utility for engineering PD resistance in grape, the advent of *Xf*-induced reporter gene expression would provide an extremely powerful tool to examine host responses in their intact cellular and tissue context. With such tools, it should be possible to examine the chemical and/or physical cues from the insect or pathogen that trigger host gene expression and the deleterious effect of the disease. For example, we have recently determined that host gene expression is induced specifically in live cells of the phloem (Cook 2005). It remains uncertain, however, whether gene expression occurs only in phloem tissue that borders infected xylem elements, or whether bacterial infection can induce host gene expression at a distance. Promoter-GFP fusions being developed in this project should help answer such questions.

OBJECTIVES

1. Produce stable transgenic grape plants containing promoter-GFP fusions that respond to *Xylella* infection. At this point 4 constructs bearing promoters derived from genes induced in *Xf* infected grape but not in healthy grape tissue will be transformed into Thompson Seedless plants by the UC Davis Plant Transformation Facility. There are four genes, G8946, G9353 G7061and G7172 to initially be expressed transgenically. These genes were shown to be induced in the phloem of infected petioles and leaves, adjacent to sites of probable *Xf* infection in the xylem as indicated in the 2005 symposium report (Gilchrist et al. 2005). Expected delivery of the first set of transgenics is November 2006.
2. Develop a rapid *in planta* assay to characterize promoter-GFP expression in a series of independent grape transformants derived from each promoter fusion via detached leaf/branch bacterial uptake system. The goal is to identify a series of independent lines for each fusion where transgene expression is strong and reproducible. GFP expression will be monitored in both excised leaves and branches (described below), as well as in stem-inoculated whole plants. The progression of induction will be assessed qualitatively in relation to location bacteria monitored by RT-PCR of both the promoter expression and the presence of the bacterium.

3. Conduct detailed analysis of promoter-GFP expression, with the following specific goals in mind: (a) Determine the extent to which the transgenic promoter-GFP fusions reproduce the patterns of expression for the endogenous promoters. Using promoter-GFP fusion constructs, confocal imaging will be used to assess temporal and spatial aspects of G8946-GFP induction at the protein level, while Taqman RealTime PCR assays will be run in parallel to quantify transcript levels from both the native genes as well as from the introduced promoter-GFP constructs. (b) Define temporal and spatial aspects of promoter-GFP expression, especially as a function of the location and quantity of bacterial colonization in the vascular tissue. We already know that each of the native genes are induced strongly in the phloem of infected leaves and petioles, but it is uncertain, for example, if there is a requirement for bacterial colonization in the physically adjacent xylem or, alternatively, if the bacterium can induce host gene expression at a distance. Developing a detailed chronology of bacterial colonization and host gene expression will not only serve to characterize the transgenic promoters, it should also help with development of models for the mechanism by which the plant perceives the bacterium.
4. Continue validation of an additional 24 genes, which also appear to be expressed only in grape tissues infected with *Xf*. This will be done using the same procedures as described below for the first four genes.

RESULTS

Promoter identification

We have characterized approximately 25,000 grape genes (Cook 2005) and found a subset of 448 genes which are up-regulated specifically in response to *Xf* infection (Gilchrist et al. 2005). Four of these genes, G8946, G9353 G7061, and G7172 were shown by PCR to have expression patterns that are strongly correlated with *Xf* infection in both greenhouse and field-grown grapes (Figure 1). Studies with an Affymetrix GeneChip determined that susceptible *Vitis vinifera* responds to *Xf* infection with a re-direction (both up- and down-regulation) of gene transcription involving over 800 genes. Analysis of 24 genes (from the subset of 448) by *in situ* hybridization established that expression occurs coincident with the presence of *Xf* in the phloem of infected petioles and leaves, adjacent to sites of probable *Xf* infection in the xylem. The Cook lab has recently obtained sequence information 5' of the transcription start for an additional 200 genes (from the subset of 448) and these represent potential differentially expressed promoters.

Promoter isolation and binary vector construction

Bacterial Artificial Chromosome (BAC) clones of *V. vinifera* that contained the four *Xf*-inducible genes and their promoters were used to isolate and sequence the 5' promoter regions in genomic clones based on comparison to full-length cDNA sequences for the respective genes. PCR primers were designed to amplify and clone approximately 1300bp of sequence immediately 5' of the transcription start site, which was predicted based on proximity to the cDNA initiation codon (Cook 2005, Gilchrist et al. 2005).

Leaf and branch inoculation methods

Recognizing the value of having a rapid, laboratory-based assay for host gene expression, we sought to develop a cut-branch and a detached leaf assay that would enable the bacteria to enter and spread though the vascular system in days to a few weeks and then assay for both the location of the bacteria and the relative level of expression of the putative promoters. In these assays, the leaf scorching indicative of Pierce's disease occurs within several weeks in most cases. Briefly, branches of the PD susceptible root stock cv. Freedom were cut under water and allowed to take up *Xf* from 1 mL of a bacteria suspension containing 2×10^7 cfu/ml for 2 hr. Stems inoculated in this manner were placed in water for 2 weeks, after which they were assayed for host gene expression by means of RT-PCR (Ref 4). As shown in Figure 2, genes G8946 and G7061 were strongly and reproducibly induced in stems inoculated with *Xf* well in advance of symptom development, consistent with our previous results using whole plants. Confocal imaging of GFP tagged *Xf* in these same tissues detected only very small amounts of bacteria in the stems (Figure 2 inset of stem cross-section) and none in the leaf lamina where the genes were detected as being expressed, suggesting that the up-regulation of these is highly sensitive to the presence of bacteria and at a distance from where the bacteria were easily detected. We conclude that this detached stem assay can provide a simple and reliable method to monitor *Xylella*-induced gene expression, significantly shortening assay time compared to whole plant assays. We are now attempting to extend the same assays with Thompson Seedless grape explanted tissue.

Branch uptake method

A terminal shoot approximately 60cm long is cut from greenhouse or growth chamber grown grape plants. The shoot is re-cut under water removing an additional 5cm. In a typical assay, approximately ten shoots are placed in a beaker of distilled water containing 2×10^7 *Xf* cells/ mL. Shoots are allowed to uptake the bacteria suspension for 2 to 48 hrs depending on the experiment. During this uptake the beaker is placed in a laminar flow hood to increase transpiration. After the uptake period the shoots are transferred to individual 50 ml glass culture tubes containing distilled water for the remainder of the experiment. Shoots are incubated at room temperature under low intensity fluorescent lights for symptom development within 2-3 weeks. The two genes assayed in the experiment illustrated in Figure 2 were expressed in the stems and in the leaf lamina. GFP-tagged bacteria were visible by confocal microscopy in the cut stem cross-sections but not in the leaf lamina where the genes were expressed. This may reflect the low sensitivity of the confocal assay to detect the bacteria or that the genes are expressed in relation to systemic signals expressed by the bacteria. Clearly, the genes are expressed in asymptomatic tissue, which is extremely encouraging in terms of being able to activate these promoters before bacterial

populations build up and before PD symptoms appear. (Figures 2 and 3).

Leaf uptake method
Young, full-sized, mature leaves were cut from greenhouse or growth chamber grown grape, the petioles re-cut under water and the leaves placed individually in 2ml plastic tubes containing $2x10^7$ *Xf* cells/mL. The remainder of the uptake and incubation was similar to the branch method. Each petiole provides 20 sections for analysis by RT-PCR and confocal microscopy. Macroscopic leaf scorch symptoms appear on cv. Freedom within 2-3 weeks (Figure 4). In this case, the assay is used to detect the induction of G7172 along with detection of the pathogen. Regions m, b, and c were analyzed independently for both G7172 and *Xf*. RT-PCR is much more sensitive for detection of *Xf* than the GFP tagged visualization by confocal microscopy. The results to date indicate that, similar to cut branch uptake, in the detached leaf assay, the G7172 promoter is *Xylella* responsive and appears near where the bacteria could be detected by RT-PCR. This assay is somewhat faster than the branch uptake but the results are similar. In both cases, it appears that, at least for the promoter assays, this method of placing the bacteria in the vascular system and detecting a response to the presence of the bacterial at the level of plant gene expression is valid, whether the expression is uniquely induced or expression is up-regulated.

Whole plant transgenics
During the current project period, promoter-GFP fusions for first four genes are being introduced into the *V. vinifera* cultivar Thompson Seedless. Embryos have been induced from transformed tissue and we anticipate having transformed plantlets for initial assays by November 2006.

CONCLUSIONS

The only effective long term strategies for protecting grape against the impact of *Xf* resident in the vascular system of susceptible grape is to genetically alter the response of susceptible grape tissue to the death induced by plant response to the bacteria. This means expression of introduced resistance genes or genes that block the plant response. The most likely means by which either of these protective measures will take place is though transgene expression. The direct products of this research are the means to express any potential therapeutic transgenes in areas of infection when and only when the bacteria are present. These promoters are one critical tool necessary for genetic resolution of Pierce's Disease.

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FUNDING AGENCIES

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Figure 1. RT-PCR analysis of the G7172 transcript from greenhouse grown grape. RNA in lanes 1 and 3 are from healthy leaves and lanes 2 and 4 are from *Xf*-infected leaves. Actin specific primers used for lanes 1 and 2 as a control and G7172 specific primers in lanes 3 and 4 indicate the presence of the promoter transcript only in the infected plant in the greenhouse grown plants.

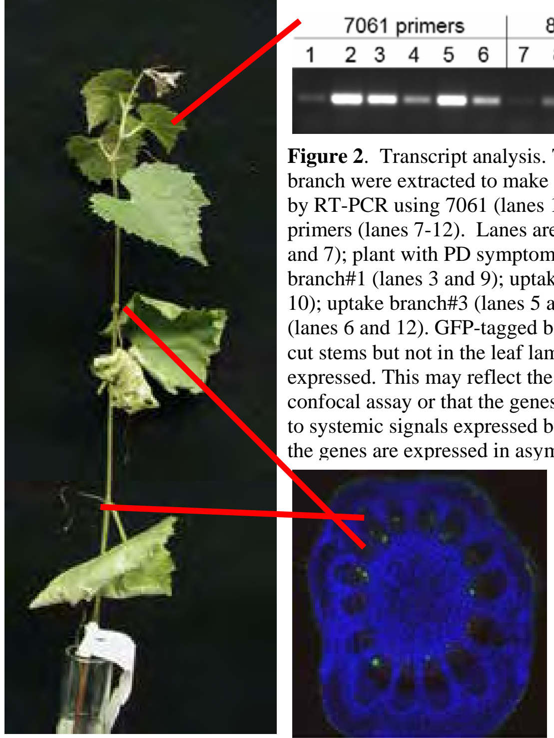

Figure 2. Transcript analysis. Two leaves from each branch were extracted to make RNA that was analyzed by RT-PCR using 7061 (lanes 1-6) or 8946 specific primers (lanes 7-12). Lanes are healthy plant (lanes 1 and 7); plant with PD symptoms (lanes 2 and 8); uptake branch#1 (lanes 3 and 9); uptake branch#2 (lanes 4 and 10); uptake branch#3 (lanes 5 and 11); uptake branch#4 (lanes 6 and 12). GFP-tagged bacteria were visible in the cut stems but not in the leaf lamina where the genes were expressed. This may reflect the low sensitivity of the confocal assay or that the genes are expressed in relation to systemic signals expressed by the bacteria. Clearly, the genes are expressed in asymptomatic tissue.

GFP-tagged *X. fastidiosa* seen in the vascular system following uptake by cut branch but not in the distal leaf lamina

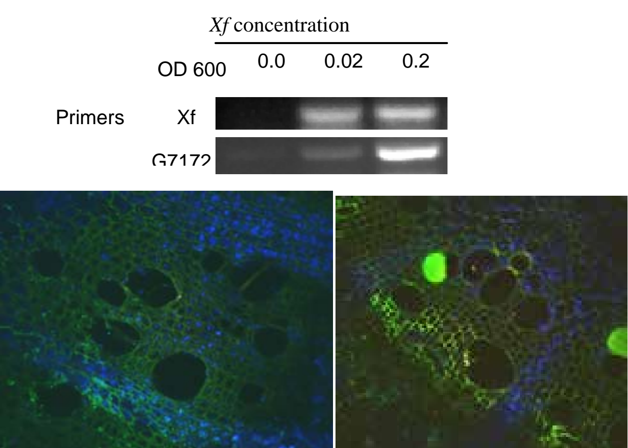

Figure 3. Branch uptake assay for expression of promoter G7172 in the presence and absence of *X. fastidiosa*, when sampled in leaf lamina. GFP-bacteria were visible 5-7 cm in the stem but only RT PCR detected the bacteria in petioles and leaf lamina in the entire branch by 3 days. Hence, the bulk of the bacteria were confined to the stem but cells had moved throughout the plant. The G7172 transcript was expressed in all tissues near where the bacteria could be detected by PCR but not in un-inoculated tissues. Expression of 7172 also appears to be positively correlated to the amount of bacteria in the tissue that was assay for the transcript.

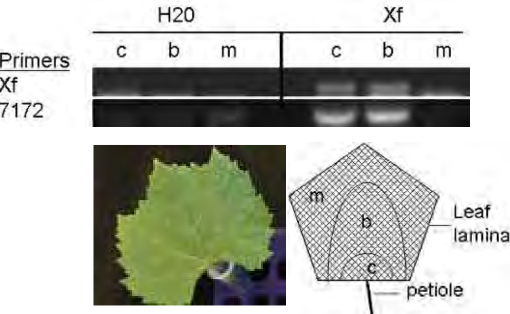

Figure 4. Example of detached leaf uptake assay for gene expression in the presence of *X. fastidiosa*. Bacteria at 10^7 CFU were introduced into the cut petiole followed by RT-PCR detection of targeted gene transcripts and the bacteria in the same tissue samples. In this case, the assay is used to detect the induction of G7172 along with detection of the pathogen in association with the bacterial but not in water control leaves. Regions m, b, and c were analyzed separately for both G7172 and *X. fastidiosa*; demonstrating that the G7172 promoter is Xylella responsive.

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

Project Leaders:

David Gilchrist
Department of Plant Pathology
University of California
Davis, CA 95616
dggilchrist@ucdavis.edu

James Lincoln
Department of Plant Pathology
University of California
Davis, CA 95616
jelincoln@ucdavis.edu

Reporting Period: The results reported here are from work conducted October 1, 2005 to September 20, 2006.

ABSTRACT

Several relatives of grape and other asymptomatic host plants can harbor high titers of *Xylella fastidiosa (Xf)* without exhibiting symptoms of Pierce's disease (PD). The basis of what is a genetic difference is unknown. We have established that leaf scorch PD symptoms in grape result from apoptosis or programmed cell death (PCD). Clearly, *Xf* does not have to kill in order colonize the vascular system leaving this endophytic association asymptomatic. We have identified from a cDNA library screen several grape genes that block PCD when over-expressed in grape tissue. Preliminary experiments indicate that one of these genes, VVPR1A, is expressed or up-regulated in situations in which PCD is blocked in humans, nematodes, hookworms and several plant species. This gene also is upregulated in the presence of *Xf*. We are testing the hypothesis that over expression of one or more of the 12 genes recovered in the anti-apoptotic screen, with an initial focus on VVPR1A, can block both PCD induced by *Xf* and disease symptoms associated with *Xf*. Preliminary results reported here indicate that grape plants over expressing VVPR1A, metallothionein, or a *Meloidogyne incognita* upregulated gene can block symptoms in a cut branch assay. Experiments with whole transgenic plants inoculated with *Xf* are in progress to assess the movement of bacteria, the induction of *Xf* responsive grape genes and if symptoms of PD are affected by the anti-apoptotic transgenes.

INTRODUCTION

Genetic strategies for disease suppression and information characterizing the bacterial-plant interaction are high priority areas in the Pierce's Disease/Glassy-winged Sharpshooter (PD/GWSS) Research Program and the National Academies report. Disease is defined as plants expressing several symptoms resulting from cell death (leaf scorch) or changes in tissue differentiation (green islands). The goal of this project is to identify novel genes from cDNA libraries of either grape or heterologous plants that, when over expressed in grape, will prevent infection, spread or symptom development due to the presence of *X. fastidiosa* (*Xf*) in the xylem (Gilchrist and Lincoln 2004). Currently, several laboratories including our own have begun to carry out systematic studies of the molecular basis of susceptibility of plants to a range of pathogens including bacteria and fungi. The objective of these studies is to identify genetic or chemical approaches that have the potential to block susceptibility in grape to PD, thereby effectively creating cells that are refractory or insensitive to the signals expressed by pathogens that lead to susceptibility. Recent published information from our laboratory established that susceptibility of several plants to a range of pathogens depends on the ability of the pathogen to directly or indirectly trigger the activation of genetically determined pathways leading to apoptosis or programmed cell death (PCD) (Gilchrist 1998, Harvey et al. 2006, Lincoln et al. 2002, Richael et al. 2001). These discoveries parallel investigations now widely reported and accepted in human medicine whereby genes, signaling pathways and chemical signals expressed by animal pathogens initiate or block infection by activating or blocking apoptosis through constitutive genes or signaling pathways present in all cells. These studies are the basis for extensive searches for apoptosis-based therapeutic approaches and agents in plants as well as animals (Greenberg and Yao 2004, Nicholoson 2000).

Dr. Tom Rost reported, both in his 2005 PD Symposium address and his annual report (Rost et al. 2005) that *Xf* moves effectively and quickly through the plant following inoculation or uptake. However, the GFP-tagged bacteria have limited dispersal in the leaf lamina expressing the marginal leaf scorch symptoms of PD. These data, obtained in part using the confocal system in our laboratory, are consistent with our own observations using GFP-tagged *Xf* to visualize the bacteria in vascular elements connected to tissue showing the marginal scorch symptoms. These data suggest two key things. First, the cell death symptom is the result of mobile signals moving from the bacteria to cells distal to the bacteria and that strategies effective in blocking the death pathways will most likely consign the bacteria to an endophytic existence in the vascular system. Consistent with this hypothesis, Dr. Steven Lindow pointed out in his 2005 PD Symposium address that *Xf* is an effective endophyte in many asymptomatic plants and can be said to be "an endophyte gone bad in susceptible grape plants". It is a fact that several *Vitis* species, including wild grape, tolerate extremely high titers of *Xf* but remain asymptomatic, while many genotypes of cultivated grapes express PD symptoms at the same or lower titers. Clearly the presence of *Xf* in the xylem is not the single determining factor in disease. In PD and many other bacterial diseases, bacteria live predominantly as endophytes or epiphytes and only occasionally as pathogens. Susceptibility of the host tissues is determined by sensitivity to the presence of the bacterium and the signals expressed by the bacteria leading to PCD. Using genetic or chemical approaches to block PCD is an viable approach in both animal and plant disease prevention (Greenberg and Yao 2004, Harvey et al. 2006).

OBJECTIVES

1. Produce transgenic grape plants over-expressing candidate anti-apoptotic plant genes obtained from functional cDNA library screens as identified in an earlier project.
2. Evaluate these 12 putative anti-apoptotic plant genes in grape for effect on bacterial population dynamics, movement in the xylem, changes in gene expression and on PD symptoms when the candidate genes are expressed constitutively at high levels.. These assays will use confocal microscopy, GFP-tagged bacteria and RT-PCR.
3. Evaluate the stem and leaf uptake procedures developed recently in our laboratory to enable rapid assessment of grape gene expression in the presence of the PD bacterium. The experiments also will use coincidental transcriptional profiling as a measure of similarity of changes in gene expression between infected whole plants and bacterial uptake assays.

RESULTS

Produce transgenic grape plants over-expressing candidate anti-apoptotic plant genes obtained through cDNA library screens

Stable full plant grape transformations of susceptible Thompson Seedless and cv. Freedom are done by the Ralph M. Parsons Foundation Plant Transformation Facility. We anticipate 10-20 transgenic plants of each construct to be evaluated. The cDNA inserts from the library screens are cloned into the binary vector B5 for direct transformation into the *A. tumefaciens.* These plants will be grown for 2 months, ramets made from cuttings, and leaves assayed for the transgene expression after cut leaf and branch uptake with bacteria (see next section for methods relating to the cut branch and leaf assays). Both Chardonnay and cv. Freedom transformations were initiated in the grant proposal but only Freedom transgenics survived in the first round. The cv. Freedom, a common rootstock, is highly susceptible to PD with the same symptoms as Chardonnay and Thompson Seedless. The Transformation Facility has successfully transformed Thompson Seedless recently and is now confident that most of the transgenics of this cultivar can be delivered by November 2006. Currently, we have begun testing lines transgenic for genes CBPR1A, CB390 and CB456. Northern analysis confirmed over-expression of the metallothionein, the *Meloidogyne incognita* upregulated gene, and the VVPR1A transgenes in the transgenic Freedom lines (Table 1). Prior to initiating full plant transformations, all of these genes in Table 1 were confirmed to block programmed cell death in transgenic roots exposed to the apoptotic inducer Fumonisin B1.

Table 1. Plant anti-apoptotic genes, derived from functional screen of cDNA libraries, for transformation into grape plants

Construct	Gene	Source
CBWG8	glutathione-S-transferase	Chardonnay
CB390*#	metallothionein	Chardonnay
CB456*#	Nematode induced gene	Chardonnay
CBWG23#	unknown function	Chardonnay
CBWG29	unknown function	Chardonnay
CBWG33	unknown function	Chardonnay
CBWG71	cytokine-like gene	Chardonnay
CBWG75#	germin-like gene	Chardonnay
CBPR1A*#	VVPR1A	Chardonnay
CBI35	Intron p35 (anti-PCD control gene)	baculovirus
CBP14LD*#	P14 (homolog of PR1A)	tomato
CB376#	Mycorrhizal induced gene	tomato

* Northern positive transgenic plants available at this time in cv. Freedom
# Scheduled to be delivered on November, 2006 as Thompson Seedless transgenic plants

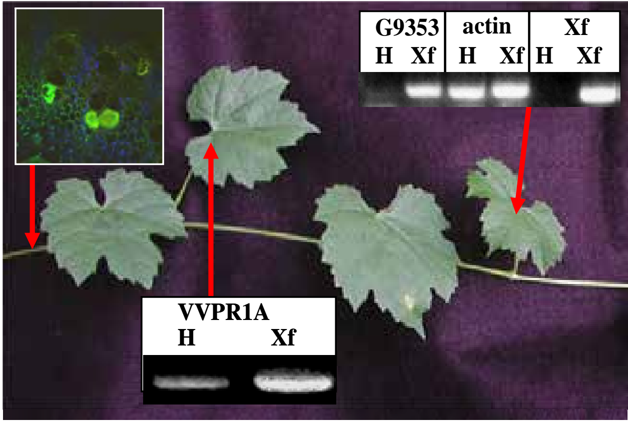

Figure 1. Analysis of transformed and untransformed plants following inoculation with GFP-tagged *X. fastidiosa.*for presence of the bacteria in relation to expression of marker genes and PD symptoms. Plants were analyzed by confocal microscopy and RT-PCR. Both infected and non-infected plants were asymptomatic at the time the assays were done. H=healthy; *Xf* = inoculated.

Evaluate effect of specific anti-apoptotic plant genes in grape on *Xf* and PD symptoms *in planta*.

The initial experiments began with CBPR1A, CB390 and CB456 using transgenic whole plants in the greenhouse, as well as branch and leaf uptake assays; the latter conducted in the laboratory. In the whole plant assays for the first three transgenes, *Xf*-GFP movement and relative concentration were assessed by PCR and confocal microscopy in individual stems, petioles and leaves, beginning with the detached branch and leaf assays (Figures 3 and 4). Initial experiments of limited numbers of stem inoculated primary transgenics in the greenhouse, however did not provide useful data. After waiting 2-3 months it was clear that the *Xf* inoculated control plants had not developed symptoms as expected nor did the transgenic plants. Both sets of plants remained asymptomatic even though the GFP-tagged bacteria were confirmed to be present throughout the plant (Figure 1). In a second inoculation, the control and transgenic plants lost most of their leaves within 3 months after inoculation, in part due to inadvertent boron toxicity in the

first month following inoculation. All current transgenic plants in pots are under a newly installed, controlled drip, irrigation with balanced nutrients and deionized water, ramets of each transgenic are ready for the experiment to be repeated with replications.

Measure the effect of blocking leaf scorch symptoms with anti-apoptotic transgenes on bacterial population and movement *in planta*

Bacterial movement and relative concentration will be monitored by RT-PCR and confocal microscopy using *Xf*-GFP. It is essential to determine the effect of blocking PCD-based symptoms in the transgenic plants on the bacterial multiplication and spread in terms of the overall impact of the transgenes. Utilization of these genes in agricultural situations requires that the impact on the pathogen and the host be quantified. This requires extensive sampling of stem, petiole and leaf tissue of all the transgenic plants. We currently sample individual plants with at least 3 stems per plant using the cut branch uptake method with 50 sites per leaf, 20 per petiole and 20 internode sites and each node on the stem. Preliminary experiments have shown that bacteria can first be detected about 1 week at the base of the leaf in the stem uptake experiments but extensive preliminary time course sampling will be required to determine the range of times needed to establish the most revealing time points in the presence and absence of the anti-apoptotic transgenes in both branch and leaf uptake studies (Figure 1).

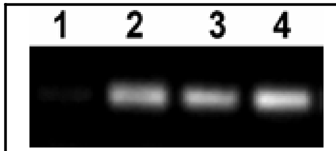

Figure 2. Transcript analysis of branch uptake of *X. fastidiosa*. RT-PCR was performed using G8946 specific primers. Lanes: 1=healthy; 2=PD symptoms; 3 and 4=PR1 transgenic grape.

Determine grape gene expression changes in transgenic compared with non-transgenic plants infected with *Xf*.

Doug Cook previously reported extensive changes in gene expression in *Xf* infected Cabernet Sauvignon on Freedom rootstock by Real-Time PCR followed by expression profiling (Cook 2005). In collaboration with Dr. Cook, we will initially assess the effect of the expression of the anti-PCD genes on a 24 gene subset of the 448 genes he reported to be upregulated only in infected tissue by Real-Time PCR. We also will assess which genetic pathway is affected by the transgenes by difference in expression profiling of inoculated transgenic and non transgenic cohorts on the full set of genes identified by Dr. Cook as differentially expressed in the presence of *Xf*. One of these genes, G8946, was up regulated only in infected stems and expressed only in the phloem and immature xylem cells adjacent to *Xf* in the mature xylem (Gilchrist and Lincoln 2005) and was up-regulated in our cut branch uptake technique described in the following section (Figure 2).

Evaluate the branch and leaf uptake procedures developed recently in our laboratory as surrogate approaches to long term greenhouse or field experiments

The goal is to use rapid response methods to induce and characterize determinants of PD compared with stem inoculation. Demonstration of changes in gene expression in the presence of the bacterial consistent with those recorded under greenhouse or field conditions would at least validate the method for preliminary characterization of plant response to bacteria at the genetic level. The experiments will use coincidental transcriptional profiling as a measure of similarity in transcriptional response of the host tissues relative to the location and appearance of scorch symptoms. In searching for a method to shorten the time from exposure of grape tissue to *Xf* and a measurable plant response, we have explored several *in planta* approaches to introducing the bacteria into the vascular system in a manner that results in changes in host gene expression and the appearance of leaf scorch symptoms. Uptake of *Xf* suspensions into cut grape branches and into cut grape leaves of susceptible grape under our experimental conditions induces typical marginal leaf scorch symptoms of Pierce's disease within 2-4 weeks compared with 12-16 weeks with whole plant stem inoculations in cv Freedom (Figures 3 and 4). Hence, experiments can be replicated many fold by repeatedly treating detached leaves as individual experimental units with clonal genetic identity compared with committing a whole plant to a single assay as one experimental unit. All of the experiments measuring bacterial dynamics and changes in gene expression in infected tissue are tedious requiring extensive serial and time course sampling and analysis. Two methods evaluated in the past few months have proven very useful in this regard are:

Figure 3. Branch uptake. Branches of transgenic grape were allowed to uptake *Xf*. After 3 weeks PR1 (A) and metallothionine (B) transgenics show reduced symptoms compared to GFP transgenic (C).

Branch uptake method. A terminal shoot approximately 60cm long was cut from greenhouse or growth chamber grown grape plants. The shoot is re-cut under water removing an additional 5cm and placed in a beaker of distilled water containing $2x10^7$ *Xf* per ml with mixing in a hood with air flow to increase transpiration. Bacteria were taken up for 2 to 48hrs depending on the experiment, then transferred to individual 50 ml glass culture tubes containing distilled water under low intensity fluorescent lights for symptom development within 2-4 weeks (Figure 3). Initial experiments confirmed that the bacterial taken up through the cut surface move into the vascular system resulting in cell death characteristic of PD leaf scorch. The plants transgenic for VVPR1A and

metallothionein exhibited little or no cell death over the duration of the experiment compared with the control branches. Analysis of the coincident changes in gene expression by transcriptional profiling between control and transformed plants is in progress.

Leaf uptake method. Young, full-sized, mature leaves were cut from greenhouse or growth chamber grown grape, the petioles were re-cut under water and the leaves placed individually in 2ml plastic tubes containing $2x10^7$ *Xf* per mL. The remainder of the uptake and incubation was similar to the branch method. Each petiole provides 20 sections for analysis by RT-PCR and confocal microscopy. Macroscopic leaf scorch symptoms appear on cv. Freedom within 2-4 weeks (Figure 4). Initial transcript analysis indicates that *Xylella*-induced genes are up-regulated in these uptake methods.

Figure 4 Leaf uptake. Leaves of untransformed (A) develop symptoms 2 weeks after uptake of *X.f.* while VVPR1A (B) and CB390 (C) transgenics are symptom free for 1 month.

CONCLUSIONS

Several relatives of grape and other asymptomatic plants can harbor high titers of *Xf* without exhibiting PD symptoms. We have established that leaf scorch PD symptoms in grape result from apoptosis or programmed cell death (PCD). Clearly, *Xf* does not have to kill in order colonize the vascular system. So, a key question addressed by this research is; are there genes in the plant that respond by triggering programmed cell death in certain grape genotypes, can this response be blocked genetically, and, if so, does this then allow the bacteria to return to the endophytic state, leaving the plant otherwise unaltered and disease symptom free? We have identified from a functional cDNA library screen several grape genes that block PCD when over-expressed. Preliminary experiments indicate that one of these genes, VVPR1A, is expressed in situations in which PCD is blocked in humans, nematodes, hookworms and several plant species. We are testing the hypothesis that over expression of genes like VVPR1A can block both PCD induced by *Xf* and disease symptoms associated with PD in both detached branch or leaf uptake assays and in inoculated whole plants.

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FUNDING AGENCIES

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THE EFFECT OF DORMANT SEASON SURVIVAL OF *XYLELLA FASTIDIOSA* IN GRAPEVINES ON PIERCE'S DISEASE EPIDEMICS IN CALIFORNIA

Project Leader:
Barry L. Hill
CDFA, PDCP
Sacramento, CA 95832
bhill@cdfa.ca.gov

Cooperators:
Jennifer Hashim
UC Cooperative Extention
Kern County, CA

William Peacock
UC Cooperative Extention
Kern County, CA

Reporting Period: The results reported here are from work conducted May 2005 to September 2006, the second year of a two-year project. The project is not yet complete, as there remains field data to be obtained in October and November 2006.

ABSTRACT

The two California Pierce's disease (PD) epidemics associated with population outbreaks of the glassy-winged Sharpshooter (GWSS), at Temecula in the mid-1990s and in Kern County, peaking in 2002, differed in the number of vineyards lost and the grapevine varieties affected. In Temecula, almost half of all vineyards of all varieties were lost to PD, whereas in Kern County only the vineyards of two varieties, Redglobe and Crimson Seedless, suffered losses; all the vineyards of the other four varieties were unaffected. A hypothetical explanation of this epidemiological pattern is that in those parts of California where the winters are more severe, dormant-season die-out of *Xylella fastidiosa (Xf)* is more likely, and only the earlier-season inoculations and infections survive the winter. The likelihood of *Xf* die-out is a function of both winter climate and varietal susceptibility. In Kern County, only the most susceptible varieties were affected by secondary (vine to vine) transmission and early season primary transmission (where insect vectors acquire *Xf* from plant sources outside the vineyard) was of little consequence. Through field experiments, this project expands our knowledge of secondary transmission in the southern San Joaquin valley. The benefit to grape producers in this area will be twofold: 1) more accurate assessment of risk of economic loss from PD, and 2) suggestion of new integrated disease-management practices to control PD.

INTRODUCTION

The glassy-winged sharpshooter (GWSS)-associated Pierce's disease (PD) epidemics in Temecula and in Kern County were the first instances of epidemic secondary transmission of PD in California since the Anaheim epidemic of 1885 – 1895. During the intervening 100+ years, losses from PD in California have resulted from primary transmission, and those losses have been economically manageable in most areas. In the General Beale epidemic in Kern county (which has a colder winter climate and longer dormant season than Temecula), only a small percentage of the vineyards were lost, and all of the lost vineyards were planted in only two of the six varieties in the area, Redglobe and Crimson Seedless.

The losses to vineyards of the other four varieties were very small—in most cases less than 1 in 10,000 vines. By contrast, all 12 of the Redglobe vineyards monitored in the General Beale area were significantly damaged, with a range of 2% to over 50% of the vines lost (Hashim, *et al*, 2003). Most of these vineyards were ultimately removed.

Grapevines acquire new *Xylella fastidiosa (Xf)* infections either by primary or secondary transmission. Primary transmission occurs when vector insects acquire the bacterium from source plants outside the vineyard, then fly into the vineyard to infect vines. Secondary transmission occurs when vector insects acquire *Xf* from an infected vine within the vineyard and then transmit the infection to other vines, known as vine-to-vine transmission.

The risk associated with these two kinds of transmission differs. The disease and vine loss pattern associated with primary transmission is linear; that is, a relatively constant number of vines per year become infected, so the yearly accumulation of PD vines increases additively and predictably. By contrast, the pattern of yearly accumulation of PD vines associated with secondary transmission is typically logarithmic, increasing as a multiple of the infected source vines that are present, so entire vineyards can be lost within just a few years.

Secondary transmission cannot begin to occur until that time in the growing season when the bacterial cells in diseased vines have multiplied and moved within the vine; the cells travel from the refuge site, where they survived the dormant season, up into the new growth where vector insects can feed and acquire them. Secondary transmission of infection can then continue until the end of the growing season. However, infection does not equal disease. The phenomenon of over-winter curing of *Xf* infections is well-documented in most viticulture areas of California (Fiel *et al*, 2003). Early-season inoculations can result in infections which survive the dormant season and progress to chronic disease and vine death. Conversely, later-season infections do not become sufficiently established to survive the dormant season, and the vines are free of infection the following year (Fiel *et al*, 2003).

In most viticulture areas of California (Napa and Sonoma Valleys, for example), secondary transmission of infection regularly occurs, but it cannot begin early enough in the season for the infection to survive vine dormancy and progress to chronic PD. In these areas, secondary transmission occurs but does not result in disease.

We propose that in the General Beale area, secondary transmission of infection occurred in all varieties, possibly infecting large numbers of vines in every vineyard. The rate of *Xf* multiplication and movement varies within plant hosts (Hill and Purcell, 1995) and among grapevine varieties. In the most susceptible varieties, Redglobe and Crimson, the rate of bacterial multiplication and movement was faster, so the result was that the bacteria had a window of opportunity some time in mid-season when secondary transmission could progress to disease. Secondary transmission of infections could not occur before this time window, and secondary transmission of *Xf* after this time window did not survive vine dormancy. Thus, in the two susceptible varieties some, but not all, of the secondary infections progressed to chronic disease.

In the resistant varieties, however, by the time secondary transmission could begin, it was too late for the infections to become well enough established to survive vine dormancy, and virtually all of those infections died out, leaving the vines free of disease the following year. This is illustrated in the two hypothetical Figures 1 & 2 below. The position and shape of these two curves can be a function of the severity of winter climate, the length of the growing season, and the varietal susceptibility. Favorable factors (such as a short, mild dormant season) would move the curves toward each other, resulting in a greater probability of overlap —thus a bigger window of opportunity when secondary transmission would result in chronic disease. In the General Beale area, most of the varieties would be “resistant” to secondary transmission of PD (curves shifted apart); thus the vineyards were not lost to disease. Those same varieties, if grown in the Temecula area, would have the curves shifted toward greater overlap, and the varieties could then be lost.

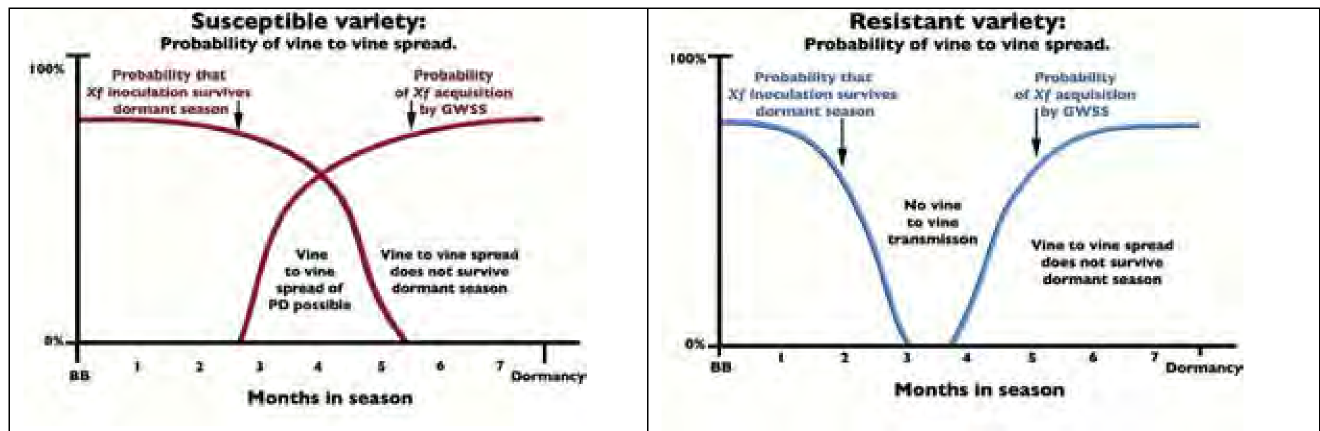

Figure 1. **Figure 2.**

This project addresses the dynamics of secondary transmission in the southern San Joaquin valley. Previous work (Fiel *et al*, 2003) has examined the left-hand curve, dormant season survival by time of inoculation. However, little is known of the right-hand curve, probability of acquisition by GWSS with regards to time. Because of concerns about the possible transmission of PD to commercial vineyards, it was not possible to pursue the best experimental designs using insects to transmit *Xf*, nor to do the experiments in commercial vineyards in either Kern or Tulare Counties. Perhaps the only possible project site, a 3.2-acre vineyard on the University of California Kearney Research and Extension Center at Parlier, CA, was available, and using this site enabled us to begin experiments that might not otherwise have been done. This site had mature vines of two varieties, Thompson Seedless and Selma Pete (a table/raisin variety similar to Thompson). For the first time, we were able to examine the effect of varietal differences on our theoretical curves.

In addition, 850 mature Thompson vines were cut about 40 cm above the ground and were grafted with Red Globe, Thompson, and Princess in 2005. In three years when these vines are mature enough, other experiments can be done to further understand the influence of varietal differences on secondary transmission and over-winter survival of *Xf*. The projects discussed herein, with other projects that build on these concepts, will help extension advisors and growers devise new integrated disease management practices for PD.

OBJECTIVES

1. Follow over-winter survival of *Xf* associated with time of inoculation by needle-inoculating 20 to 35 vines at a time, of each variety, at twice-a-month intervals for 4 months beginning on May 1, 2005. Confirm all resulting infections by ELISA testing of each vine during the year that they are inoculated. Test all vines in late season 2006 to determine whether the infections persisted over the dormant season.
2. Determine the time of detection of *Xf* in foliage in 2006. In May 2005, 60 vines of each variety would be needle-inoculated. At 2X per month intervals in 2006, all 120 vines to be sampled where *Xf* is most likely to appear in the new foliage to determine when *Xf* is detectible. Test all samples by ELISA, and store a part of each sample at minus 80^0F for possible future PCR testing.

3. Graft 850 mature Thompson vines with 3 varieties of differing PD susceptibility to enable future experiments in this vineyard about the influence of varietal differences on secondary transmission.

RESULTS

Objective 1: The 180 Selma Pete vines used in these over-winter survival experiments were grafted in 2001 about 30 cm above the ground on to mature Thompson vines. These Selma Pete vines, now in their fourth growth year, and another 220 Mature Thompson Seedless vines were needle-inoculated in 2005 at twice-per-month intervals beginning at the May 1 through the middle of August, for a total of eight inoculation times. The inoculated vines were tested in late 2005, and the inoculations were 100% effective in producing infections in the vines. Each vine was inoculated in two places on opposite sides of the vine (different cordons) on first-year growth about 15 cm from old wood. At each inoculation site, both a petiole and the stem were inoculated with droplets containing ca. 10^7 *Xf* cells from a 9-day-old culture. The over-winter survival of the resulting infections is shown in Figure 3.

Objective 2: The samples for testing the time of *Xf* detection in the new foliage in 2006 were petioles taken from the site considered most likely to be where the bacterium would appear first, whenever possible from the base of the cane that was inoculated the previous year. Because each vine had two inoculation sites, two sites were sampled for each vine, and 60 vines produced 120 samples. In many vines, one side of the vine began testing positive several weeks before the other side. Even on August 18, the samples from one side were still testing negative in 24 vines of Thompson and four vines of Selma Pete, respectively.

On June 14, petiole samples were collected from six vines that had tested positive on June 1 (three vines each of Thompson and Selma Pete). One basal petiole was tested from each new shoot, growing from old wood within 15 cm of the trunk. A positive petiole would mean that *Xf* was present in the basal portion of the cane. The Thompson and the Selma Pete had *Xf* in 5% (3 of 58) and 19% (12 of 62) of the canes respectively.

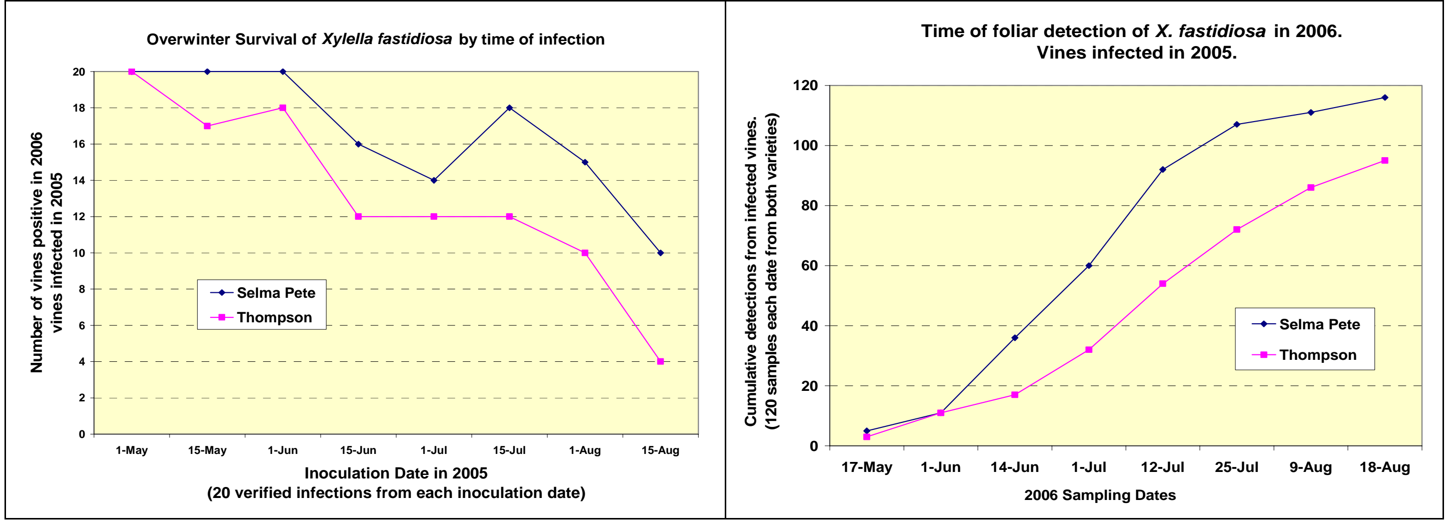

Figure 3. **Figure 4.**

DISCUSSION

The over-winter survival experiment (Figure 3) was designed to represent the worst-case possibility, and therefore the results do not represent what might occur in an actual field situation, nor do the results agree with previous work at Kearney (Feil et.al., 2003). We chose to inoculate the base of the canes vs. more distal sites because the severity of the GWSS vectored PD epidemics has been in part attributed to the possibility that GWSS can feed (and therefore acquire and inoculate) at the base of the cane. Mid season basal inoculations are more likely to result in infections that survive the winter than more distal inoculation sites. We inoculated each site with a very large number of cells to insure that all inoculations would result in infections. We inoculated multiple millions of bacterial cells per inoculation into the xylem, compared to inoculations by GWSS or another vectors that might introduce a few (<100) cells. Our resultant curves (Figure 3) were skewed far to the left in comparison to previous work at Parlier. Feil et.al. (2003) found that infections resulting from basal insect inoculations in July survived the winter, but none of their August inoculations, whether by insect or needle, or basal or distal, resulted in infections that survived. Our work is, however, the first case of comparing the differences in over-winter survival of *Xf* as a function of varietal susceptibility, supporting the idea that more susceptible varieties result in over-winter survival curves that are shifted to the right. It may be that irregularities in the shape and position of the curves in Figure 3 are the result of using an excessive number of bacterial cells per inoculation. We will address these aspects in future inoculation experiments.

The "time of foliar detection" curves in Figure 4 are probably not affected by the decision to inoculate with a worst-case design, and probably do represent actual field epidemic situations. These curves show a difference between varieties in when the bacteria become detectable in the new growth, and this is consistent with the hypothesis about secondary transmission that is represented in Figures 1 & 2.

Putting together the information from Fiel et.al. (2003) and our Figure 4, we would predict that the window of possibility for secondary transmission that survives the following winter may begin in early June and end by early August. However possibility is not the same as probability, and epidemics are stochastic phenomena. When *Xf* is first detected it is present in only a small part of the total canopy; and it is highly patchy. Also in mid June only a small proportion of the canopy of chronically infected vines have detectible *Xf* in the foliage, where it would be available for vector acquisition. Therefore the target area, both in the vineyard and on the vine, where acquisition feeding might occur in mid June is a very small part of the total vineyard or canopy, especially compared with the target area in August and beyond. Also in mid June to August the target area where an infective vector must feed in order to inoculate a vine with an infection that survives the winter is a small and continuously shrinking portion of the canopy of a vine.

The fact that GWSS can feed at the base of canes in July and August does not speak to the probability that GWSS would prefer to feed at these target sites and search for them preferentially. Furthermore we know of no evidence that in mid summer GWSS prefers a basal feeding site (where either acquisition or inoculation might be successful) over the more available and vigorously growing outer parts of the canes. GWSS flying onto an infected vine in July would have a very small probability of randomly encountering a target feeding site that would result in acquisition. This raises the question why did secondary transmission play such a big role in the Temecula epidemic and in the susceptible varieties in the General Beale epidemic? We propose that the most important epidemiological factor, in addition to the ability of GWSS to feed at the base of the canes, is simply the extraordinarily high numbers of GWSS that occurred in these epidemics. One or a few GWSS landing on a vine may be very unlike to acquire *Xf*, but when hundreds or even thousands of GWSS per vine are feeding and actively moving among the vines, the probability of *Xf* acquisition and transmission by a percentage of these GWSS becomes larger. This may be enough to explain the kind of secondary transmission that was observed. Also the effect of variety on shifting the shape and position of the curves as represented in Figures 1 & 2 may explain the varietal difference observed in the General Beale epidemic.

Figure 4 represents new information. It does not however quantify the probability (vs. the possibility) that GWSS will acquire *Xf* by feeding on an infected vine. Our future efforts will be directed toward determining the geometric features of the target feeding area in an infected vine, and in exploring the behavioral feeding preferences of GWSS in the mid season. This will help to interpret the curves in Figure 4 and to come closer to predicting a more realistic position and shape for the theoretical acquisition curve postulated in Figure 2. The research vineyard at Kearney provides an opportunity to pursue these goals.

CONCLUSIONS

The results of these experiments support the hypothesis for secondary transmission that is represented in Figures 1 & 2 above, namely that the two curves which represent: (1) the probability that an *Xf* infection survives the dormant season, and (2) the probability of *Xf* acquisition by a vector must overlap for secondary transmission of *Xf* to survive dormancy and progress to PD. The experiment concerning time of foliar detection of *Xf* in previously infected vines provides some limits on when such overlap of these curves can begin, and previous work suggests the probable end of the window. We now better understand the severe losses of the two recent Kern and Temecula epidemics, and strategies are emerging for timely, effective, and affordable control practices to predict and avoid such losses in the future. The benefit to grape producers in this area will be twofold: 1) more accurate assessment of risk of economic loss from PD, and 2) suggestion of new PD management practices. For example protecting vines during the window of overlap might reduce or eliminate secondary transmission of PD. Practices that use this epidemiological knowledge may be thought of as Integrated Disease Management, a concept analogous to Integrated Pest Management that has been so widely adopted and successful.

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FUNDING AGENCIES

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THE PIT MEMBRANE BARRIER TO *XYLELLA FASTIDIOSA* MOVEMENT IN GRAPEVINES: BIOCHEMICAL AND PHYSIOLOGICAL ANALYSIS

Project Leader:
John Labavitch
Dept. of Plant Sciences
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

Cooperators:
Mark Matthews
Dept. of Vitic. and Enol.
University of California
Davis, CA 95616

L. Carl Greve
Dept. of Plant Sciences
University of California
Davis, CA 95616

Qiang Sun
Dept. of Plant Sciences
University of California
Davis, CA 95616

Tom Rost
Section of Plant Biology
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted July 1, 2006 to September 30, 2006.

ABSTRACT
Studies planned for this proposal will (1) examine further the impacts of cell wall-degrading proteins on pit membrane integrity, (2) describe what our uses of the *Xylella fastidiosa* (*Xf*) cell wall-degrading enzymes tell us about the pit membrane polysaccharide network, and (3) specifically examine the relationship between pit membrane disruption, grapevine ethylene production, and xylem water conduit obstruction. Of particular interest because of its potential for identifying a new mechanism for a vine's resistance to PD, will be tests of the role of *Xf* cell wall xyloglucan-degrading endo-β-1,4-glucanases (EGases) in increasing the pit membrane's porosity and efforts to identify natural pant proteins that are inhibitors of those EGases.

This is a new project, approved in Spring 2006, with funding beginning July 1. Dr. Alonso Pérez-Donoso, who had recently finished his Ph.D. work in our laboratory was to have been the primary bench scientist in the project. However, he was offered a faculty position in Santiago, Chile and left to assume that position in early Spring 2006. Therefore, progress toward meeting our objectives has been slow. We are fortunate in that Dr. Quang Sun will be taking a position as a postdoctoral researcher in the project, beginning October 1, 2006. We anticipate rapid progress on Objectives 2 and 3 once Dr. Sun has become comfortable with his new laboratory environment. We have been able to begin testing of xyloglucanase-inhibiting proteins (XGIPs) on *Xf* EGase activity. Unfortunately, no inhibition was detected.

INTRODUCTION
For five years, Labavitch and the listed collaborators have been testing a model proposed to describe the development of Pierce's Disease (PD) in grapevines (Labavitch et al., 2001, 2002; Labavitch and Matthews, 2003; Labavitch et al., 2004, 2005; Pérez-Donoso, 2006; Pérez-Donoso et al., 2006). Findings reported in the last two PD Symposia strongly suggest that enzymes, likely produced by *Xylella fastidiosa (Xf)* resident in xylem water-conducting cells (also Roper et al. 2004) are important contributors to the escape of the pathogen from the vessels into which it has been introduced by GWSS, thus initiating its systemic spread through the vine and the subsequent development of PD symptoms. However, observations made only in the past year have suggested that seasonal changes in normal grapevine development may also contribute to the systemic spread of *Xf*, beginning in late Spring. These observations may be linked to those made by collaborators Rost, Matthews et al. (Thorne et al., 2006) suggesting that relatively long xylem conduits, likely to be of primary xylem origin, may allow relatively long distance passage (i.e., the length of 2-3 internodes) of *Xf* into grape leaves. While this pathway is not likely to facilitate long distance systemic spread of the pathogen through stems, it may facilitate rapid movement from stems into which *Xf* has moved, into leaves where disease symptoms then become evident. Work planned for this project will examine aspects of these reports, with a strong focus on factors that might affect the integrity of the pit membranes in grapevine xylem water conduits.

OBJECTIVES
1. Characterize the biochemical action of *Xf* EGase, *in vitro* and *in planta* and determine if it is inhibited by plant proteins that have been identified as xyloglucan-specific endoglucanase (EGase)-inhibiting proteins.
2. Examine the full range of effects on grapevine pit membrane porosity that result from introduction of cell wall-degrading polygalacturonase (PG) and EGase.
3. Repeat our 2005 observations of a late Spring, dramatic increase in the porosity of grapevine pit membranes.

RESULTS
Objective 1. Characterization of the biochemical action of *Xf* EGase, *in vitro* and *in planta* and determine if it is inhibited by plant proteins that have been identified as xyloglucan-specific endoglucanase (EGase)-inhibiting proteins. We have reported that the introduction of PG and EGase to the xylem of explanted grapevine stems causes breakdown of pit membrane structure (see the report for the project "The contribution of the pectin-degrading enzyme polygalacturonase (PG) in transmission of *Xf* to grape

and the use of PG-inhibiting proteins for transgenic resistance to Pierce's disease" in these *Proceedings*) while increasing pit membrane porosity (Labavitch et al. 2005). Our colleagues Cecila Aguero and Abhaya Dandekar have reported that the expression of the gene encoding the pear fruit PG-inhibiting protein (PGIP) in transgenic grapevines slows the development of PD in the modified genes. We have also shown that the *Xf* EGase is active in digesting xyloglucan, a primary cell wall polysaccharide that is likely to be the pit membrane target of the EGase. Thus the *Xf* EGase can be considered to be a xyloglucanase (XGase). Therefore, if the tomato protein that has been identified as an XGase-inhibiting protein (XGase-IP) is able to inhibit the *Xf*-XGase, then expressing it in combination with the pear PGIP in transgenic grapevines could provide substantially enhanced PD tolerance.

The tomato XGase-IP was provided by our colleague, Dr. Will York, at the Complex Carbohydrate Research Center at the University of Georgia. *Xf* EGase/XGase was isolated from *E. coli* transformed by Dr. Caroline Roper to express one of the pathogen's -1,4-glucanase-encoding genes (Labavitch and Matthews, 2003). We also tested the ability of the tomato XGase-IP to block the activity of a purified GWSS β-1,4-glucanase and a fungal XGase provided by colleagues at Novozymes (positive control) (Figure 1).

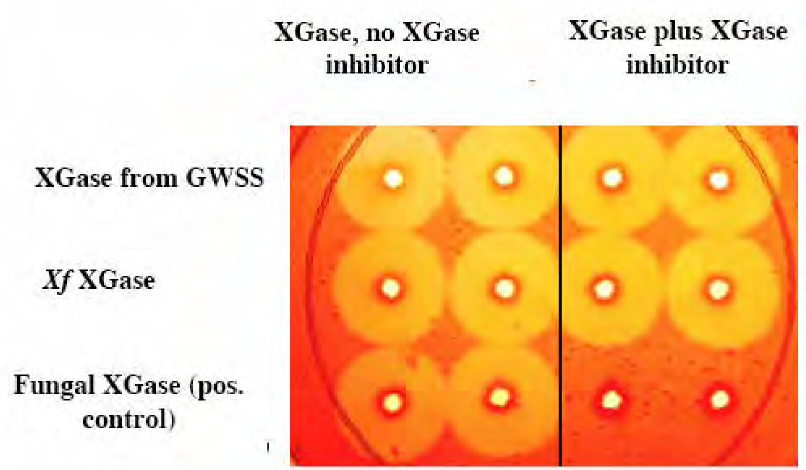

Figure 1. Shown is a radial diffusion assay of XGase activity. The xyloglucan (XG) substrate is dissolved in buffer and then mixed with melted agar. The agar is poured into a Petri dish and hardens. Wells are cut in the agar and then samples of the GWSS, *Xf* or fungal XGases are placed in the wells (left half). As the enzyme diffuses into the substrate-containing agar it digests it. The agar is stained with the dye Congo red to reveal the presence of undigested XG. The bigger the clear zones (shown above in yellow) the greater the XGase activity. The XGases were mixed with the tomato XGase-IP (right hand half) and the XGase activity was determined as described above. If the addition of the XGase-IP has caused inhibition of the XGase (i.e., reduces the size of the clear zone, as for the positive control) then it is an effective inhibitor. However, neither the GWSS nor the *Xf* XGase was inhibited (i.e., the clear zones are the same size whether the XGase-IP is present or not).

While the absence of inhibition of the *Xf* BGase/XGase indicates that the tomato XGase-IP will likely not be useful for enhancing tolerance of PD, this result does not eliminate the idea from consideration. We have studied the PG-inhibiting proteins (PGIPs) of plants for many years. They are very selective in the PGs that they inhibit (Stotz et al., 2000). Some PGs are strongly inhibited by a given PGIP while other PGs are not inhibited at all. It is reasonable to think that XGase-IPs display the same selectivity. We are not engaged in studies to discover new sources of XGase-IPs. However, as additional inhibitors are reported we will attempt to obtain them in order test their action against the *Xf* XGase.

Objective 2. To examine the full range of effects on grapevine pit membrane porosity that result from introduction of cell wall-degrading polygalacturonase (PG) and EGase.

Objective 3. To repeat our 2005 observations of a late Spring, dramatic increase in the porosity of grapevine pit membranes.

Work on these objectives has not begun. Dr. Sun will be joining the lab soon and work will begin on these objectives at the start of Spring, 2007.

CONCLUSIONS

The only concrete conclusion that we can report for this new project at this time is that tomato XGase-IP does not inhibit the *Xf* and GWSS β-1,4-glucanases that we have previously purified.

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FUNDING AGENCIES

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

LINKING THE MODEL OF THE DEVELOPMENT OF PIERCE'S DISEASE IN GRAPEVINES TO AN UNDERSTANDING OF THE DYNAMICS OF GLASSY-WINGED SHARPSHOOTER TRANSMISSION OF *XYLELLA FASTIDIOSA* TO GRAPEVINES AND GRAPEVINE GENE EXPRESSION MARKERS OF PIERCE'S DISEASE

Project Leaders:

John Labavitch
Dept. of Plant Sciences
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

Elaine Backus
USDA ARS PWA
Crop Dis., Pests and Gen.
Parlier, CA 93648
ebackus@fresno.srs.usda.gov

Mark Matthews
Dept. of Vit. & Enology
University of California
Davis, CA 95616
mamatthews@ucdavis.edu

Ken A. Shackel
Dept. of Plant Sci.
University of California
Davis, CA 95616
kashackel@ucdavis.edu

Cooperators:

L. Carl Greve
Plant Sciences, UC Davis

Alonso Pérez
Plant Sci, UC Davis

Holly Shugart
Crop Dis., Pests, ARS Parlier

Bruce Kirkpatrick
Plant Pathol., UC Davis

David Morgan
CDFA
Riverside, CA 92501

Rex Cocroft
Div. of Biological Sciences
University of Missouri-Columbia

Reporting Period: The results reported here are from work conducted October 1, 2005 to Sept. 30, 2006.

ABSTRACT

For several years we have been studying the development of Pierce's disease (PD) in grapevines. Our studies have been guided by a model of PD development proposed with our initial application for funding. The Model proposed several "steps" in disease development following introduction of the PD causal agent, the bacterium *Xylella fastidiosa* (*Xf*):

***Xf* introduction to vessels =>vessel cavitation =>initial water deficit => *Xf* population increase => production of enzymes by *Xf* =>cell wall digestion => oligosaccharide signals => ethylene synthesis rise => a "wave" of vessel occlusion beyond the infection site => collapse of vine water transport => leaf abscission => vine death**

Our hypotheses have proven quite accurate, although aspects of the model are still being tested. We have shown that xylem vessel obstruction (tyloses, plant cell wall component-derived gels, and bacterial extracellular polysaccharides) and consequent reductions in stem water transport capacity are early consequences of infection with *Xf*, before bacterial populations are substantial and have spread far from the inoculation point. We have shown that ethylene treatment of vines also triggers vessel obstruction development and reduced water movement and that ethylene emanation from vines may increase following infection. We have also developed data for xylem vessel length distributions in grapevines and shown that *Xf* must pass through vessel pit membranes if the bacterial population is to develop systemically, thus suggesting that digestion of cell wall polymers in the pit membranes is likely to be important to disease spread. These findings are reported in several reports at the annual PD Symposium (Labavitch et al., 2001, 2002, 2004, 2005; Labavitch and Matthews, 2003) and, more recently, at disciplinary scientific society meetings (Perez et al., 2004; Roper et al., 2004) and in refereed reports (Stevenson et al., 2004). We describe herein the continuing studies that have made clear that the *Xf* genome contains genes that encode cell wall-degrading polygalacturonase (PG) and endo-β-1,4-glucanase (BGase) and that these two enzymes are sufficient to open the pit membrane network, suggesting that this is the mechanism used by the pathogen to permit systemic development in infected grapevines.

INTRODUCTION

Overall, many of the investigators listed above are involved in three CDFA-supported projects that are centered in the Labavitch lab. Two of these projects are outgrowths of our earlier project that was designed to test our proposed model for Pierce's disease (PD) development. Thus, it is difficult to avoid discussing some of the work in our other two projects in this report for the third project, which is an expansion of the primary model to link it to the studies of other PD researchers.

OBJECTIVES

1. Complete testing of our model of PD development in grapevines.
2. Determine whether glassy-winged sharpshooter (GWSS) feeding on grapevines is accompanied by xylem vessel cavitation.
3. Determine whether the grapevine "regulators" that we have identified as important to development of PD affect the expression of grapevine genes that have been shown to be important markers of *Xf* presence/PD infection.

RESULTS

Objective 1. Complete testing of our model of PD development in grapevines.

Efforts in this research year have examined four aspects of the model not previously tested. The first is the hypothesis that cell wall breakdown caused by the action of bacterial enzymes, like the pectin-degrading enzyme, PG contributes to the ability of *Xf* to systemically colonize the grapevine xylem which ultimately leads to disease. We demonstrated that the open reading frame encoding a putative PG did, in fact, encode a functional PG. Furthermore, *Xf* mutants lacking PG did not move from the point of inoculation and did not cause PD when inoculated into grapevine. The second is related to work designed to show whether *Xf* wall-digesting enzymes are present in the xylem of infected vines. The third pertains to descriptions of the porosity of the pit membranes that separate one vessel from its neighbors. The fourth pertains to the idea, discussed by many but never actually demonstrated, that *Xf* produces an exopolysaccharide (EPS) and that this EPS is associated with the pathogen in infected grapevines. While the first three of these questions are clearly relevant to the examination of the "*Xf* enzymes and cell wall digestion" section of our model (above) they are also addressed in a second project that grew out of our model testing efforts. The title of that project is "The contribution of the pectin-degrading enzyme polygalacturonase (PG) in transmission of *Xf* to grape and use of PG-inhibitor proteins for transgenic resistance to Pierce's Disease" and the specific data that pertain to *Xf*'s PG and the pathogen's β-glucanase and their role in Pierce's disease development, specifically the opening of pit membranes, can be found in the report for that project in these *Proceedings*. The final question in this section, related to the production of EPS by *Xf*, is addressed below.

Does *Xf* produce an extracellular polysaccharide (EPS) and is this associated with bacteria that have colonized grapevine xylem water conduits? The sequence information for the *Xf* genome suggests that the pathogen should produce an EPS like that produced by *Xanthomonas campestris.* Because the *X. campestris* EPS is important for development of diseases caused by the pathogen it seemed reasonable to determine if the predicted similar EPS of *Xf* contributes to PD development. Thus answers to the questions posed above are potentially crucial to understanding how PD develops. Caroline Roper contacted Prof. L. Ielpi of the University of Buenos Aires who had reported on his studies of EPSs produced by a number of *X. campestris* strains with mutations in the genes encoding proteins involved in synthesis of its EPS. One of these was predicted to produce an EPS with a structure like that predicted for the putative *Xf* EPS. Prof. Ielpi kindly provided a sample of this mutant EPS variant and it was used to raise polyclonal antibodies. These were used to produce an immunoaffinity chromatography column that was used to purify cross-reactive polysaccharide from gel-like material that accumulated on the walls of flasks used for liquid culture of *Xf*. The structural analysis of this affinity-purified polysaccharide is now underway. The anti-*Xf* EPS antibodies were also used in a Protein A double sandwich ELISA assay now being used to quantify EPS production by the pathogen. These results are not presented in this report but are described in Caroline's dissertation (Roper, 2006) and in a manuscript that is now in rough draft form.

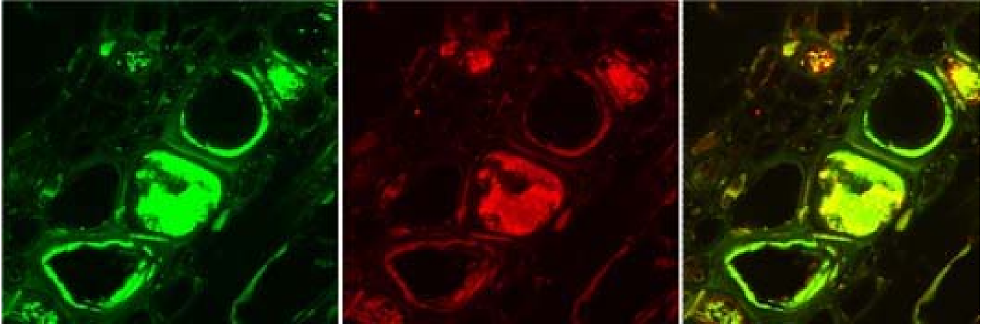

Figure 1. The co-localization of *Xf* cells and EPS. In the left panel a confocal laser-scanning microscope was used to show the presence of GFP-tagged *Xf* in the petiole xylem of a leaf from an infected grapevine. In the center panel the rabbit anti-*Xf* EPS antibodies were used to bind the EPS and then a red fluorescing dye (AlexaFluor 568)-tagged anti-rabbit serum to show the EPS based on the red fluorescent signal. In the right panel the two images are merged to give a yellow to orange signal indicating the co-localization of *Xf* cells and EPS.

The anti-*Xf* EPS antibodies were also used to demonstrate the presence of cross-reactive material, presumably *Xf* EPS, in *Xf* biofilms formed *in vitro* and *in planta*. The EPS often co-localized with *Xf* cells and appears to contribute to xylem vessel occlusion although the exact role of EPS in virulence is unclear (Roper 2006).

Objective 2. Determine whether GWSS feeding on grapevines is accompanied by xylem vessel cavitation.

This Objective is addressed by a combination of researchers with expertise in (1) electrical penetration graph (EPG) monitoring of sharpshooter feeding on grapevine xylem (Backus and colleagues, USDA), and (2) water-moving capacity of

xylem vessels (Shackel, Matthews and Labavitch, UCD). Progress was made this year in developing the protocols needed for both the insect and plant portions of the project. However, these tests have proven to be more challenging than was foreseen at the time the proposal was written.

Ph.D. candidate Alonso Pérez developed the MRI techniques that were used to determine whether vessels that the insect has been ingesting from become air-filled (i.e., cavitated) following the end of feeding. Pérez had previously attended Backus' EPG Workshop at California State University-Fresno in July, 2005, where he learned recording and analysis of sharpshooter EPG waveforms. Perez also tuned and tested a classical acoustic emissions (AE) monitor that has been used for over 40 years to record the ultrasonic vibrations of vessel cavitation. With this monitor, Perez visited the Backus laboratory in October 2005 to gather preliminary data using smoke tree sharpshooter (STSS) on cowpea, some of which was presented at the 2005 Pierce's Disease Research Symposium. Backus then lent her EPG equipment to Labavitch, and Perez used various combinations of EPG and AE monitors with both STSS and GWSS on grape, plus MRI imaging, during November 2005 to January 2006 at UCD. Unfortunately, the AE monitor proved unreliable for use with insects, because it was impossible to tune out vibrations made by insect movements during feeding. MRI images consistently showed no cavitation, even when many AE signals were recorded. It was therefore decided to try recording cavitations with a different, more modern approach. Perez completed his Ph.D. in March, 2006 and returned to Chile to take a position at the Catholic University in Santiago.

Backus enlisted the aid of her former colleague at the University of Missouri, Rex Cocroft, an expert in acoustic recording of insect sounds using highly sensitive, laser accelometry. Backus's head technician, Holly Shugart, a former Master's student of Cocroft's, traveled to Missouri for 3 weeks in July 2006, and attempted to use Cocroft's laser to record GWSS on both cowpea and grape. Shugart was successful in tuning the laser to detect cavitations in cut stems of cowpea, but only after drastic measures. She found that the laser detects ultrasonic vibrations as trains of extremely short pulses, each approx. 2-5 μsec in duration. The computerized recording equipment could not simultaneously record insect EPG and laser pulses, because sample rates in excess of the software maximum of 250,000 samples per sec (Hz) were required. Therefore, Backus hired colleagues at Sable Systems, Inc. to build a pulse-stretcher on rush order (over one weekend), which lengthened the pulses, allowing a more achievable sample rate of 70,000 Hz. After much tuning of the laser plus the pulse stretcher, Shugart achieved very clear, noise-free recordings of cavitation (Figure 2), which were experimentally verified by refilling cut, cavitating stems with water, observing loss of cavitations, then cutting the stems again and observing re-occurrence of cavitations (data not shown). Also, a single, successful recording was made of GWSS EPG feeding on cowpea, simultaneous with laser recording (data not shown). The laser was set at a much more sensitive level than for cut-stem cavitation controls, and did pick up trains of pulses. However, they were clearly correlated with waveforms known to represent stylet sawing, as well as walking and other insect movements. No signals as loud as cavitation were recorded during insect feeding, only during non-probing movements. These results suggest that sharpshooters do not cause cavitation during stylet penetration to the xylem, and support the MRI findings of Perez. However, this work will need to be repeated, with simultaneous recording of EPG and laser, plus before-and-after images by MRI. A laser accelerometer will need to be purchased to continue the work in California.

Objective 3: Determine whether the grapevine "regulators" that we have identified as important to development of PD affect the expression of grapevine genes that have been shown to be important markers of *Xf* presence/PD infection.

As discussed in this and previous PD research reports, we have now developed a substantial data set describing events in the development of PD in grapevines. We will work in the coming year to focus on two important PD development steps proposed by the model, but not yet fully tested. These relate to the potential roles of the plant hormone ethylene and oligosaccharides digested from grapevine cell walls in influencing the spread of the *Xf* population in vines or the vine's response to *Xf* presence. We have obtained the supplies needed to follow expression of the set of 4 grapevine genes that are expressed relatively early following *Xf* introduction into vines (Cook et al., 2003). The testing of the timing of expression will be based on real-time PCR of these 4 genes in relation to the appearance of early PD symptoms, most specifically the growth and spread of *Xf* in the weeks early after inoculation and development of vascular system occlusions as followed by MRI.

CONCLUSIONS

Our group of cooperating PD researchers feels that the best way to effectively deal with the threat caused by the disease is to fully understand its development in grapevines. A full understanding of the interaction of GWSS, *Xf* and *Vitis vinifera* should identify aspects of disease development that can be targeted by control measures that can be exploited using genetic approaches or new field management practices. We feel that the identification of a key role for *Xf* PG in PD development may provide a useful target toward which PD control measures could be directed. Should work to determine the extent to which grapevine ethylene production is a response to infection confirm our model's prediction, we will have another valuable marker of PD development as well as another potential target to exploit in terms of interrupting the systemic spread of *Xf*.

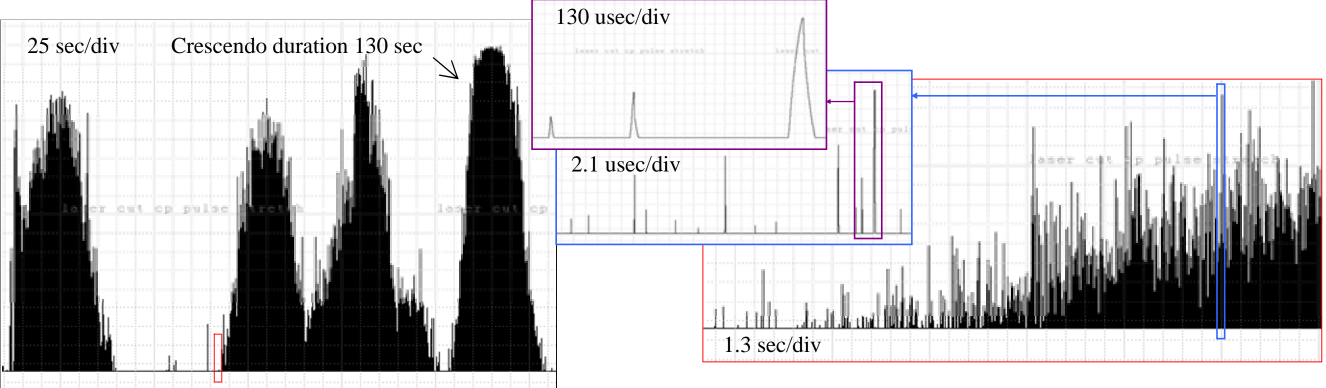

Figure 2. Control recordings of cut, cavitating cowpea stem using laser accelerometry. Rapid trains of pulse crescendos occur sequentially (black box), perhaps indicating separate groups or bundles of xylem cells cavitating. When the recording is temporally expanded, trains of thousands of individual pulses become discernible (red box in left view, expanded on right). Further expansion (blue then purple boxes) reveal individual, stretched pulses.

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Roper, M.C. 2006. The characterization and role of *Xylella fastidiosa* plant cell wall degrading enzymes and exopolysaccharide in Pierce's disease of grapevine. Ph.D. dissertation.

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

THE CONTRIBUTION OF THE PECTIN-DEGRADING ENZYME POLYGALACTURONASE (PG) IN TRANSMISSION OF *XYLELLA FASTIDIOSA* TO GRAPE AND THE USE OF PG-INHIBITING PROTEINS FOR TRANSGENIC RESISTANCE TO PIERCE'S DISEASE

Project Leaders:

John Labavitch	Elaine Backus	David Morgan
Dept. of Plant Sciences	USDA ARS PWA	Calif. Dept. Food. Agric
University of California	Crop Dis., Pests and Gen.	Mt. Rubidoux Field Sta.
Davis, CA 95616	Parlier, CA 93648	Riverside, CA 92501
jmlabavitch@ucdavis.edu	ebackus@fresno.srs.usda.gov	dmorgan@jps.net

Cooperators:

L. Carl Greve	Alonso Pérez	Caroline Roper	Bruce Kirkpatrick
Dept. of Plant Sciences	Dept. of Plant Sciences	Dept. of Plant Pathology	Dept. of Plant Pathology
University of California	University of California	University of California	University of California
Davis, CA 95616	Davis, CA 95616	Davis, CA 95616	Davis, CA 95616
Ken Shackel	Abhaya Dandekar	Tom Rost	Mark Matthews
Dept. of Plant Sciences	Dept. of Plant Sciences	Dept. of Plant Biology	Dept. of Vit. and Enology
University of California	University of California	University of California	University of California
Davis, CA 95616	Davis, CA 95616	Davis, CA 95616	Davis, CA 95616

Reporting Period: The results reported here are from work conducted October 1, 2005 to Sept. 30, 2006.

ABSTRACT

Work this year has shown that the *Xylella fastidiosa (Xf) p*olygalacturonase (PG) is an endo-acting enzyme. This provides us a reference that we can now use to biochemically characterize the interaction between the pathogen's PG and PG inhibiting proteins (IPs) that is implied by the fact that transgenic expression of PGIPs protects vines against PD. We also report that PG, in combination with a bacterial β-1,4-glucanase enzyme (BGase) that has also been discussed in previous reports can act to cause tears in the pit membranes that are likely to be barriers slowing the systemic spread of the *Xf* population. We also report on a few set-backs that have slowed our examination of the roles of insect plant cell wall-degrading enzymes in PD development. Fortunately, the set-backs are now being corrected so that important questions can be addressed in work supported by a no-cost extension.

INTRODUCTION

In a companion report we have discussed continuing work that has been done to test the model we have proposed to describe the development of Pierce's disease (PD) in grapevines. That model proposes a key role for a pectin-degrading enzyme, polygalacturonase (PG). Over the past few years we have demonstrated that *Xylella fastidiosa* (*Xf*) has a PG encoding gene and that the *Xf* PG is important for disease development. This project is a spin-off from our "model" project, based on the observation that, when expressed in transgenic grapevines, plant PG-inhibiting proteins (PGIPs) provide some protection against PD.

OBJECTIVES

1. Determine whether the pectin-degrading enzyme of *Xf* contributes to the systemic spread of the bacterial population in inoculated grapevines (first priority).
2. Determine whether the pectin-degrading enzyme(s) in the salivary secretions of the glassy-winged sharpshooter (GWSS) contributes to inoculation success of *Xf* into grapevines (second priority).

RESULTS

Objective 1

Determine whether the pectin-degrading enzyme of *Xf* contributes to the systemic spread of the bacterial population in inoculated grapevines

This question is related to a major component of our model for PD development and prior results in the project focused on testing that model led to this project. We had previously shown that the *Xf* PG gene coded for some sort of PG activity. This conclusion was based on isolation of the protein from *E. coli* transformed to express the putative PG gene. This year's work involved the development of a PG assay based on identification of the products of enzyme action. If the products included a series of oligosaccharides, rather than only monosaccharide galacturonic acid, the PG would be an *endo*-acting PG (Figure 1).

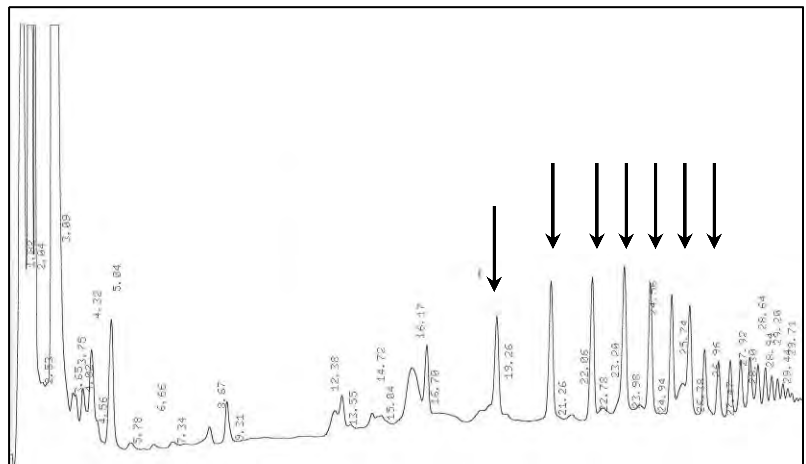

Figure 1. Protein was isolated from *E. coli* transformed to express the *Xf* DNA sequence thought to encode PG. When the protein was incubated with the PG substrate polygalacturonic acid, several oligosaccharide digestion products (arrows) were produced. Shown is an HPLC analysis of these products; oligomer size of peaks increases as they emerge later from the column (i.e., further to the right). There were no digestion products present when HPLC was done with the substrate incubated with no protein or with protein from untransformed *E. coli*. (Data from Roper, 2006; Roper et al., 2006)

When PGs are incubated with polygalacturonic acid substrate in the presence of PGIP a complex forms between the PGIP and the PG. This slows the rate of substrate digestion by PG and shifts the oligomeric digestion products to larger sizes; i.e., further to the right in the chromatogram. The next step is to isolate more PG protein from the transformed *E. coli* and test the ability of the pear PGIP that has been expressed in grapevines (Aguero et al., 2005) and PGIPs from several other plant species to see if they inhibit *Xf* PG. These tests may identify PGIPs that are more active than the pear PGIP in blocking the pathogen PG's action.

In work reported last year (Labavitch et al., 2005) we indicated that the combined action of two *Xf* cell wall-degrading enzymes, PG and BGase was sufficient to increase the porosity of grapevine pit membranes, allowing the passage of 20 nm gold particles through explanted stem segments. Our colleagues Tom Rost and Mark Matthews and their postdoctoral researcher, Qiang Sun have been carrying out studies involving electron microscope examinations of the grapevine xylem system and have obtained detailed images of pit membranes. We flushed solutions containing fungal PG and the *Xf* BGase into the xylem system of grapevines and then looked at the impact of the enzyme treatment on pit membrane integrity (Figure 2).

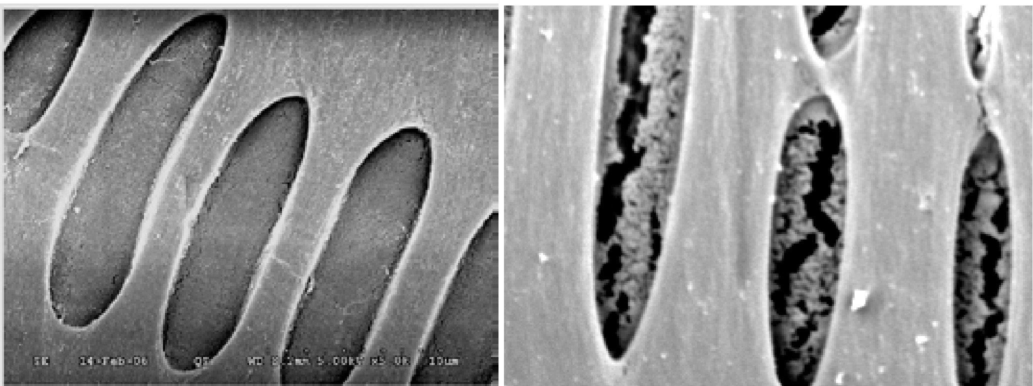

Figure 2. In the left-hand image pit membranes are seen as somewhat grainy-looking, oval surfaces framed by the lignified secondary wall of a xylem water conduits. The right-hand image is a similar view of pit membranes after the vessels have been flushed with PG and BGase. The magnification is approximately. 5,000X for the two images.

It is clear that the combined actions of the two enzymes has caused a substantial erosion of the pit membranes. Although the images have been cropped to make this side-by-side comparison, all of the pit membranes in the image of enzyme-treated stem tissue were extensively damaged.

Objective 2. Determine whether the pectin-degrading enzyme(s) in the salivary secretions of GWSS contributes to inoculation success of *Xf* into grapevines.

In the past project year we were not able to obtain a sufficient supply of GWSS for use in isolating salivary glands and their enzyme complements. Thus, little progress was possible. However, it appears that a more reliable insect supply may be developing and we will be continuing the insect side of the project in the 2006-2007 project year. We requested a no-cost extension to support that work beyond the June 30, 2006 end date of this project.

CONCLUSIONS

PG is an important *Xf* virulence factor supporting the bacterium's colonization of grapevine xylem and the development of PD. Work in the past year will be useful in determining the specific nature of the protection against PD symptom development that has been reported to result from expressing PGIPs in grapevines. The assay used to demonstrate the endo-nature of *Xf*'s PG will be of value when testing the relative action of several PGIPs against *Xf* PG. Once a reliable GWSS supply is available, similar tests of the GWSS PG can be made and studies can be carried out to determine whether PGIP-expressing transgenic grapevines are useful in suppressing disease development resulting from *Xf* introduced during GWSS feeding.

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FUNDING AGENCIES

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MICROARRAY GENE EXPRESSION ANALYSIS OF GRAPE PLANTS IN RESPONSE TO *XYLELLA FASTIDIOSA* INFECTION

Project Leader:
Hong Lin
Crop Diseases, Pests & Genetics
USDA-ARS
Parlier, CA 93648
hlin@fresno.ars.usda.gov

Cooperators:
Harsha Doddapaneni
Department of Viticulture and Enology
University of California,
Davis, CA, 95616

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

Jiqiang Yao
Citrus Research Board
Visalia, CA 93279

Reporting Period: The results reported here are for work conducted October 2005 to September 2006.

ABSTRACT
Transcriptional profiling using a custom high-density microarray chip of 20,020 *Vitis* transcripts showed significant variations in responses between the susceptible and resistant genotypes to *Xylella fastidiosa (Xf)* infection. Differentially expressed transcripts reflecting spatial and temporal responses to Pierce's disease (PD) involved in metabolic processes such as diseases resistance, water stress, photosynthesis and cell wall synthesis were identified. The results suggest that *Vitis* responses to *Xf* are genotype and tissue dependent, and are stage specific. VitisExpDB is an online MySQL-php driven relational database that houses annotated EST data. The database will provide genomic resource to grape community for functional analysis for both vinifera and non-vinifera grape varieties and aid in the grape genome annotation.

INTRODUCTION
The impact of Pierce's disease (PD) on the California grape industry has been significant since the introduction and establishment of a more effective vector, *Homalodisca vitripennis*, the glassy-winged sharpshooter (GWSS) (Almeida and Purcell 2003). Development of resistance in grape is stymied by the relatively limited amount of genetic and molecular information regarding genotype specific resistance to PD infection (Davis et al. 1978). From genotypic screening and genetic mapping studies, it was concluded that a dominant allele controls PD resistance and recently, Krivanek et al. (2006) identified a major quantitative trait locus that controls PD resistance and denoted it as 'Pierce's disease resistance 1' (*PdR1*). The above studies confirm that the genetic basis of PD resistance in grapes varies from tolerance to resistance and suggest that host responses to the pathogen are genotype dependent. Further, in the PD resistant genotypes, differential responses between stem and leaf tissues were also noted (Krivanek and Walker, 2005). The results from these studies prompted study of molecular basis of this host / pathogen interaction.

Plants respond to pathogen attack through a variety of signaling pathways consisting of a large number of regulatory as well as effector genes. Microarrays facilitate automated analysis of transcriptional profiling data to enable complete understanding of such gene function and interactions. The goal of this study was to identify and characterize the molecular events in the grape / *Xylella fastidiosa* (*Xf*) interaction using genome wide transcriptome profiling between resistant and susceptible genotypes and among the different tissue types.

OBJECTIVES
1. Microarray gene expression analysis.
2. Develop of a grape transcriptional relational database.

RESULTS
Objective 1 - Microarray gene expression analysis.
***Custom microarray chip design*:** Previously, we have characterized transcriptomes (5,794 ESTs) from 12 tissue specific (stem, leaf and shoot) subtractive suppression hybridization (SSH) libraries. All the sequenced ESTs that are at least 100 bp in length (5421 ESTs) were submitted to the NCBI's ESTdb under the accession numbers DN942225 to DN947645. These ESTs and all the other EST sequences publicly available till July of 2005 were analyzed to deduce a non-redundant set of 20,020 ESTs with 1,947 from the SSH libraries, including 40 from the cDNA-AFLP experiments, 10,014 from *V. vinifera*, 5,470 from *V. shuttleworthii*, 1,219 from *V. aestivalis*, 780 from *V. rupestris* x V. sp and 588 from *V. riparia.* Nine individual 60-mer probes were designed for each EST. A total of 191,450 probes were selected for the entire set and there were two spots for each probe on the slide totaling 382,900 spots per slide.

Experimental set-up: Total RNA from stem and leaf tissues was hybridized to 36 slides (eighteen for each explant) in a two-color experiment using the monochromatic dyes Cy5 and Cy3. RNA from three time points: early (1 week), mid (6 weeks) and late (10 weeks) stages of disease development from both infected and non-infected tissues of resistant and susceptible genotypes was analyzed. For each time point, there were three slides (biological replicates) including a dye flip.

Data analysis: For each gene and for each explant (stem and leaf) there were 54 data points per each stage (18 per slide x 3 biological replications) of disease development. Data representing raw spot intensities generated by the GenePix software were analyzed using the SAM microarray analysis software to generate fold differences and q-values. Clustering of the significantly differentially expressed genes was carried out using TMEV software.

Overview of transcriptional responses: A total of 8926 transcripts (5,299 individual ESTs and the rest are overlaps) showed statistically significant differentially regulation in the above experiments, with nearly 30 % of those being cloned from the SSH libraries (Table 1). Out of 5,299 individual transcripts that were responsive, 58.65 % (3,108 ESTs) were specific to a certain stage. Below we briefly describe the expression pattern of the major categories of these differentially expressed genes.

Table 1. Microarray hybridization identified differential expression in grape stem (**A**) and Leaf (**B**) tissues in response to *Xf* infection in both susceptible and resistant genotypes. Microarray analysis was carried out using the SAM (Significance of Microarray Analysis) software, with a q-value cutoff of 0.5 %. Values are presented for genes that are at least two-fold regulated.

(A) Stem tissue

		Genotype			
Stage	**Response**	**9621-67**		**9621-94**	
		# Of ESTs	Fold-Change	# Of ESTs	Fold-Change
Week-1	Up-regulated	294	2.0 - 6.44	9	2.0 - 2.92
	Down-regulated	421	0.49 - 0.09	2	0.48, 0.4
Week-6	Up-regulated	230	2.0 -5.05	938	2.0 – 38.9
	Down-regulated	55	0.49 - 0.22	665	0.49 - 0.025
Week-10	Up-regulated	451	2.0 -18.16	459	2.0 – 37.26
	Down-regulated	291	0.49 - 0.14	995	0.49 - 0.03

(B) Leaf tissue

		Genotype			
Stage	**Response**	**9621-67**		**9621-94**	
		# Of ESTs	Fold-Change	# Of ESTs	Fold-Change
Week-1	Up-regulated	269	2.0 – 7.47	0	-
	Down-regulated	43	0.49 - 0.28	6	0.48-0.36
Week-6	Up-regulated	82	2.0 -5.68	151	2.0 – 14.7
	Down-regulated	18	0.49 - 0.33	37	0.49 - 0.23
Week-10	Up-regulated	328	2.0 -15.63	1363	2.0 - 53.02
	Down-regulated	590	0.49 - 0.05	1229	0.49 - 0.04

1. Disease related proteins

Selective induction of 19 transcripts known to be associated with defense responses was observed in stem tissue of the resistant genotype with 2 to 6.5 fold upregulation. This includes transcripts such as MAP kinase, transcription factor EREBP1, Disease resistance protein ADR1, mannitol dehydrogenase among others. Similarly, in the leaf tissue, several defense related transcripts were differentially upregulated with 2 to 3.4 fold (PR1, PRB1-3, ABC transporter-like protein). In the susceptible genotype, only the leaf tissue showed selective induction of defense related transcripts. A large number of transcripts belonging to serine/threonine kinasePR5K, along with others were several fold induced. Transcripts such as defense-related protein, Germin-like protein subfamily 3, endochitinase B precursor were expressed in both the genotypes and could indicate broad genotype independent response.

2. Photosynthesis

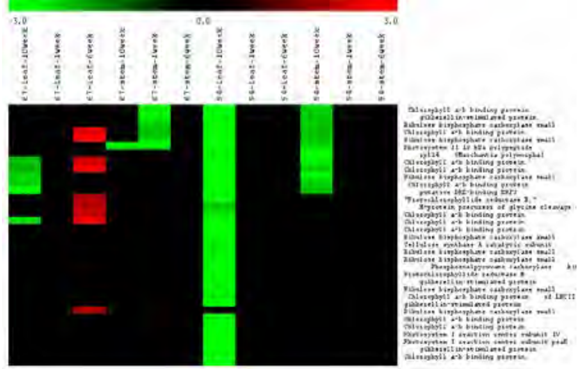
Expression levels of leaf tissue transcripts involved in photosynthesis from the susceptible genotype showed a clear indication of down regulation (Figure 1A). Some of the down regulated transcripts included RUBISCO (0.303-fold) Photosystem I reaction center subunit III, chloroplast precursor (0.31-fold) and Chlorophyll a-b binding protein AB80 (0.26-fold). On the other hand, expression of the DREbinding ERF3 was upregulated (4.26-fold). This suggests an increased stress in the susceptible genotype compared to the resistant genotype at this stage of disease development.

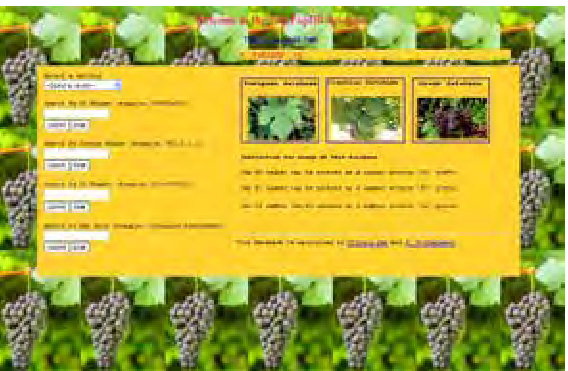
3. Water stress proteins

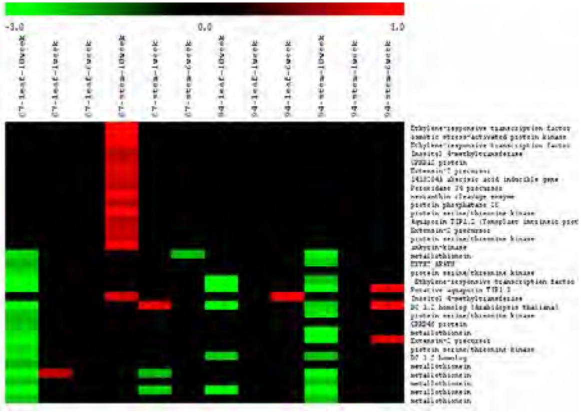
In the leaf tissue of the susceptible genotype, 10 weeks after infection, expression of several of the drought and water stress associated transcripts was upregulated such as Betaine aldehyde dehydrogenase (6.23-fold), heat shock transcription factor 1 (2.5-fold) and dehydrin (3.55) (Figure 1B). Expression of these transcripts was unchanged in the resistant genotype at the same stage of disease development. On the other hand, some of the transcripts in the leaf tissue showed several fold up regulation in the susceptible genotype compared to the resistant genotype, such as galactinol synthase a protein induced by water stress (15.1-fold and 38.4-fold in the 9621-67 and 9621-94 genotypes respectively) and the cold and drought regulated protein (CORA; 2.4-fold and 9.77-fold respectively). In the stem tissue from the same genotype, up regulation of transcripts belonging to osmotic stress-activated protein kinase (6.49-fold), neoxanthin cleavage enzyme (8.32-fold) and DT-regulated protein (3.66) was observed.

4. Cell wall and xylem proteins

Genes involved in cell wall degradation such as pectinesterases, exopolygalactruonase and other senescence associated proteins such as caffeoyl-CoA O-methyltransferase were several fold upregulated in the susceptible 9621-94 genotype compared to the resistant 9621-67 genotype. Expression of cellulases such as endoglucanase and that of xyloglucan endotransglucosylase was several folds higher in the 9621-94 genotype (0.3-fold and 37.95-fold respectively in the resistant 9621-67 and susceptible 9621-94 genotypes). Similarly, down regulation of ESTs such as Germin like proteins and monocopper oxidase precursor in the 9621-94 genotype was more pronounced than in the 9621-67 genotype. Expression of glucanases was downregulated in both the genotypes after 10 weeks of infection.







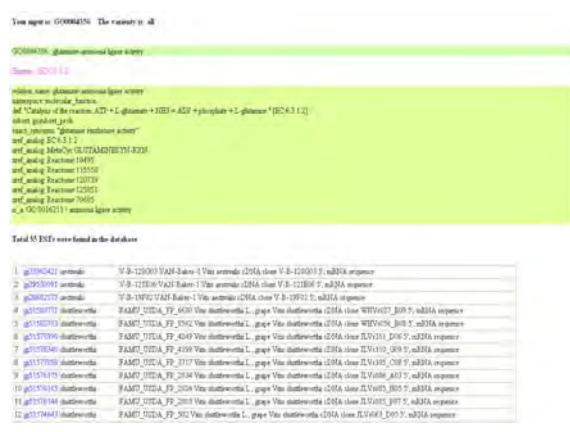


Figure 1. Expression profiling of the differentially regulated transcripts. Red indicates transcriptional activation and green represents repression. Transcripts that are not significantly regulated are shown in black. **(A)** Photosynthesis related transcripts. **(B)** Water-stress related transcripts. Hierarchical clustering was performed using TMEV. The results show a clear segregation of the genotype and tissue types in response to pathogen infection. **(C & D)** VitisExpDB is an online MySQL-php driven relational database that houses annotated EST and gene expression data for both vinifera and non-vinifera grape varieties.

Objective 2 - Develop of a grape transcriptional relational database:

VitisExpDB is an online MySQL-php driven relational database that houses annotated EST data for both vinifera and non-vinifera grape varieties. The database includes all the EST data in the public domain from both vinifera and non-vinifera varieties. In the present study, using the latest Gene Ontology (GO) terminology, a uniform structural vocabulary was

developed for the above grape varieties. Further, extensive cross referencing is allowed to retrieve the data using multiple search indices. Future plans include expansion of the database to incorporate all the microarray expression data from our as well as other reported studies. ESTs of *V. vinifera* and *non-vinifera* grapes (*Vitis vinifera*, *V. shuttleworthii*, *Vitis hybrid cultivar*, *V. rupestris* x *V. arizonica*, *V. aestivalis*, *V. riparia*, *V. pseudoreticulata*, *V. cinerea* x *V. rupestris*, *V. cinerea* x *V. riparia* are currently included.

Database architecture and Web interface: The relational database is powered by an Apache 2.0 server and was developed using MySQL 5.0 as the database management system on Red Hat Enterprise Linux 4 RPM (x86). EST sequence sets were downloaded from NCBI GenBank (UniGene, dbEST) and annotated with GO terms Sequence similarity search was carried out using the default blast parameters and a cut off E value of 10-4. On the main search page, a drop down menu that lists the variety (s) to be queried is provided. The database can be searched by Gene Ontology ID, GenBank ID, enzyme number, or by inputting key word (s) (Figure 1C). The result page displays the number of ESTs matching the query, individual EST sequence, its description, EC number, and its Gene Ontology classification (Figure 1D). VitisExpDB database is available at http://cropdisease.ars.usda.gov/~fruit_tree/.

CONCLUSIONS

Characterizing the molecular basis of the grape response to *Xf* is critical to understanding the mechanisms of PD resistance and pathogenesis. Based on our transcript profiling, it is clear that grape plant response to *Xf* infection is different among tissues between resistant and susceptible genotypes, and early and late stages. While a broad spectrum and presumably non specific plant response was observed for defense and water stress related protein expression in the susceptible genotype, a majority of this did not overlap with the resistance genotype response. Further, transcript profile also indicated a higher level of water deficiency in the susceptible genotype compared to resistant genotype.

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FUNDING AGENCIES

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COMPARATIVE PROTEOMIC ANALYSIS OF STEM TISSUE AND XYLEM SAP FROM PIERCE'S DISEASE RESISTANT AND SUSCEPTIBLE GRAPEVINES

Project Leaders:

Hong Lin
Crop Dis., Pests, & Genetics Res. Unit
USDA/ARS
Parlier, CA 93648
hlin@fresno.ars.usda.gov

Felix Fritschi
Crop Dis., Pests, & Genetics Res. Unit
USDA/ARS
Parlier, CA 93648
ffritschi@fresno.ars.usda.gov

Cooperators:

Litao Yang
Crop Dis., Pests, & Genetics Res. Unit
USDA/ARS
Parlier, CA 93648
lyang@fresno.ars.usda.gov

Andrew Walker
Department of Viticulture and Enology
University of California
Davis, CA, 95616
awalker@ucdavis.edu

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT

Analyses of host plant resistance / susceptibility mechanisms to *Xylella fastidiosa* (*Xf*) infection are critical for understanding host-pathogen interactions. Proteomic analyses of stem tissue and xylem sap samples were initiated to complement genomic approaches employed in elucidating Pierce's disease resistance mechanisms. Samples from one highly resistant and two susceptible grape genotypes were collected at multiple time points post-inoculation from control and *Xf*-inoculated plants. Two-dimensional gel electrophoresis revealed numerous proteins that were differentially expressed and dependent on plant genotype and/or inoculation treatment. Proteins identified by oMALDI-TOF comprised a wide range of functional types. The importance of these proteins with respect to host-pathogen interactions will be investigated further.

INTRODUCTION

While numerous factors including temperature, fertilization and time are known to affect xylem sap chemistry (Andersen and Brodbeck, 1989a, 1989b, 1991; Andersen et al., 1995, 2004b), the protein composition of grape xylem sap in response to *Xylella fastidiosa (Xf)* infection has not been investigated in detail to date. In other host plant-pathogen systems, xylem sap proteins were shown to be important in suppression of disease development (Ceccardi et al., 1998; Guo et al., 1993; Nemec, 1995; Reimers and Leach, 1991; Reimers et al., 1992; Rep et al., 2002; Young et al., 1995). Without a doubt, due to its xylem limited growth habit, *Xf* 's growth and development are influenced by xylem sap characteristics. Thus manipulation of the xylem sap composition presents a promising venue to interfere with *Xf* infections.

Disease expression in stems of grape vines results in blocking of water flow to the shoot and, thus, is critical to the lethal nature of Pierce's disease (PD). *Xf* infection results in uneven cane maturation which expresses itself in the formation of green-islands. The irregular nature of green-islands suggests the involvement of localized rather than systemic signals in the formation of the observed spatial symptomology. The importance of *Xf* populations in stems in respect to PD resistance of grape genotypes was also illustrated in recent studies (Krivanek et al., 2005; Krivanek and Walker, 2005). Thus, examination of stem tissue provides an opportunity to identify important aspects of plant-pathogen interactions.

Examination of xylem sap and stem protein makeup is a new approach that allows us to complement our genomic studies conducted on the same susceptible and resistant sibling genotypes employed in this study.

OBJECTIVES

1. Discover xylem sap and stem proteins differentially expressed in PD resistant and susceptible grapes in response to *Xf* infection.
2. Identify differentially expressed proteins from xylem sap and stem induced by *Xf*.

RESULTS

Objective 1. Discovery of differentially expressed proteins.

PD resistant (9621-67) and PD susceptible (9621-94) genetic lines selected from a segregating population of *V. rupestris x V. arizonica* as well as *vinifera* grape, Chardonnay were used in this comparative study. We completed expression experiment conducted in the greenhouse where treatment and control grapevines were mechanically inoculated with *Xf* suspension respectively. Leaf and stem tissues were then collected at ten time points from as early as day one post inoculation up to three months when PD symptom was fully expressed in susceptible grapes. A separate set of grapes (same genotypes and treatments as above) was grown in the greenhouse for xylem sap protein extraction at 2 time points post inoculation. The xylem sap was extracted using a pressure chamber following the same sampling scheme as above. Samples (stem, leaf and

xylem sap) collected at each time point were immediately store at –80°C for later protein extraction. After protein extraction, 2-DE separation of protein from all treatments was carried out to characterize differentially expressed proteins.

Xylem sap samples

Two-dimensional gel electrophoresis revealed apparent differential expression of numerous proteins in xylem sap collected from resistant (9621-67) and susceptible (9621-94) genotypes. In addition, infection with *Xf* also appears to affect xylem sap protein expression (Figure 1).

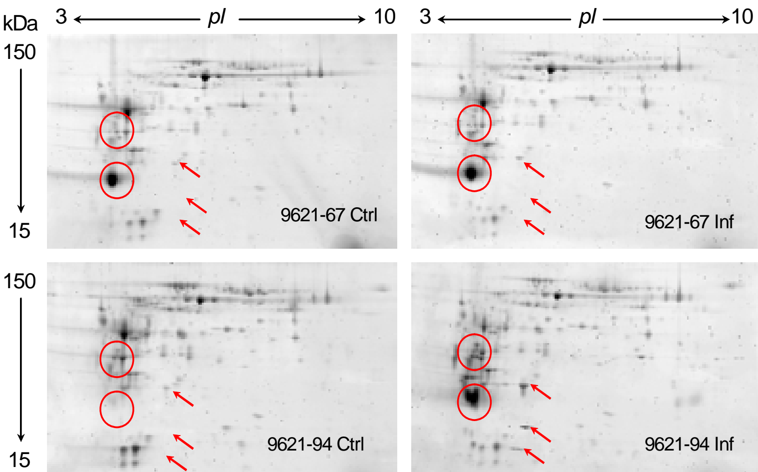

Figure 1. Comparison of the 2-DE gels from xylem sap from the highly resistant (9621-67) and susceptible (9621-94) genotype. Xylem sap samples were collected 6 weeks post-inoculation. Examples of putative differentially expressed proteins are indicated by circles and arrows.

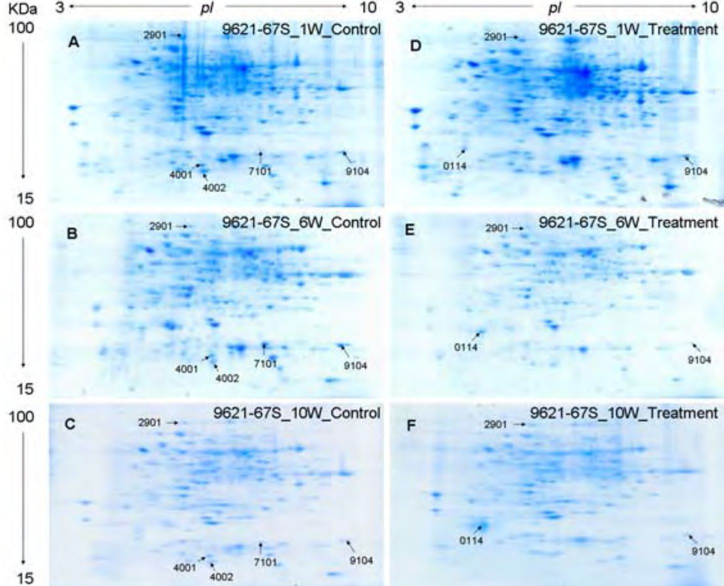

Figure 2. Three gels for each sample from the same stem tissue were run using Bio-Rad 2-DE systems. The resulting 2-DE gel patterns in all three repeats were consistent and reproducible. More than 200 protein spots could be clearly distinguished in the stem protein gels. Approximately 50 of those proteins appear to be differentially expressed (minimum of 2-fold difference in expression). Protein profiles were influenced by genotype, infection status, and stage of disease development (Figure 2). The most profound differences in expression were found in the resistant genotype (9621-67).

Objective 2. Identification of selected proteins.

Twenty-three differentially expressed proteins identified by this approach are listed in Table 1. The identified proteins comprise a wide functional range and their importance in respect to PD pathogenesis / resistance will be investigated in more detail.

Table 1. Differential protein expression was detected between infected and healthy stem tissues in PD resistant and susceptible genotypes. Proteins showing altered expression levels were excised from 2-DE and identified using peptide mass fingerprinting. Database searches using MASCOT algorithms based on the peptide mass fingerprint data identified the differentially expressed proteins.

SSP No (a)	Score (b)	Masses Matched	Protein *Mr/pI*	Accession No (c)	Protein description	Seq. cov. %
3601	440	8	41915 / 5.25	gi|11276972	T51184 actin [imported]-rape	24
8702	844	18	57266 / 7.19	gi|19070130	AF236127_1 catalase [*Vitis vinifera*]	44
3605	284	4	43516 / 5.81	gi|18157331	S-adennosylmethionine synthetase [*Phaseolus lunatus*]	13
2304	229	3	31459 / 4.64	gi|128207	Nitrogenase iron protein 2 (Nitrogenase component II)	18
4001	148	5	15448 / 5.22	gi|19114954	putative transcriptional activator hypothetical protein	38
4702	112	6	45160 / 6.09	gi|127546	UDP-N-acetylglucosamine enolpyruvyl transferase	20
4703	201	7	48823 / 6.33	gi|9858547	ribulose bisphosphate carboxylase large subunit	23
9102	234	3	17104 / 8.40	gi|729762	17.0 kDa class II heat shock protein (HSP 18)	36
6002	339	4	17357 / 6.39	gi|11182124	pathogenesis-related protein 10 [*Vinifera*]	35
7101	423	3	17813 / 6.92	gi|2674179	kinesin-related protein KRP1 [*Rattus norvegicus*]	18
2902	164	8	63122 / 5.19	gi|861170	heat shock protein 70 [*Zea mays*]	22
2703	356	12	49249 / 5.19	gi|4388533	"F1-ATP synthase, beta subunit [sorghum bicolor]"	33
9001	280	5	12602 / 8.42	gi|8248145	ORF 109 [*Lactocococcus lactis*]	49
7602	144	4	42245 / 6.96	gi|29125898	putative nitrous oxide reductase	20
7201	154	4	26752 / 6.98	gi|1708287	HO_PORPU Heme oxygenase	19
3403	128	3	34214 / 5.69	gi|26397694	PDXK_ARATH Pyridoxal kinase	14
6605	199	4	43542 / 644	gi|23039358	hypothetical protein [*Trichodesmium erythraeum* IMS101]	14
8506	175	6	37039 / 8.12	gi|120675	G3PC_SINAL Glyceraldehyde 3-phosphate	28
206	117	5	23353 / 4.71	gi|9507691	Putative mobilization protein	30
4001	197	6	17935 / 5.01	gi|29841427	Similar to NM_025435 RIKEN cDNA 150	60
4002	87	3	16088 / 4.94	gi|138317	VGR_BPP1 TailL fiber protein	24
9104	140	7	18914 / 8.08	gi|15896178	HD superfamily hydrolase [*Clostridium acetobutylium*]	29
7101	73	4	17802 / 7.56	gi|25091005	PPIA_HELPJ Peptidyl-prolyl cis-trans isomerase	32

(a) The SSP no. is a number designed by PDQuest Software and is used in matching spots for all gels.
(b) The Score is calculated by the BioAnalyst software
(c) Accession no. according to SWISS-PROTGreater

CONCLUSIONS

Differentially expressed proteins were discovered among genotypes, infection status, and sampling times using a 2-dimensional gel electrophoresis approach. Identification of the differentially expressed proteins is ongoing, however, selected spots were successfully identified using peptide-mass-fingerprinting. The identified proteins fall within a broad range of functional classes, including pathogenesis related proteins. Continued detailed characterization of identified proteins in respect to their potential role in host-pathogen interactions and resistance mechanisms will be necessary to understand their functions and possible utility in controlling PD.

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BREEDING PIERCE'S DISEASE RESISTANT TABLE AND RAISIN GRAPES AND THE DEVELOPMENT OF MARKERS FOR ADDITIONAL SOURCES OF RESISTANCE

Project Leaders:

David W. Ramming
Crop Dis., Pests, & Genetics Res. Unit
USDA/ARS
Parlier, CA 93648

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

Cooperator:
Hong Lin
Crop Dis., Pests, & Genetics Res. Unit
USDA, ARS
Parlier, CA 93648

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

ABSTRACT

This year six crosses for Pierce's disease (PD) resistant table and raisin grapes using seedless parents were made, resulting in 2,398 ovules and 747 embryos. These crosses were based on *Muscadinia* and *V. tiliifolia* sources of resistance. Nineteen seeded crosses consisting of 3,950 emasculations and 49 bagged clusters were made. These crosses were based on *V. arizonica, Muscadinia,* and southeastern United States (SEUS) sources of resistance. Over 120 selections have been made based on fruit quality and are ready for greenhouse screening for resistance to PD. Two BC2 families from 89-0908 *V. arizonica* source of resistance segregated in a 1:1 ration for resistance:susceptibility, based on molecular markers associated with the *PdR1* locus. A smaller family from the same source of resistance had an unexpectedly small number of resistant seedlings. DNA samples have been collected from 154 seedlings from the C33-30 x BD5-117 family and are ready for SSR marker analysis. Additional seedlings are being produced to increase probability of identifying markers associated with this source of resistance.

INTRODUCTION

Pierce's disease (PD) has existed in California at least since the late 1800s when it caused an epidemic in Anaheim. A number of vectors for PD already exist in California causing its spread. The introduction of the glassy-winged sharpshooter (GWSS) to California in the 1990's increased the spread and damage caused by PD. Other vectors exist outside California and are always a threat. All of California's table and raisin grape cultivars grown commercially are susceptible to PD. An effective way to combat PD and its vectors is to develop PD resistant varieties so that PD epidemics or new vectors can be easily dealt with. PD resistance exists in a number of *Vitis* species and in *Muscadinia*. PD resistance has been introgressed into grape varieties in the southeastern United States, but fruit quality does not match the *Vitis vinifera* table and raisin grape cultivars grown in California. Greenhouse screening techniques have been improved to expedite the selection of resistant individuals (Krivanek et al. 2005, Krivanek and Walker 2005). Molecular markers have also been identified that make selection of PD resistant individuals from *V. arizonica* in these families even quicker (Krivanek et al. 2006). The USDA, ARS grape breeding program at Parlier, CA has developed elite table and raisin grape cultivars and germplasm with high fruit quality. This collaborative research gives the unique opportunity to develop high quality PD resistant table and raisin grape cultivars for the California grape industry.

OBJECTIVES

1. Develop PD resistant table and raisin grape germplasm/cultivars with fruit quality equivalent to standards of present day cultivars.
2. Develop molecular markers for *Xylella fastidiosa (Xf)*/PD resistance in a family (SEUS) other than those from *V. arizonica.*

RESULTS

Objective 1

This year the seedless embryo culture crosses concentrated on using the *Muscadinia* source of resistance and a unique source of resistance from *V. tiliifolia*. Six crosses were made for a total of 2,398 ovules cultured (Table 1). A total of 748 (31%) embryos were extracted and transplanted on fresh medium for growth into plants. Nineteen seeded crosses were made for PD resistance (Table 2). For five of the crosses, 3,950 emasculations were made and 49 clusters were bagged for 14 additional crosses which had female flowered parents. Fruit has been harvested and seeds are being extracted for germination in January. The number of seeds produced from each resistant source was: 1,881 from *V. arizonica*; 1,643 from *Muscadinia*, and 2,071 from SEUS with an additional 184 from BD5-117 BC1. Over 120 selections have been made based on fruit quality from populations made for PD resistance. These families are from resistant sources different than the *V. arizonica* source of resistance. These selections are in line for PD testing in the greenhouse. Three families (89-0908 *V. arizonica* source of resistance) produced in 2005 were tested for molecular markers associated with PdR1 locus on chromosome 14

(Table 3). Families 05-5551 and 05-5501 segregate in a 1:1 ratio. Family 05-5502 does not fit the same segregation ratio having only 16.7% resistant plants.

Table 1. 2006 table and raisin grape PD resistant seedless x seedless crosses and the number of ovules and embryos produced.

Female	Male	Type		No. berries Picked	No. berries Opened	No. Ovules	No. Embryos
Muscadinia source of resistance							
A90-45	Scarlet Royal	Table	BC1	171	171	319	86
A90-45	A85-40	Table	BC1	353	353	433	114
A90-45	Diamond Muscat	Raisin	BC1	260	247	275	76
A90-45	B82-43	Raisin	BC1	404	390	350	85
V. tiliifolia source of resistance							
C33-30	IAC572	Table	F1	593	536	610	118
B82-43	IAC572	Raisin	F1	315	315	411	158
SEUS source of resistance							
C33-30	BD5-117	Genetic family		460	430	631	118

Table 2. 2006 table and raisin grape PD resistant seeded x seedless crosses and the number of seeds produced.

Female	Male	Type		No. Emasculations	No. Seeds
89-0908 *V. rupestris* x *V. arizonica*					
A81-17	B28-126	Table	BC2	4 bags (a)	446
A81-17	C45-64	Table	BC2	4 bags	390
A81-17	Y150-14	Table	BC2	3 bags	396
A81-17	Y315-54	Table	BC2	4 bags	96
A81-138	A95-21	Raisin	BC2	3 bags	106
A81-138	B82-43	Raisin	BC2	3 bags	124
A81-138	C81-116	Raisin	BC2	3 bags	193
A81-151	B82-43	Raisin	BC2	3 bags	9
A81-151	C81-116	Raisin	BC2	3 bags	8
A81-151	A95-21	Raisin	BC2	3 bags	113
Muscadinia source of resistance					
A90-37	C45-64	Table	BC1	5 bags	787
Bloodworth	A51-50	Raisin	F1	5 bags	856
SEUS source of resistance					
Z74-26-1	Autumn Royal	Table	F1	1005	375
Z74-26-1	A63-85	Raisin	F1	997	820
Z74-26-1	B82-43	Raisin	F1	1072	800
Z70-8-1	C57-94	Table	F1	3 bags	76
Z70-8-1	B82-43	Raisin	F1	3 bags	-
SEUS BD5-117 source of resistance					
A104-29	B28-126	Table	BC1	451	103
A104-29	C45-64	Table	BC1	455	81

(a) Parents with female flowers were not emasculated, only bagged and pollinated.

Table 3. Determination of seedling resistance based on molecular markers for 89-0908 BC2 families.

Family	Type Cross	No. Resistant (a)	No. Resistant? (b)	No. Susceptible (c)	Off type
05-5551	Raisin	40	13	45	8
05-5501	Table	28	12	26	0
05-5502	Table	4	8	20	0

(a) Resistant = marker on both sides of *PdR1* region.
(b) Resistant? = marker on one side of *PdR1* region.
(c) Susceptible = no markers.

Objective 2

The PD resistant grape selection BD5-117 from Florida was hybridized with the seedless table grape selection C33-30 and a family with 154 individuals produced. Initially, 20 plants were evaluated in the greenhouse for resistance to PD and 10 were found resistant with very low bacteria counts and PD symptoms. One hundred fifty-four DNA samples have been extracted and are ready for screening against SSR primers. Fruit from these individuals has been collected and is being evaluated for berry size, seed/trace weight and fruit characteristics. The cross was repeated this year to increase the number of individuals in the family (Table 1). A total of 631 ovules from the seedless parent C33-30 were cultured and 118 embryos have been extracted and sub-cultured to be grown into plants.

CONCLUSIONS

Populations for the development of PD resistant seedless table and raisin grape cultivars continue to be produced from seedless and seeded parents. Sources of resistance in addition to *V. arizonica* are being used. Over 120 selections have been made based on fruit quality and are ready for greenhouse testing for resistance to PD. Two BC2 families from 89-0908 *V. arizonica* show a 1:1 segregation ratio for resistance:susceptibility resulting in a total of 68 resistant seedlings from these two families.

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FUNDING AGENCIES

Funding for this project was provided by the University of California Pierce's Disease Grant Program and the Consolidated Central Valley Table Grape Pest and Disease Control District.

MECHANISMS OF PIERCE'S DISEASE TRANSMISSION IN GRAPEVINES: XYLEM PATHWAYS AND MOVEMENT OF *XYLELLA FASTIDIOSA*

Project Leaders:
Thomas L. Rost
University of California
Davis, CA
tlrost@ucdavis.edu

Mark A. Matthews
University of California
Davis, CA
mamatthews@ucdavis.edu

Cooperator:
J.W. Hudgins
University of California
Davis, CA
jwhudgins@ucdavis.edu

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ABSTRACT
This progress report shows that open pathways likely exist for *Xylella fastidiosa* (*Xf*) movement across grafts in grape stems via xylem pathways. Studies thus far have been conducted on grafted and non-grafted three-year-old *Vitis vinifera* cv. Chardonnay plants. The movement of air was used to determine if open xylem conduits were present through grafts into canes, and the length of these pathways was measured. It was determined that connections via xylem vessels are generally about twice as long in non-grafted plants (450 mm) compared with grafted plants (225 mm). Current investigations are underway with dilute use latex paint and tagged *Xf* to understand the pathways for bacterial movement across grafts.

INTRODUCTION
Grapes are one of the important crop plants in which the shoots of one variety are grafted to root stocks of another to generate plants with the desired characteristics of both. Reports have clearly shown the presence of long, open xylem conduits that connect stems to leaves in chardonnay (Thorne et al., 2006; Chatelet et al, in press). Anatomical studies have also indicated that *Xylella fastidiosa (Xf)* appears to be primarily restricted to xylem vessels in canes, however little is known about the vessels, and subsequently the movement of *Xf* across grafts. The capacity for *Xf* to move in plants differs among species ranging from generally unrestricted throughout the major organs, to only a few centimeters from the original inoculation point. The objectives of this study are to examine the connection of vessels from canes into stems through grafts, and determine if it is possible for *Xf* movement to occur freely across these grafts. To meet these objectives a strategy of air and latex paint are being implemented to study open anatomical systems, and most importantly the use of *Xf* to examine movement across grafts.

OBJECTIVES
1. Conduct a study of connections in grafted *Vitis vinifera* cv. Chardonnay, and determine if open vessel systems allow movement of *Xf* across grafts via air pressure.
2. Conduct an anatomical study of connections in grafted *Vitis vinifera* cv. Chardonnay, and determine if open vessel systems allow movement of *Xf* across grafts with latex paint.
3. Use PCR to determine the presence of *Xf* across graft unions after inoculation at known positions relative to the graft.

RESULTS
Following inoculation in grapevine, *Xf* moves in the nutrient poor xylem vessels and eventually causes disease symptoms that result plant death by unknown mechanisms. Previously, reports from our labs have indicated that bacteria can move freely in canes and from petioles into leaves during a systemic infection process. It is of interest to determine the movement across grafts to clarify movement into stems and possibly into root systems. Our preliminary results indicate that the graft unions of *Vitis vinifera* cv. Chardonnay do indeed contain continuous vessels; however, the open system length into canes is about ½ of that when compared with non-grafted plants of the same cultivar (Figure 1A). Measurements collected of cane length and associated open conduits appeared significantly different between grafted and non-grafted plants (Figure 1B). Differences were not found to be significant in stem length between grafted and non-grafted plants (Figure 1C). These results indicate that graft unions would not be an impediment to bacterial movement, and that *Xf* would be able to move further distances across in non-grafted areas of the plant because of the presence of continuous vessels. This study of the xylem structure will be further evaluated with current studies to determine the connective pathway of air movement by latex paint, and confirm that *Xf* can be moved through the vessels in the presumed transpiration stream.

CONCLUSIONS
From our preliminary results, graft unions in stems do not appear to restrict the movement of *Xf* in *Vitis vinifera* cv. Chardonnay. Although the length of open vessels is reduced by about ½, open vessels cross the graft union as determined by air movement. However, the numbers of vessels that cross the graft are less than 10% in distribution when compared with non-grafted plants (data not shown). In order for *Xf* to move from a cane or leaf across a graft it would need to be inoculated into a vessel that happens to extend through the graft union, or the bacteria would need to degrade membranes to move into adjacent vessels through bordered pits. Current studies with paint and PCR detection of *Xf* will confirm these results.

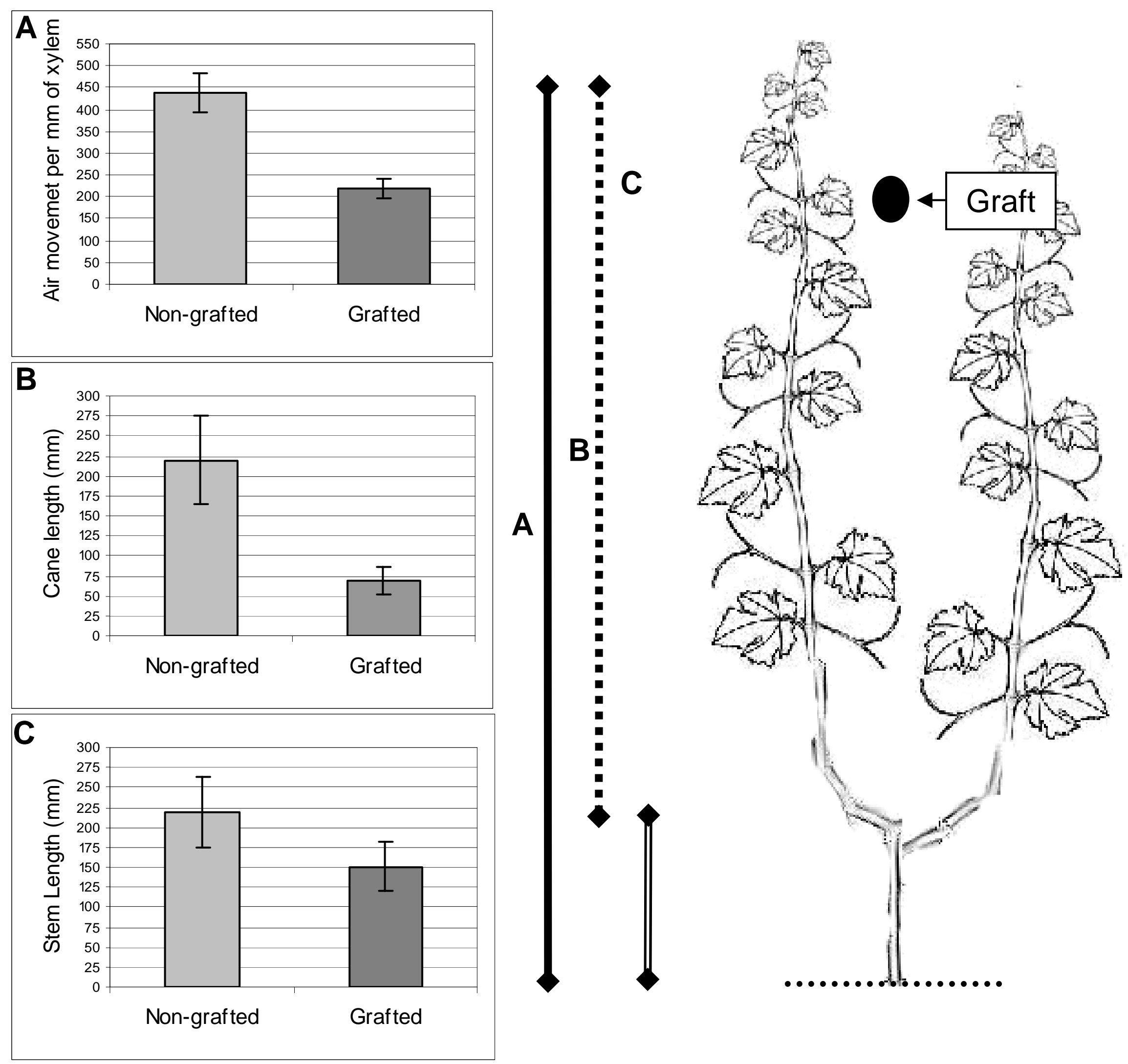

Figure 1. Air movement through graft system. Results indicate that the graft unions of *Vitis vinifera* cv. Chardonnay contain continuous vessels. **(A)** The open system length into canes is about ½ of that when compared with non-grafted plants of the same cultivar. **(B, C)** Cane length measurements are significantly different between grafted and non-grafted plants, however differences were not found to be significant in stem length between grafted and non-grafted plants **(C)**.

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FUNDING AGENCIES

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

MECHANISMS OF PIERCE'S DISEASE TRANSMISSION IN GRAPEVINES: THE XYLEM PATHWAYS AND MOVEMENT OF *XYLELLA FASTIDIOSA*. XYLEM STRUCTURE AND CONNECTIVITY IN GRAPEVINE SHOOTS PROVIDES A PASSIVE MECHANISM FOR THE SPREAD OF BACTERIA IN GRAPE PLANTS INFECTED WITH PIERCE'S DISEASE

Project Leaders:
Thomas L. Rost
Section of Plant Biology
University of California
Davis, CA 95616
tlrost@ucdavis.edu

Mark A. Matthews
Dept. of Viticulture & Enology
University of California
Davis, CA 95616
mamatthews@ucdavis.edu

Cooperator:
David S. Chatelet
University of California
Davis, CA 95616

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ABSTRACT
This progress report shows that there is a difference in the structure of xylem pathways available for *Xylella fastidiosa* (*Xf*) movement in host plants known to support systemic bacterial movement and those that don't. In addition to a reduced inter-organ connectivity, non-systemic species also show a shorter path available for bacterial movement in the leaves. However, systemic and non-systemic species show similar rates of tylose formation, signifying that tyloses don't seem to be responsible for the lack of *Xf* movement in the non-systemic plants. To be more conclusive, more xylem characteristics from the different hosts are being examined

INTRODUCTION
Xylella fastidiosa (Xf) capacity to move in plants differs greatly among species (Purcell, 2004), ranging from moving everywhere in the stem and leaves to only a few centimeters from the infection point. Our lab showed the presence of long xylem conduits from stem to leaves in grape cultivars chardonnay and cowart (Thorne et al., 2006; Chatelet et al, in press) and we recently reported that these conduits seemed to be shorter in alternate hosts in which bacterial movement is limited. A higher number of tracheids, shorter and narrower vessels, spatial organization of the vessel and of the paratracheal parenchyma cells could be a passive strategy to limit bacterial movement. Another strategy of the non-systemic species could be to confine the bacteria to a limited area by a more timely production of tyloses or, in the case of asymptomatic species showing systemic bacterial movement, to limit the population size under a harmless threshold. The objectives of this study are to carefully study the comparative anatomy of different species of plants which support a range of *Xf* population sizes and movement characteristics. Our hope is to understand how the xylem network might control bacterial movement in susceptible plants.

OBJECTIVES
1. Conduct an anatomical comparison of plant species that support high, medium and low population sizes of *Xf*.
2. Conduct an anatomical comparison of plant species that show systemic movement of *Xf* vs. those that do not.

RESULTS
A range of species was examined: with a high infection rate, high bacterial population and showing systemic movement: *Vitis vinifera* cv. Chardonnay and *Vitis vinifera* cv. Cabernet sauvignon; one species with a high infection rate, medium bacterial population and showing systemic movement: *Ipomoea purpurea* (morning glory), *Vinca major* (periwinkle), *Citrus sinensis* (Orange), *Prunus anygdalus* (Almond), and species showing non-systemic movement: *Alnus rhombifolia* (white alder), *Umbellularia californica* (california laurel), *Artemisia douglasiana* (mugwort) and *Chenopodium quinoa* (quinoa), *Datura wrightii* (datura), *Eucalyptus globules* (eucalyptus).

Stem-petiole-leaf lamina connectivity - Grape shoots have open xylem conduits that allow the passive movement of GFP-*Xf* from the stem to 50-60% of the leaf length through the primary xylem (Rost et al., PD symposium report 2005; Chatelet et al., in press). The xylem of several different plant species harboring *Xf* was examined using air and paint injection to determine if similar xylem conduits exist. When loaded at the base of the petiole, air and paint traveled to various extents into the leaf blade of all examined species (Figure 1).

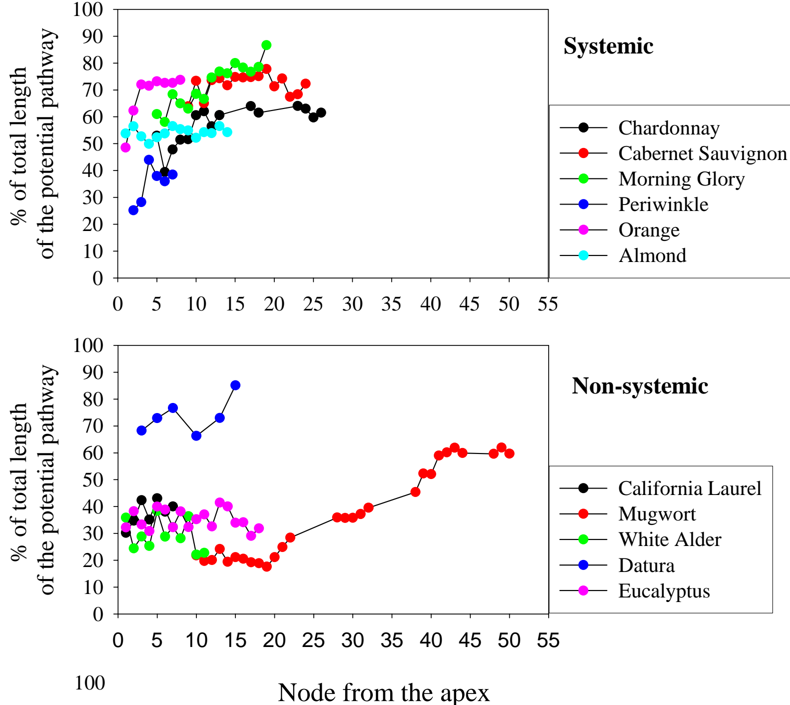

Figure 1. Air and paint position in the leaf veins calculated as percentage of the total length of the vascular pathway from the base of the petiole to the tip of the leaf. Air and paint were loaded at the base of the petiole of leaves from different nodes.

Although variations from the mean were large, species categorized as supporting the systemic spread of *Xf* seemed to have longer open conduits compared to the non-systemic plants. Another important difference between systemic and non-systemic species resides in the continuity of these conduits between the stem and the leaves. In most of the non-systemic hosts, air or paint moved only into the first leaf above the stem loading point as opposed to several leaves in systemic species. A more detailed investigation of the anatomy of the stem-leaf connection is currently undertaken.

Leaf shape and vascular network

In addition to the importance of the stem-leaf connection, the shape and venation pattern of the leaves might also be important criteria to the movement of *Xf* into the leaves. The comparison of leaves of the different hosts showed a variety of shape and venation pattern and didn't reveal any obvious relation between bacterial movement and leaf morphology.

Xylem structure of the stem and the petiole

Similarly, xylem structure was variable among species. There was no evident relation between the stem and petiole xylem structure and bacterial movement. The only difference could be in the size and number of vessels. This is currently being investigated.

Tyloses formation

In addition to the xylem connectivity (see previous reports), comparison of tylose formation and development between grapevine and alternate hosts was studied. Bacterial movement in the alternate hosts could be impeded by rapid tylose formation as opposed to grapevine where tylose development occurs after colonization by the bacteria. Stems of similar age from the species mentioned above were wounded with a needle to imitate the insect. Stem segments were collected at 0, 1, 3 and 6 days after wounding and the presence of tylose in the vessels was observed in cross-sections made within the wounded area, 5 mm above it, 10 mm above and 100 mm above the wound. For each distance, the proportion of vessels partially filled or completely occluded by tyloses was calculated. The results showed that there are no differences between systemic and non-systemic plants.

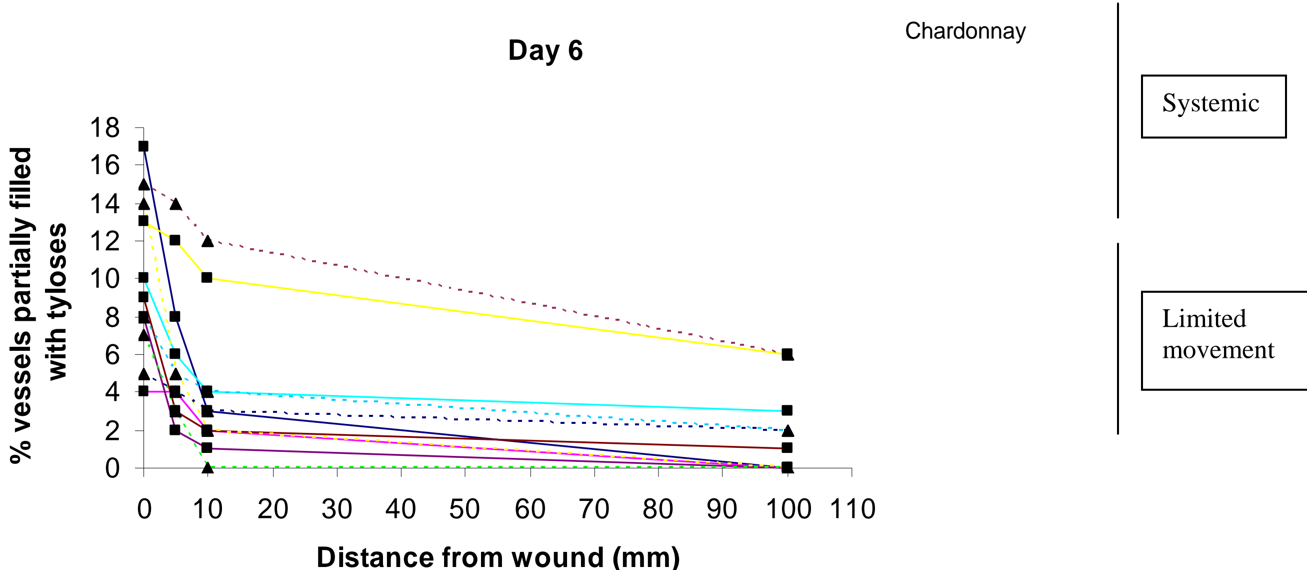

Figure 2. Percentage of vessels partially filled with tyloses (A, C, E) six days after the wounding.

CONCLUSION

Our results show that there are differences in xylem structure between systemic and non-systemic species. Indeed it seems that non-systemic species have shorter and narrower vessels in the stem, a lower connectivity between stem and leaves and a shorter path within the leaves. In addition, tylose formation didn't seem to be the cause for the limited *Xf* movement in the non-systemic plants. The species examined didn't produce more tyloses or faster.

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FUNDING AGENCIES

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

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

Project Leaders:

Ken A. Shackel
Dept. of Plant Sciences
University of California
Davis, CA 95616
kashackel@ucdavis.edu

John Labavitch
Dept. of Plant Sciences
University of California
Davis, CA 95616
jmlabavitch@ucdavis.edu

Cooperators:

Mark Matthews
Dept. of Vitic. and Enol.
University of California
Davis, CA 95616

L. Carl Greve
Dept. of Plant Sciences
University of California
Davis, CA 95616

Jeffrey Walton
NMR Facility
University of California
Davis, CA 95616

Alonso Pérez
Dept. of Plant Sciences
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted October 1, 2005 to September 30, 2006.

ABSTRACT

Our report in the 2005 Pierce's Disease Research Symposium (Shackel and Labavitch, 2005) demonstrated the value of using MRI to follow the development of cavitations in grapevine xylem following introduction of *Xylella fastidiosa* (*Xf*) via needle inoculation. Strong correlations between increasing proportions of stem xylem conduits that were cavitated, visualized using MRI, and decreased water conductance. Similar observations were made on stems after treatment of vines with ethylene, supporting, but not proving, the suggestion of our Pierce's disease (PD) development model (Labavitch et al., 2005) that vine ethylene production was an important factor in the development of *Xf* infection. This year's effort has been devoted primarily to the replication of the observations of the first two years of this project. In addition, we have continued with the development of conventional, destructive anatomical approaches that we will use to define the nature of the more permanent xylem obstructions, gels and tyloses, whose presence in the water conduits of infected vines may be associated with the occurrence of cavitations. Finally, after considerable delay, we can report that our collaboration with colleagues in the University of California, Davis (UCD) NMR Facility to develop an NMR probe designed for ease of use in grapevine imaging and greater resolution appears to be on course.

INTRODUCTION

Results from several Pierce's Disease (PD) research programs reported in the 2001 to 2005 PD research symposia in San Diego have supported the idea that obstructions in the grapevine's water-transporting xylem tissue develop rapidly post-inoculation, before an appreciable bacterial population has been established. Thus, careful analysis of the timing of changes in xylem element anatomy and function relative to *Xylella fastidiosa (Xf)* introduction and the appearance of the external symptoms of disease development, is important for establishing reliable indicators of the "stage" of PD development. Because the more conventional destructive analyses of xylem function made it impossible to fully understand the progression of internal symptoms and loss of grapevine water-conducting capacity with symptom appearance, we began testing the possibility of using MRI to follow xylem function in individual vines over time. In the course of this study we have developed imaging techniques for obtaining quantitative information about xylem function in individual vine internodes over time, defined the limitations of these techniques, and demonstrated that both PD infection and ethylene treatment trigger decreases in vine water-moving capacity (Shackel et al., 2005; Pérez-Donoso, 2006; Pérez-Donoso et al., 2006). The techniques we have developed also have been used in our tests of a model for PD development (Figure 1; Labavitch et al., 2005). We are currently attempting to define the relationship of the tracheid and vessel cavitations revealed by MRI and other, less transient occlusions that develop in the xylem of *Xf*-infected grapevines.

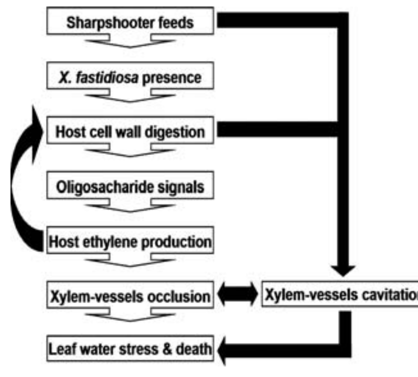

Figure 1. Hypothetical model for PD development. PD starts with infection caused by the glassy-winged sharpshooter's introduction of *Xf* locally (i.e., into one or a few vessels). Once *Xf* is in the xylem the bacteria become systemic, which implies that *Xf* must be able to cross (digest away?) the 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 an increase 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.

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 *Xf* 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 *Xf* inoculation or introduction by the glassy-winged sharpshooter.

RESULTS

Objective 1. Optimization of MRI for visualizing water transport deficiencies in PD-infected grapevines.
Some progress has been made on this objective in the last few months. In fact, this objective has been a problem for two years. We had planned to have a prototype of an MRI coil optimized for use in imaging water in grapevine xylem at the end of year one of this project. This part of the work was developed with cooperator Dr. Jeffrey Walton of the UCD NMR Facility. Jeff developed the plans for the coil and then a series of losses of suppliers of key parts and the failure of the outside group that was to do the fabrication to deliver have stalled things. We are now nearing the end of the project's final year and there is no coil thus far. However, Dr. Walton recently redrew the detailed plans for the new coil and they are now being examined by the group that will do the fabrication. We are hopeful that the "advanced" coil will be available before much more delay.

Alonso Pérez has now finished his Ph.D. program (Pérez-Donoso, 2006) and returned to a faculty position in Santiago, Chile. We will have funds left in the project budget and will ask for a no-cost extension. Dr. Qiang Sun, who has been working with Professors Tom Rost and Mark Matthews in their PD research projects, will join our group on October 1, 2006. Qiang has some experience with MRI and will take the MRI training program offered by the UCD NMR Facility in Fall quarter. He will then continue with the remaining work in this project and work in a new project ("The pit membrane barrier to *Xf* movement in grapevines: Biochemical and physiological analysis") that was approved this year.

Objective 2. Test for correlations of observed vascular system obstructions with predictions based on MRI data and **Objective 3. Use of MRI to follow the development of grapevine obstructions over time in vines infected with *Xf* or treated with ethylene.**
A series of greenhouse-grown 'Chardonnay' grapevines was inoculated with *Xf*, enclosed in chambers and treated with ethylene for 48 h, or left untreated (controls). MRI was used to follow the development of cavitations over time. At intervals, imaged stems were marked to indicate where cavitations had been observed, excised fixed and sectioned. The intent is to develop a thorough histochemical analysis sections taken along the lengths of these stems to determine whether there is a correlation between the positions of tyloses and vascular system gels and points along the stems where a great deal of cavitation has been seen. This effort was begun as a collaboration involving Pérez, Dr. Katy Pinney in Prof. Vito Polito's lab (Plant Sciences Department), and undergraduate researcher Joshua Lenhof. Following Pérez' departure, Pinney and Lenhof have continued the work. The final correlations of cavitations, tyloses and gels have not been developed because that requires completion of the full histochemical analysis. However, the histochemical work has made clear that PD and ethylene treatment also influences the formation of readily distinguishable xylem obstructions (Figure 2).

A manuscript describing much of the work done in the three years of this project (Pérez-Donoso et al., 2006) is now being reviewed by the journal *Plant Physiology*.

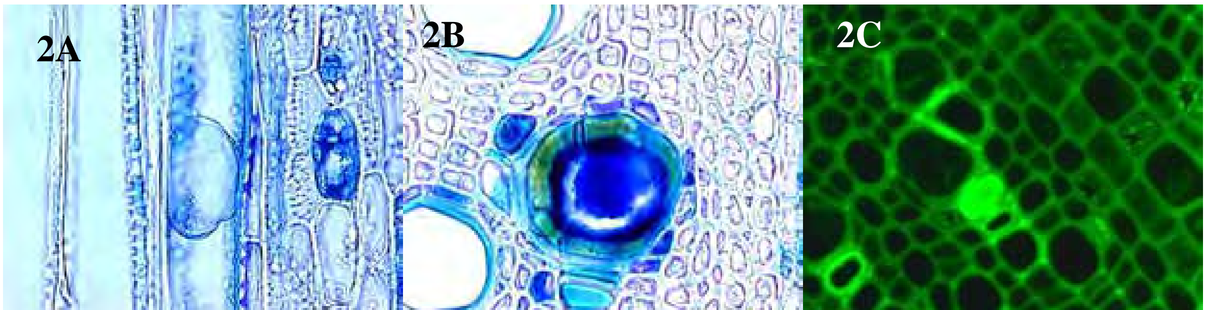

Figure 2. Sections were taken from a region of a PD-infected stem in which MRI analysis had shown a relatively high amount of xylem cavitation. **A.** A longitudinal section of the stem showing an open vessel adjacent to a vessel blocked with a tylose. **B.** A cross-section of the stem showing a xylem conduit blocked with what appears to be a non-cellular, vascular system gel. Panels **A** & **B** are stained with the non-specific stain toluidine blue. **C.** A cross section through stained with coryphosphine-O. The bright green fluorescence indicates the presence of pectin. The conduit in the center of the panel is filled with a pectin-rich gel.

Objective 4. Use of MRI to determine whether localized xylem cavitation occurs at the site and time of *Xf* inoculation or introduction by the glassy-winged sharpshooter.

The work to determine whether GWSS feeding on grapevines is accompanied by xylem cavitation has begun (see the report for the project "Linking the model of the development of PD in grapevines to an understanding of the dynamics of GWSS transmission of *Xf* to grapevines and grapevine gene expression markers of PD" in these *Proceedings*). The work has not progressed to the point where MRI analysis is needed.

CONCLUSIONS

We expect that our combined approach (use of non-destructive and destructive methods) to study xylem function will determine which kinds of disruption (tyloses, pectin gels, or air embolisms) predominate in PD-infected stems and describe the developmental progression of vascular system occlusions that occur during the different stages of the disease.

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

Project Leaders:

Andrew Walker
Dept. of Viticulture and Enology
University of California
Davis, CA 95616
awalker@ucdavis.edu

Summaira Riaz
Dept. of Viticulture and Enology
University of California
Davis, CA 95616

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT

Development of a framework simple sequence repeats (SSR) genetic linkage map based on the 181 genotypes of 9621 family, which segregates for Pierce's disease (PD) resistance is complete. The current genetic linkage map consists of 236 non-AFLP markers (SSR, EST-SSR and ESTP-RFLP) in 19 linkage groups. The PD resistance locus, *PdR1*, maps to linkage group 14 (LG - essentially a chromosome) of the male parent (F8909-17), which now consists of 30 markers, nine of which are localized within 10 cM (very closely) of *PdR1*. The 9621 mapping population was expanded from 181 to 457 genotypes. A total of 13 markers polymorphic for F8909-17 mapped to LG 14 and were added to 276 segregants (core population set is 457). We also screened an additional 400 seedlings with two markers (one on either side of *PdR1*) and a total of 50 unique recombinant plants were planted in the field. To avoid confounding affects of resistance inherited from D8909-15 (which is also highly resistant, but with a very different form of resistance) the 04-190 population was selected and a map of LG 14 with 220 genotypes was completed. 04-190 is a cross of *V. vinifera* F2-7 (Cabernet Sauvignon x Carignane) x F8909-08 (sibling of F8909-17). We have used F8909-08 extensively in PD resistant wine and table grapes, therefore it is necessary to validate that *PdR1* gene segregates 1:1 in progeny from its crosses. We completed greenhouse screening of 160 genotypes from the 04-190 population to verify the molecular marker results. The *PdR1* resistance locus segregates 1:1 and mapped to the same position with surrounding markers ctg1025882 and VMCNg2b7.2. We also increased the core population of 04-190 from 220 to 395 seedling plants. Leaf tissue for DNA extraction and green cuttings for greenhouse testing and ELISA screening from the additional 175 plants were collected in late summer, and results are expected in early spring 2007.

Efforts to construct a bacterial artificial chromosome (BAC) library from b43-17 (the basis of the *PdR1*) were initiated. A total of 200 green cuttings were collected that resulted in 160 plants that are being cultivated for young etiolated shoot tips that provide an excellent source of DNA for the BAC library. This BAC library is being developed to provide markers from BAC end sequencing for LG 14, so that we can create a physical map of the *PdR1* gene family, which will lead to genetic engineering efforts. We are also working to add resistance gene analogs (RGA) markers, which are generalized genetic sequences involved in a wide range of pest and disease defense responses in plants, to our genetic maps. The addition of these markers may identify common regions of disease resistance and possible functions of the *PdR1* gene family.

In order to understand the stability and segregation of PD resistance from different sources, work on six different mapping populations was completed. We are also continuing mapping efforts in the 0023 population, a cross of D8909-15 x *V. vinifera* B90-116, to identify quantitative trait loci (QTL) and then saturate linkage groups with these QTLs with more markers. This population is important because we have extensive data for cluster and berry traits, and *Xylella fastidiosa* (*Xf*) resistance data for about 200 plants. We completed the characterization of Mexico collection, the source of the exceptional resistance to *Xf* and collected by Dr. Olmo in 1960. We are using these unique selections in our genetic and molecular breeding to produce PD resistant table and wine grape cultivars.

INTRODUCTION

We have been mapping resistance to *Xylella fastidiosa (Xf)* in three (9621, 0023, and 04-190) populations, and to *Xiphinema index*, the dagger nematode in two (9621 and 0023) populations. The preliminary AFLP-based 9621 genetic map has been published (Doucleff et al. 2004). The 9621 population was then mapped with the more informative microsatellites or SSR markers, which provide a more reliable and repeatable framework for initial mapping of candidate genes and QTLs. In addition, tightly linked SSR markers are ideal for marker-assisted selection (MAS) due to their applicability across different genetic backgrounds and ease of use. This year, mapping efforts within the 9621 and 04-190 populations have concentrated on linkage group 14 that harbor the *PdR1* resistance locus (Krivanek et al. 2006; Riaz et al. 2006). The addition of SSR markers to this linkage group was greatly aided by the existence of other SSR-based genetic maps of grape that have been developed within *V. vinifera* populations and by the availability of expressed sequence tag polymorphism (ESTP) markers developed by other grape researchers and available on various genetic databases. e are now initiating construction of a BAC library. A high quality BAC library with good coverage is essential for the isolation of the BAC clones that harbor *PdR1* resistant genes. BAC end sequencing of these clones will allow us to develop a physical map in conjunction to genetic map, develop more markers around the *PdR1* region, and lead genetic engineering of susceptible *V. vinifera* grapes with the *PdR1* gene.

OBJECTIVES

1. Develop a fine scale genetic linkage map around the *Xf* resistance locus in D8909-15 x F8909-17 (9621) segregating for *Xf* resistance. COMPLETED
2. Add markers associated with *PdR1* from linkage group 14 (9621 map) to 400 additional 9621 individuals (more individuals, more recombinants, more refined genetic map).
3. Screening of additional EST derived SSR markers for which functions are known and shift focus to EST-SSR markers isolated from the resistant genotype D8909-15. COMPLETED
4. Screen resistant gene analogue markers (RGA), if polymorphic add them to the core of 9621 map.
5. Initiate development of a BAC library from the resistant genotype b43-17 (the source of *PdR1*).
6. Study marker segregation linked to *PdR1* in different genetic backgrounds. Initiate genetic mapping of the 04-190 population (*V. vinifera* F2-7 x F8909-08) with markers on linkage group 14. Apply this information to further refine the MAS process and assist the ongoing winegrape breeding efforts. Increase the core population of 04-190 population to 400 plants.

RESULTS

Objective 1

Completed. This project was initiated with an AFLP-based genetic map developed from 116 individuals from the 9621 population (Doucleff et al. 2004). The framework map of the 9621 population is now complete with 236 markers (primarily SSR, 210 mapped and 26 linked). The consensus map spans 1154 cM in 19 linkage groups. LG 14 is largest group with 30 markers. Fifteen markers were closely associated to the PdR1 locus. The average distance between markers is 5.5 cM (Riaz et al. 2006). The framework map contains 60 new functionally associated EST-SSR and EST-RFLP markers that have not been mapped on any other published grape map.

Objective 2

We previously reported on the genetic map based on original core set of 181, which we are expanding to 457 genotypes, the largest population maintained for map-based positional cloning of genes in the grape research community. We used tightly linked markers to screen an additional 276 genotypes and chose a sub set of 60 genotypes (primarily recombinants with a few resistant and susceptible genotypes as controls), currently being screened with results expected fall 2006. This increased number of individuals should help us refine the position of *PdR1* locus. Fine scale placement of markers in relation to a resistance locus is the first step toward the screening of BAC library clones that contain the resistance gene. The "map-based positional cloning of genes" approach relies on solid genetic map and it has been effectively used in many organisms to clone genes of interest. We also screened an additional 400 9621seedlings for two markers flanking *PdR1* to find more recombinants. Fifty recombinants were found, they were planted in the field, and screening is underway to determine the linkage phase of markers to the *PdR1* locus. Markers from LG 14 will be added to these genotypes and they will become part of the core population. A complete map of LG 14 with 450 genotypes will be presented in spring 2007 report.

Objective 3

Completed. The 9621 framework map has more than 40 EST-SSR markers developed by the Genome facility, University of California, Davis; seven out of 30 markers on LG 14 are EST-SSR markers. These markers have been annotated with known functions after being compared to available databases. The nucleotide sequence of these larger fragments of DNA will help with the BAC library screening to isolate clones that harbor the *PdR1* resistance locus. We completed screening of an additional 50 EST-SSR markers with known function and polymorphic markers were added to the 9621 and 04-190 populations.

Objective 4

Resistant gene analogue (RGA) markers have now been reported for many organisms. The theory behind RGAs is that a surveillance system of receptors encoded by R genes reacts in a general way against all pathogens (viruses, bacteria, fungi or nematodes). The R gene products react with the products of *Avr* genes or with general bacterial elicitors. Once binding of a ligand modifies the receiver domain, NBS or STK domains become available for down-stream components of a signal transduction and initiate defense responses. Two reports have been published on grape using degenerate primers of conserved sequences from different classes of R genes to isolate RGA homologs, and then develop these homologs into STS (sequence tagged sites) markers (Di Gaspero and Cipriani 2003, Donald et al. 2002). We chose a subset of 20 RGA-STS primers to screen parental samples for polymorphism. The majority of these RGA markers amplified successfully, however they were not polymorphic. We used a subset of five different restriction enzymes to find restriction site based polymorphism. Three markers were polymorphic with different restriction enzymes. They were added to the core 9621 population, but none resided on LG 14. Additional work on RGA markers is on hold until we find a better system to run the gels, such as a single strand conformational polymorphism gel system.

Objective 5
Now that we have constructed a strong genetic map for the *PdR1* locus, the next step is to develop a BAC library, which enables the isolation of the *PdR1* resistance gene(s). We choose the resistant genotype b43-17, the *V. arizonica / candicans* source of *PdR1* and *Xf* resistance, to develop this BAC library. The selection of a genotype for development of a BAC library is very critical. From our genetic analyses, we know that *Xf* resistance from b43-17 segregates as a major single locus (*PdR1* segregates in this way in both 9621 and 04-190). However the exact number of genes involved can only be determined from a physical map. Molecular marker studies of b43-17 allele sizes indicate that *PdR1* might be a cluster of very tightly linked genes. Our studies also indicate that b43-17 is heterozygous for three of the markers that are tightly associated to *PdR1*. F8909-08 and F8909-17, which both have strong *Xf* resistance, are progeny of b43-17 and they inherit different resistance alleles with these markers. This information suggests that there might be a cluster of genes associated with resistance and that the F8909-08 and F8909-17 siblings inherited different copies of the resistance genes. This makes b43-17 an even better candidate for BAC library development. We collected 200 cuttings of b43-17 to produce 160 plants. Young leaves, flower clusters and tendrils are ideal for the isolation of high quality DNA, and these tissues from the 160 plants are almost ready for extraction.

Objective 6
Because both parents of the 9621 population are *Xf* resistant, and because the D8909-15 parent contains a different, and as yet unmapped *Xf* resistance loci (derived from *V. arizonica/girdiana* b42-26), more mapping was necessary. This led to the mapping of the 04-190 population, a cross of *V. vinifera* F2-7 (Carignane x Cabernet Sauvignon) x F8909-08. We completed DNA extraction from 220 plants in the 04-190 population and a set of 37 SSR and EST-SSR markers from LG 14 were tested on small subset of eight samples (including both parents) to verify polymorphisms. Markers that were polymorphic for the parents were used on the entire 04-190 set, and the plants were greenhouse screened to verify the molecular marker results. Marker order for LG 14 was consistent between F8909-17 (9621 paternal map) and F8909-08 (04-190 paternal map) except for one marker, VMC6e1 (Figure 1). Both genotypes inherit different resistant alleles of b43-17 that might represent different copies of resistant genes. The F8909-08 LG 14 map spans 92 cM and the closest markers to *PdR1* were six cM on each side. This molecular marker work with two mapping populations developed from full sibling parental genotypes helped us to evaluate the stability, penetration and efficacy of PD resistance. It enabled us to choose easily scored, highly polymorphic markers for use in MAS for breeding PD resistant winegrapes. The results of MAS and genetic map of LG14 for the 04-190 population have been submitted for publication (Riaz et al. 2006, submitted). In addition, we expanded the 04-190 population size from 220 to 400. Leaf tissue for DNA extraction and cuttings for greenhouse testing were recently taken from 175 of these plants. DNA will be extracted from these plants and markers from LG14 will be analyzed, and they will be greenhouse tested. We hope to complete the expanded map of 04-190 population in spring 2007.

We also initiated work to study the expression, penetration; segregation and stability of resistance to PD from different genetic sources to better predict the durability of resistance in crosses. So far we have used two resistance sources (b42-26 and b43-17). The populations and genotypes examined are noted in Table 1, and their segregation patterns are reported below.

Expected or Known Segregation Patterns:
1. 9621 Population: *PdR1* single locus for F8909-17 and multiple QTLs for D8909-15.
2. 0023 Population: multiple QTLs.
3. 03-300/5 population: *PdR1* resistance segregates 1:1 (single gene model), both marker and greenhouse screen.
4. 04-190 population: *PdR1* segregates 1:1, both marker and greenhouse screen.
5. 04-191 population: *PdR1* resistance segregates 1:1
6. 04-373 population: All plants should be resistant with assumption that b43-17 is homozygous resistant for PD
7. 04-5554 population: progeny are 93.75% *V. vinifera* and an excellent test of *PdR1*expression through four backcross generations to *V. vinifera*.

It is much easier to manipulate resistance when it is inherited as a single major locus, both in terms of traditional breeding and for map based positional cloning of genes. Therefore, it is essential to understand how resistance from different sources segregates in populations. The greenhouse and marker testing of the six populations in Table 1 (9621, 0023, 03300, 04-190, 04191, and 04373), which derive *Xf* resistance from our two highly resistant backgrounds (b42-26 and b43-17), indicates that resistance coming from b43-17 segregates as single major locus and it is very unique to this genotype. Resistance from b42-26, in the 0023 population, is quantitatively inherited and appears involve multiple genes that might be present on multiple chromosomes. We used six SSR markers, tightly associated to *PdR1* in F1 populations of b42-26 and b40-14 (another resistant *V. arizonica* genotype). Both genotypes were heterozygous for all six markers and both alleles of six markers were associated to resistance in the F1 progeny. This is a very important finding, indicating that *Xf* resistance involves different mechanisms of resistance, different genes, and that resistance genes are specific to certain genotypes. Understanding of the single locus resistance mechanism in b43-17, will help us to elucidate the complex mechanisms of resistance in b42-26 and b40-14. The addition of multiple markers to the 0023 population is nearly complete. The map will contain about 230 markers, and should provide enough coverage for QTL analysis. Once linkage groups with QTLs for PD resistance are identified, we can focus on those linkage groups and saturate them with more markers.

We have completed analysis of the Olmo Mexican *Vitis* Collection, verifying the identity of these complex species and the extent to which *Xf* resistance and the *PdR1* locus exist in the population; a manuscript is in preparation. This work resolved confusion between the original campus collection and the USDA National Clonal Repository collection. Fifty-one genotypes were with the six SSR markers linked to *PdR1*, and they were greenhouse screened for *Xf* resistance. We are correlating this data to identify new resistance alleles for breeding purposes, and determine the distribution of known resistance alleles in the entire set. A set of 24 SSR markers was added to the 51 genotypes to study correlations among taxonomic descriptions, geographic location and *Xf* resistance. This manuscript is also in preparation.

Table 1. Parentage and species information for populations and genotypes being used to map PD resistance.

Population / Genotype	Species / Parentage
b42-26	*V. arizonica/girdiana*
b43-17	*V. arizonica/candicans*
D8909-15	*V. rupestris* A. de Serres x b42-26
F8909-08 and F8909-17	*V. rupestris* A. de Serres x b43-17
F2-7 and F2-35 (both females)	*V. vinifera* (Carignane x Cabernet Sauvignon)
9621	D8909-15 x F8909-17
0023	F8909-15 x *V. vinifera* B90-116
03300/5	101-14Mgt (*V. riparia* x *V. rupestris*) x F8909-08
04-190	F2-7 x F8909-08
04191	F2-7 x F8909-17
04373	F2-35 x b43-17

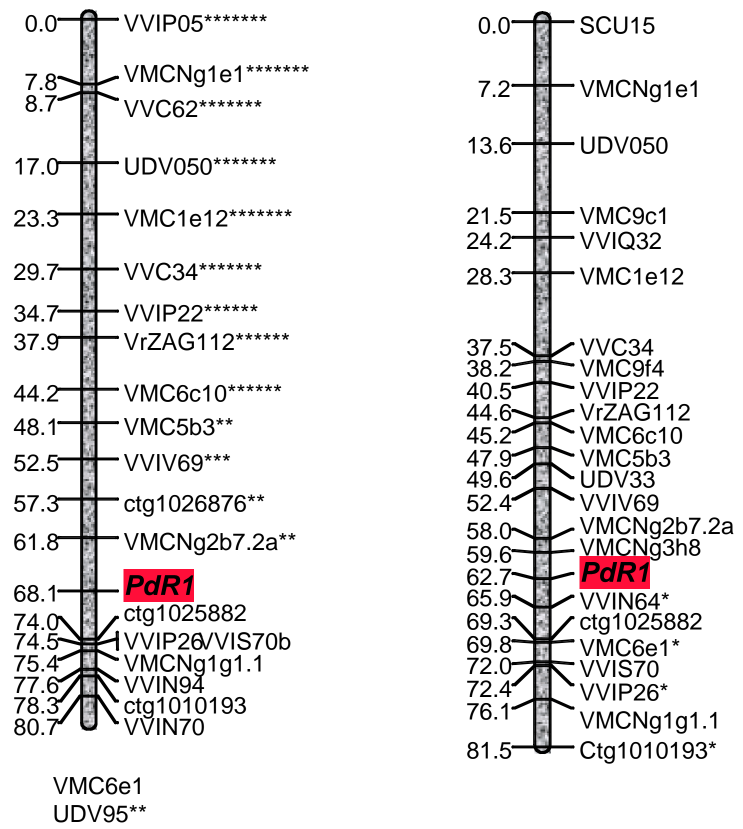

Figure 1. Genetic map of LG14 of two populations (9621 and 04-190).

CONCLUSIONS

Results from this project have allowed us to: 1) understand the segregation of PD resistance in two different backgrounds; 2) develop a framework genetic map for *Xf* resistance; 3) select markers for effective MAS for grape breeding; 4) begin development of a physical map of genomic fragments that carry resistance genes; and finally 5) work towards map-based positional cloning of genes. We are focusing on LG 14 in a variety of genetic backgrounds to verify the single gene nature of *PdR1* expression, and are using QTL analysis in the 0023 population to study resistance from b42-26. These genetic linkage maps will enable us to characterize and clone different variants of genes conferring resistance to PD, and ultimately lead to the genetic transformation of susceptible grape varieties with grape resistance genes. PD resistance makers generated in this study are also used in our breeding program to optimize and expedite selection, allowing us to screen larger populations and make more rapid progress in the production of resistant winegrapes.

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BREEDING PIERCE'S DISEASE RESISTANT WINEGRAPES

Project Leaders:

Andrew Walker
Dept. of Viticulture and Enology
University of California
Davis, CA 95616-8749
awalker@ucdavis.edu

Alan Tenscher
Dept. of Viticulture and Enology
University of California
Davis, CA 95616-8749

Reporting Period: The results reported here are from work conducted October 2005 to September 2006.

ABSTRACT

We continue to make strong progress breeding Pierce's disease (PD) resistant winegrapes. We have incorporated marker-assisted selection (MAS) for the PD resistance gene, *PdR1* (see companion report), into our breeding and reduced the seed to seed breeding cycle to three years, allowing very rapid progress towards PD resistant winegrapes. This year's crosses were focused on broadening the *V. vinifera* winegrape base in our breeding lines. We produced thousands of seed with 87.5% and 75% *vinifera* progeny. Many of our current populations have the *PdR1* allele from F8909-08; we made many crosses this year to include the alternate *PdR1* allele from F8909-17. Crosses were also made to produce a new mapping population for a collaborative project with the USDA-Parlier to allow mapping of PD resistance from the resistant Florida selection BD5-117, which will help with comparative evaluations of PD resistance genes. The best sources of PD resistance allow very low levels of *Xylella fastidiosa* (*Xf*) to develop in xylem vessels. If these were grafted onto phylloxera resistant rootstocks, the *Xf* in them might kill the rootstocks. Thus, we have made crosses to produce PD and nematode resistant rootstock, and can use MAS for both *PdR1* and the *Xiphinema index* resistance gene, *XiR1*. We made crosses to develop a number of additional mapping populations for fine-scale mapping efforts with *PdR1*. We also replicated advanced 87.5% Syrah and Chardonnay selections with *PdR1* to produce enough fruit for wine evaluation studies next fall. Finally, we are studying wine making and quality parameters at the 1L, 20L and 2,000L levels to determine which quality parameters are predictive at all scales and which will be best suited to large scale winemaking tests on single vine selections.

INTRODUCTION

This project is directed at breeding Pierce's disease (PD) resistant winegrapes with *Vitis vinifera* fruit quality and the ability to greatly suppress *Xylella fasidiosa (Xf)* populations and movement within the vine while preventing PD. California's *V. vinifera*-based vineyards are susceptible to PD and resistant varieties provide the best long-term solution to this disease. PD resistance exists in a number of *Vitis* species and in the related genus, *Muscadinia*. In addition, many resistant cultivars exist, which derive their resistance from these sources, but they lack *V. vinifera* fruit quality and the genetics of their resistance is complex, and considered to be controlled by at least three independently inherited genes (Mortensen 1968). This complex genetics greatly limits the number of resistant progeny they produce when crossed to *V. vinifera* cultivars, which dramatically slows breeding progress. However, we have discovered a unique form of resistance to *Xf* that is controlled by a single dominant locus (*PdR1*) derived from forms of *V. arizonica* (Riaz et al. 2006 and see companion report in this Proceedings by Walker and Riaz), and are using this resistance source and *PdR1* markers to rapidly backcross PD resistance into high quality *V. vinifera* winegrapes via marker assisted selection. At the same time we continue to incorporate other resistance sources to broaden the base of PD resistance.

We are uniquely poised to undertake this important breeding effort. We have developed rapid screening techniques for *Xf* resistance and have optimized ELISA and PCR detection of *Xf* (Buzkan et al. 2003, Buzkan et al. 2005, Krivanek et al. 2005a 2005b, Krivanek and Walker 2005). We have unique and highly resistant *V. rupestris* x *V. arizonica* selections, as well as an extensive collection of southeastern grape hybrids, that allow the introduction of extremely high levels of *Xf* resistance into commercial grapes. We have seed that is 87.5% *V. vinifera*, from winegrape cultivars, with resistance from our b43-17 *V. arizonica/candicans* resistance source. There are two sources of *PdR1*, siblings from b43-17. These selections – F8909-08 and F8909-17 have been introgressed into a wide range of winegrape backgrounds over multiple generations. We are also maintaining a number of lines with resistance from southeastern United States (SEUS) species. Although these lines have complex genetics and we have not been able to develop markers associated with their resistance, we maintain these lines for later crosses to broaden PD resistance.

OBJECTIVES

1. Breed PD resistant winegrapes through backcross techniques using high quality *V. vinifera* winegrape cultivars and *Xf* resistant selections and sources characterized from our previous efforts.
2. Continue the characterization of *Xf* resistance and winegrape quality traits (color, tannin, ripening dates, flavor, productivity, etc.) in novel germplasm sources, in our breeding populations, and in our genetic mapping populations

RESULTS
Objective 1
We have reduced the breeding cycle for the development of PD resistant grapes to three years (seed to seed) using the marker-assisted selection (MAS) with the b43-17 resistance sources and their progeny. Last year we attempted to reduce this cycle to two years by converting tendrils to clusters on seedlings known to have *PdR1* using cytokinins. This technique has been used in the past on male vines and on plants generated from dormant cuttings. Of the six plants treated, two produced clusters: one was female flowered which we hoped to cross to the other, but it produced malformed flowers with non-shedding adherent petals which prevented us from gathering pollen. We will expand our tendril conversion efforts to gather pollen for Spring 2007 primarily with advanced selections possessing the F8909-17 *PdR1* allele.

The 2006 pollination season focused on broadening the use of winegrapes in the F8909-08 *PdR1* allele background, and over 9,500 seeds were produced. Many more crosses were made to incorporate *M. rotundifolia* powdery mildew and PD resistance genes from *vinifera*/*rotundifolia* (VR) sources from Olmo collections. These crosses were also made to test for the presence of the *Run1* (powdery mildew resistance) marker. We also made crosses for our recently re-funded collaborative project with David Ramming (USDA- Parlier) focused on PD resistant table grapes.

2006 Crosses
The first group (Table 1a) utilized the F8909-08 allele originally from the b43-17 *V. arizonica/candicans* resistance source in a third generation backcross to produce progeny with 87.5% *V. vinifera* parentage. The second group (Table 1b) also utilizes the b43-17 resistance source and its progeny will contain 75% *V. vinifera*.

The third group of crosses (Table 1c) utilized the F8909-17 allele form also originally from the b43-17 *V. arizonica/candicans* resistance source in a second generation backcross to produce progeny with 75% *V. vinifera*.

The fourth group (Table 1d) continues our efforts to use a broad range of SEUS PD resistant cultivars. These crosses included crosses with the BD5-117 resistance source (Daytona x Stover with resistance from *V. shuttleworthii* and other SEUS species). This cross of two highly resistant selections a cross of a seedless larger berried *vinifera* table grape (C33-30) by BD5-117 and selections from the embryo rescued progeny 03187 population were crossed. This population will be used in a collaborative project with the USDA-Parlier to map PD resistance originating from BD5-117. We also crossed the resistant female 03187-80 with *vinifera* winegrapes. Other winegrape crosses utilized sibling selections from *vinifera* x the highly PD resistant DC1-39 and a series of crosses with the VR hybrid NC-11J with Cabernet Sauvignon, Chenin blanc and Tempranillo. The fifth group (Table 1e) consists of crosses we made to support our mapping efforts (detailed in the fine-scale mapping report) and increase the number of individuals in two specific mapping populations (further detailed in our companion report on fine-scale mapping of PD resistance genes).

The final group of crosses (Table 1f) was made to produce PD resistant rootstocks. These rootstocks are not necessarily expected to induce PD resistance, but are to be used with PD resistant varieties that may carry enough *Xf* to infect and potentially kill the rootstock, although the scion would be unaffected by PD. The first set of these crosses utilized PD resistance from the F8909-08 and F8909-17 source, which are also resistant to the dagger nematode vector of fanleaf degeneration. We have an excellent DNA marker for this trait, *XiR1*, allowing us to screen for both markers at once. We also used 9365-85, a rootstock selection scheduled for release in Spring 2007 with exceptional resistance to root-knot, dagger, citrus and lesion nematodes, the ability to resist multiple nematode strains, and maintain nematode resistance at 80F soil temperatures (a very rare trait). Florilush (Dog Ridge x Tampa) is a PD resistant rootstock from Florida reported to be resistant to PD and nematodes. It was crossed with two selections both containing *PdR1* and *XiR1* so we can expedite screening using MAS for two traits.

The 2006 plantings were primarily seedlings that had been pre-screened for PD resistance with *PdR1* and constitute one of the few examples of MAS in grape (for more detail see our April 2006 report). Table 2 presents these progeny from the 2005 crosses that went to the field for fruit evaluation and follow-up *Xf* resistance testing. We also planted additional individuals for mapping populations being used in our companion mapping and characterization of *PdR1* project. An additional 373 PD resistant siblings of genotypes from populations screened in the greenhouse, but that do not carry PdR1are detailed in Table 3.

Objective 2
Table 3 presents the percentage of seedlings in each of two possible resistance categories (strong resistance <150K cfu/ml, or moderate <500K cfu/ml) from crosses based on *Xf* resistance from Midsouth and DC1-39. Although these progeny are expected to be PD resistant, they host higher levels of *Xf* than progeny based on the b43-17 *V. arizonica/candicans* source. The crosses include multiple combinations of resistant (R) and susceptible (S) selections. The results of these tests helped confirm that the greenhouse screening system is well-adapted to a wide range of genotypes, allowed use to make decisions about how many seedlings to plant in the field (see Table 3), and guided our crosses for 2006.

Table 4 presents testing results on seedling populations in the field. These seedlings were from populations in alternative mapping populations; for the production of PD resistant rootstocks; to verify *PdR1* screening results and advance to wine evaluations; to produce wine grapes based on SEUS resistance sources or from *V. arizonica* that has not yet been evaluated for *PdR1*, but is known to be PD resistant; and to complete evaluation of remaining members of the 89 series *V. arizonica/candicans* population.

Table 5 presents the horticultural characteristics of progeny that are 75% *vinifera*. These progeny were evaluated for typical *vinifera* winegrape appearance, with reference to their winegrape pollen parents. We also screened them for erect cane growth, a beneficial character in terms of reduced disease pressure, and brushy habit so that laterals and canopy density could be reduced. Evaluations of powdery mildew on the leaves, canes and clusters are being recorded, but are not yet complete. The selections with *PdR1* and optimal leaf form and growth habit will be used in the next generation crosses.

This spring/summer we planted eight promising PD resistant selections with *PdR1* that are 87.5% *vinifera*; four from a cross with Syrah, and the other four from a cross with Chardonnay, with six to seven replicates of each. We should get some fruit from these vines summer 2007 and will micro-vinify the fruit to begin testing wine quality of our advanced selections. In preparation of these winemaking tests, we have a MS student comparing wine making at 1L, 20L and 2,000L levels to see what quality parameters are most accurate and predictive of high quality across the three quantities. These results will help us evaluate wine quality on a single-vine basis.

We continue to use the Beringer Yountville site to field test selection for PD resistance. In May 2006 we inoculated a wide range of PD resistant materials from the *PdR1* and SEUS resistance sources. ELISA sampling of these vines is scheduled for October 2006. We are also currently testing 60 recombinants from the 9621 mapping population in collaboration with the fine scale mapping of *PdR1* project with results expected in November.

CONCLUSIONS

This project continues to breed PD resistant winegrapes with the primary focus on the *PdR1*resistance source so that progress can be expedited with MAS. Populations with *Xf* resistance from other sources are being maintained and expanded, but progress is slower with these sources. We continue to supply plant material, conduct greenhouse screens and develop new mapping populations for our project on fine-scale mapping of PD resistance leading to the characterization of the *PdR1* resistance. Next year will see the first testing of wine from advanced selections with 87.5% *vinifera* from winegrapes.

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Table 1. 2006 PD breeding program crosses and the number of seeds collected or expected (in italics).

Female	Male	Seeds	Comments
1a. Monterrey *V. arizonica/candicans* resistance source to produce progeny with 87.5% *V. vinifera* parentage.			
A81-138	Alicante Bouschet, Cab Sauv, Carignane, Chard, Chenin blanc, Colombard, Sauv. blanc, Symphony, Syrah, Tannat	1224	Highly resistant table grape selection by classic wine grape cultivars
1b. Monterrey *V. arizonica/candicans* resistance source (F8909-08) to produce progeny with 75% *V. vinifera* parentage.			
504-60	Chenin blanc, Colombard, Symphony	*750*	504-60 is 50% *V. vinifera*, resistance from 8909-08 has *PdR1*.
03188-06	Chenin blanc, Colombard, Symphony, Tannat	*650*	03188 population is 50% *V. vinifera* resistance from 8909-08 has *PdR1*.
03188-07	Chenin blanc, Colombard	*300*	" "
1c. Monterrey *V. arizonica/candicans* resistance source (F8909-17 allele) to produce progeny with 75% *V. vinifera* parentage.			
F2-35	04191-019	*100*	Breeding population from F8909-17
Malaga Rosada	04191-019, 04373-49, 04373-56	*600*	"
F2-7	04373-49	*150*	Eliminates *V. rupestris* and uses 8909-17 allele
F2-7	04373-56	*150*	"
04373-64	Chenin blanc	*75*	"
1d. Other resistance sources.			
03187-080	03187-143	*100*	R x R, BD5-117 resistance for mapping by USDA
03187-080	Aubun, Chenin blanc	*225*	R x S, BD5-117 source
0110-050	0110-090	*200*	DC1-39 resistance source and >50% *V. vinifera*
0110-092	0110-090, 0126-13	*500*	DC1-39 resistance source and >50% *V. vinifera*
NC-11J	Cab Sauv, Chenin blanc, Tempranillo	*450*	*M. rotundifolia* resistance and >75% *V. vinifera*
1e. Mapping populations with *V. arizonica* PD resistance sources.			
F2-7	F8909-17	*600*	Remake of 04191 mapping population
F2-35	b40-14	*1050*	Alternate to b42-26 resistance source
F2-35	b43-17	*1000*	Remake of 04373 mapping population
1f. Rootstock crosses to combine PD and nematode resistance.			
03300-018, 9621-050	9365-85	*240*	8909-08 or 8909-17 allele form of PdR1 with broad nematode resistance
03300-088, 03305-07	9621-152, 9621-161	*215*	Combines 8909-08 and 8909-17 allele form of PdR1 with Xi1
Florilush	9621-161, 9621-244	*1000*	Combines broad nematode and PD tolerance with 8909-17 allele form of PdR1 and XiR1

Table 2. 2005 progeny that went to University of California, Davis breeding blocks for evaluation.

Seedling Percent Vinifera	Seed Parent	Pollen Parents	Seeds	# PD Resistant Seedlings Planted	% R plants seed to field
2a. Genotypes tested for PdR1 prior to planting					
88%	A81-138	Cab. Sauvignon, Chard, Sauv blanc, Syrah	307	66	21%
75%	03188-06	Airen, Barbara, Chard, Tempranillo, Viognier	419	86	21%
75%	03188-07	Barbara, Syrah, Viognier, Zinfandel	472	122	26%
75%	03188-12	Alicante B., Barbara, Cab Franc, Syrah, Viognier	664	178	27%
75%	03188-32	Airen, Syrah, Viognier	331	20	6%
75%	F2-7, F2-35	03188-01, 03188-25, 03188-30, AT0062-81	701	131	19%
75%	Cab. Sauv, S. blanc	03188-25, 03188-30, AT0062-81	163	10	6%
2b. Additions to mapping populations (genotypes were not marker screened)					
50%	F2-7	F8909-08		175	
50%	F2-35	b42-26		65	
50%	D8909-15	b42-26		55	breeding
2c. Plantings of *rotundifolia* resistance source (genotypes were untested)					
>75%	F2-35	b59-45		10	
>75%	NC-11J	Cabernet Sauvignon		5	

Table 3. Percentages of resistant seedlings from Midsouth and DC1-39 in greenhouse testing for *Xf* resistance and number of untested siblings planted in field.

Cross	Female	Male	Cross Type	%<150k cfu/ml	%<500k cfu/ml	# planted
05-357	0110-092 (G11-32)	0126-13 (G12-49)	RxR	40%	50%	15
05-355	0028-44 (J28:44)	0028-35 (J28-35)	RxR	6%	35%	50
05-378	0110-050 (G10-45)	0126-13 (G12-49)	RxR	20%	35%	65
05-375	0110-092 (G11-32)	5025-102 (G32-18)	RxR	10%	30%	30
05-308	0028-44 (J28:44)	0058-09 (J27-09)	RxR	10%	25%	15
05-374	5025-073 (G31-51)	0126-13 (G12-49)	SxR	0%	25%	5
05-361.1	0124-37 (G15-37)	0126-13 (G12-49)	IxR	0%	20%	18
05-379	0110-050 (G10-45)	5025-102 (G32-18)	RxR	0%	20%	25
05-371	5025-073 (G31-51)	5025-102 (G32-18)	SxR	14%	14%	20
05-359	0110-092 (G11-32)	9967-52 (B11-52)	RxS	6%	12%	30
05-358	0110-092 (G11-32)	9967-03 (B11-03)	RxS	5%	11%	35
05-363	0110-092 (G11-32)	5025-033 (G31-21)	RxS	8%	8%	15
05-362	0124-37 (G15-37)	5025-033 (G31-21)	IxS	0%	0%	10
05-372	5025-073 (G31-51)	5025-033 (G31-21)	SxS	0%	0%	15
05-380	0110-050 (G10-45)	5025-033 (G31-21)	RxS	0%	0%	25
		Total Planted				373

Table 4. Evaluations of *Xf* resistance under our greenhouse screen completed Fall/Winter 2005- 2006. Five replicates of each were tested.

Group Name	Resistance source	Genotypes tested	Comments
0023	D8909-15	75	b42-26 mapping population
03305	b43-17	20	Production of PD resistant rootstock
03188	b43-17	33	Resistant winegrape breeding and also verifies *PdR1* markers
04190	b43-17	114	Resistant winegrape breeding and mapping to refine *PdR1* markers
89 Series untested	*V. rupestris* x *V. arizonica /candicans* types	56	Completes *Xf* resistance survey
Misc. types	SEUS or *V. arizonica*	32	Types for wine breeding
045554	b43-17	26	Confirms resistance of PdR1 at 87.5% *vinifera* level

Table 5. Horticultural characteristics of progeny resulting from crosses of elite *vinifera* wine cultivars and breeding genotypes with *PdR1*. All progeny are 75% *vinifera*.

Female	Pollen Parent	Vigor (Ave) High=3 Dead=0	Vigor (n)	% with Vinifera-like leaves	% with Erect canes	Not brushy
03188-06	Airen	2.4	23	15%	36%	14%
03188-06	Chardonnay	1.7	22	20%	32%	14%
03188-06	Tempranillo	2.2	22	15%	59%	50%
03188-06	Viognier	1.4	17	10%	60%	20%
03188-07	Barbara	1.5	33	12%	35%	54%
03188-07	Syrah	1.9	46	10%	59%	71%
03188-07	Viognier	2.0	23	0%	79%	53%
03188-07	Zinfandel	1.8	20	29%	88%	59%
03188-12	Alicante Bouschet	1.9	33	10%	58%	55%
03188-12	Barbara	1.7	100	18%	57%	39%
03188-12	Cabernet Franc	1.9	29	8%	50%	17%
03188-12	Syrah	1.9	8	50%	50%	50%
03188-12	Viognier	2.0	8	0%	50%	33%
03188-32	Airen	1.7	7	14%	29%	43%
03188-32	Viognier	1.9	12	17%	42%	17%
F2-35	03188-01	2.3	22	43%	57%	33%
F2-35	03188-25	1.1	15	0%	92%	8%
F2-35	0062-81	2.5	23	24%	76%	57%
F2-7	03188-01	2.6	18	17%	72%	22%
F2-7	03188-25	1.3	21	15%	35%	15%
F2-7	03188-30	2.3	30	23%	63%	30%

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